Eucalyptus globulus Labill. regeneration from seeds in Portugal's mainland

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Abstract

***Eucalyptus globulus* Labill. regeneration from seeds in Portugal's mainland**

PhD: Forestry Engineering and Natural Resources

Student: Ana Sofia Nunes do Carmo Águas

Eucalyptus globulus Labill. is a plant species native to SE Australia, Tasmania and adjacent islands. It was introduced in Portugal in the mid-19th century. In 2005/06, it was the most abundant tree species in 23% of the afforested area in Portugal's mainland. Forests dominated by this species are one of the most fire-prone forest types in Portugal. This thesis was aimed to contribute to a better understanding of the naturalization process in Portugal's mainland, with a special focus on the role of fire in this process. A multiscale approach was used to address the problem. Natural regeneration of *E. globulus* from seeds occurs in every natural region of the territory and may reach high densities in some locations. Spatial distribution of both wildling occurrence and density is not uniform on national (mainland), regional, local, and stand scales. The existence of seed sources (reproductive trees) is of primary importance. Climatic and soil conditions affect the broad scale distribution of this regeneration and its performance. Site quality and forest management are fundamental on a local scale. Fire clearly plays a relevant role, inducing seed release from burnt trees, providing safe microsites for plant recruitment and development, and allowing for the establishment of plants in the mid-term. Moreover, litter charring enables the early development of *E. globulus* in otherwise toxic conditions. Maximum wildling densities observed were 0.3 plants m^{-2} and 9.9 plants m^{-2} inside unburnt and burnt plantations, respectively. Portuguese plantations, from a region with nationally moderate levels of seminal regeneration, had mean wildling densities 3.1 times higher than Australian plantations, from seven regions either inside or outside the native range. In summary, cultivated trees are able to produce offspring, which grows, establishes and may produce seeds next to parent trees, in many parts of Portugal's mainland. Therefore, naturalization is in progress and widespread in this territory, and fire does facilitate it.

Keywords: *Eucalyptus globulus* Labill.; seminal regeneration; fire; forest stand; naturalization.

Resumo

Regeneração seminal de *Eucalyptus globulus* Labill. em Portugal continental
(*Eucalyptus globulus* Labill. regeneration from seeds in Portugal's mainland)

Doutoramento em Engenharia Florestal e dos Recursos Naturais

Aluna: Ana Sofia Nunes do Carmo Águas

Eucalyptus globulus Labill. é uma espécie nativa do sueste da Austrália, Tasmânia e ilhas adjacentes. Foi introduzida em Portugal em meados do século XIX. Em 2005/06, já era a espécie arbórea mais abundante em 23% das florestas de Portugal continental. As florestas onde é dominante são um dos tipos de floresta mais propensos ao fogo em Portugal. Esta tese visou contribuir para a compreensão do processo de naturalização em Portugal continental, com especial foco no papel do fogo neste processo. Nesta investigação, foi utilizada uma abordagem multiescala. A regeneração seminal de *E. globulus* ocorre espontaneamente em todas as regiões naturais do território e pode atingir altas densidades nalguns locais. A distribuição espacial das ocorrência e densidade das plantas espontâneas não é uniforme em nenhuma das seguintes escalas: nacional (continental), regional, local ou dos povoamentos. A existência de fontes de sementes (árvores reprodutivas) é de importância primária. As condições edafoclimáticas afetam a distribuição geográfica da regeneração e seu sucesso. A qualidade da estação e a gestão florestal são fundamentais à escala local. O fogo desempenha um papel importante, induzindo a liberação de sementes de árvores queimadas e criando microlocais seguros para o recrutamento, desenvolvimento e estabelecimento dos descendentes, que podem atingir a idade de reprodução. O aquecimento severo de folhada fitotóxica retira-lhe a capacidade de inibir o desenvolvimento inicial de *E. globulus.* As densidades máximas observadas de regeneração foram 0,3 plantas m^{-2} nas plantações não queimadas e de 9,9 plantas m^{-2} nas queimadas. As plantações portuguesas, de uma região com níveis nacionalmente moderados de regeneração, tinham densidades médias de eucaliptos espontâneos 3,1 vezes superiores às das plantações australianas de sete regiões (dentro e fora do território nativo). Em suma, em muitas partes do território continental de Portugal, existem eucaliptos cultivados com capacidade de produzir naturalmente descendentes, que se desenvolvem, estabelecem e podem produzir sementes nas imediações das árvores-mãe. Assim, a naturalização está em curso e bastante difundida neste território, sendo o fogo um facilitador do processo.

Palavras-chave: *Eucalyptus globulus* Labill.; regeneração seminal; fogo; povoamento florestal; naturalização.

Resumo alargado

Regeneração seminal de *Eucalyptus globulus* Labill. em Portugal continental
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Aluna: Ana Sofia Nunes do Carmo Águas

Eucalyptus globulus Labill. é uma espécie nativa do sueste da Austrália, Tasmânia e ilhas adjacentes. Apesar de ter uma distribuição geográfica restrita no seu território nativo, é uma espécie muito cultivada nas regiões temperadas fora desse território. A espécie foi introduzida em Portugal em meados do século XIX mas só começou a ser plantada de forma extensiva cerca de 100 anos mais tarde. Atualmente, é uma das espécies mais representadas nas florestas portuguesas do continente. As suas florestas servem essencialmente para abastecer a indústria da celulose e do papel. Sendo uma espécie com interesse económico, há muito conhecimento acumulado sobre a sua produtividade e melhoramento genético. Os conhecimentos existentes sobre a sua ecologia como espécie exótica estão associados, sobretudo, às finalidades produtivas. Tratando-se de uma espécie exótica amplamente cultivada e com um tempo de residência relativamente longo, os relatos da ocorrência de regeneração seminal natural já têm mais de 70 anos. No entanto, esta realidade não se encontrava sequer estudada quantitativamente ao início desta década, por não ser considerada de interesse. Os estudos que existem são sobretudo sobre o risco de invasão. Existem alguns outros sobre aspetos específicos que afetam a disponibilidade de sementes, tais como a predação e a libertação de sementes induzida pelo fogo. É, pois, necessário um estudo que aborde o fenómeno que está a montante da invasão, a naturalização. É este o processo que permite que uma espécie exótica se torne independente do controlo humano para poder subsistir naturalmente num território diferente daquele em que evolui. Esta espécie pertence ao género *Eucalyptus* que evoluiu num ambiente onde o fogo tem sido um importante agente seletivo. Atualmente, em Portugal, observa-se uma alteração do regime de fogo associada às alterações climáticas e às alterações de uso do solo. As florestas dominadas por esta espécie são um dos tipos florestais que mais frequentemente arde em Portugal. Os estudos sobre o comportamento reprodutivo desta espécie em áreas ardidas também são escassos. Assim, esta tese tem como objetivos principais: (1) contribuir para a compreensão do processo da naturalização de *E. globulus* em Portugal continental; (2) avaliar a influência do fogo na regeneração seminal natural desta espécie. Para o efeito, foi utilizada uma abordagem multi-escala, contemplando escalas espaciais que vão da transcontinental e à microlocal. Procurou-se ainda identificar alguns mecanismos através dos quais o fogo pode influenciar o potencial de regeneração seminal da espécie.

Foi feito um estudo exploratório em que se procurou verificar a distribuição geográfica das ocorrências de regeneração de *E. globulus*. Foram utilizados dois métodos: a análise das bases de dados dos 4º e 5º Inventários Florestais Nacionais; e a observação direta de bermas de estradas nacionais e municipais. Verificou-se que a regeneração seminal espontânea está dispersa por todo o território, mas quase sempre nas proximidades de fontes de sementes detetáveis (árvores com copas reprodutoras). A variação da distribuição não é uniforme e está limitada por condicionantes edáficas e, sobretudo, climáticas. As densidades mais elevadas ocorrem em regiões onde o clima é mais ameno e com pluviosidades moderadas a altas, nomeadamente na faixa ocidental das regiões norte e centro do país.

O estudo propriamente dito dividiu-se em quatro partes, onde foram feitos estudos parcelares que se enquadravam em escalas espaciais e temporais progressivamente menores.

Primeiramente, foi feita uma abordagem biogeográfica em que se comparou a densidade de regeneração natural nas bordaduras de plantações de Portugal (região ocidental do vale do Tejo) e da Austrália (sete regiões no sul da Austrália, uma em território nativo da espécie e seis fora). Foram também considerados fatores climáticos, características das(os) estações/povoamentos florestais e respetiva gestão. Na região portuguesa: as plantas espontâneas eram mais abundantes nas exposições mais húmidas, exploradas em talhadia (2ª rotação ou superior), com plantas cujo fuste tinha idades superiores a seis anos e localizadas em estações com índice de qualidade intermédio. As densidades de regeneração eram significativamente mais altas em Portugal do que em praticamente todas as regiões australianas. Num subconjunto de povoamentos comparável (1ª rotação e não ardidos), observaram-se densidades médias 3,1 vezes superiores em Portugal do que na Austrália (sete regiões). A identificação dos fatores de natureza biogeográfica que podiam explicar estas diferenças revelou-se complexa. Foi possível identificar a rotação dos povoamentos e a idade do fuste ou das varas como positivamente associadas às densidades de plantas espontâneas. O tempo de residência da espécie nas regiões estudadas e a pressão de propágulos associada a este tempo podem ter contribuído para as diferenças observadas.

Em segundo lugar, foi feita uma avaliação da ocorrência, densidade e estrutura (classes de tamanho) da coorte de regeneração seminal natural em florestas ardidas. O objetivo era analisar os fatores associados ao estabelecimento de regeneração seminal natural a médio prazo (5–7 anos após o fogo). Foram feitos levantamentos em 284 povoamentos puros de *E. globulus* e de *Pinus pinaster* e em povoamentos mistos de ambas as espécies. Foram efetuados modelos para tentar avaliar a relação entre esta regeneração e variáveis ambientais (associadas ao potencial produtivos das regiões) e variáveis de gestão dos povoamentos. A regeneração seminal natural de *E. globulus* foi encontrada em 93.1% dos povoamentos puros de eucalipto, 19.0% dos pinhais puros e em 98.6% dos povoamentos mistos. Em povoamentos onde foi detetada regeneração, a densidade mediana foi 0.20 plantas m^{-2} e a máxima foi 4.55 plantas m^{-2}. A altura (h) mediana das plantas foi 2,0 m. Entre estas plantas, 95.3% tinham h > 1,30 m e diâmetro à altura do peito igual ou inferior a 5 cm. A probabilidade de estabelecimento, a densidade de plantas e a altura mediana eram mais elevadas nas regiões

consideras de alta produtividade para a espécie (critérios edafoclimáticos). Houve três variáveis de gestão que foram identificadas com influenciadoras da regeneração. O corte de matos diminuiu a ocorrência de regeneração. A lavoura fez baixar a densidade e a altura das plantas. O corte de madeira queimada favoreceu a densidade.

Em terceiro lugar, foram analisadas as condições microlocais da ocorrência de regeneração seminal natural após o fogo e o corte de árvores queimadas, com vista à caracterização do nicho de regeneração da espécie em situações de pós-fogo. Foram recolhidos dados em quatro povoamentos. Em cada povoamento foram amostrados microlocais de 20 plantas espontâneas da espécie-alvo, cuja altura (h) era 30 < h < 100 cm, e outros tantos microlocais sem plantas espontâneas desta espécie. Num dos quatro povoamentos, foram ainda analisados os microlocais de 20 plantas com h < 18 cm e de 20 plantas com h >130 cm. Foram identificadas duas coortes recrutadas após o fogo. Uma primeira recrutada logo após a ocorrência do fogo e uma segunda recrutada após as operações de corte. As sementes terão provindo das árvores queimadas em pé, no primeiro caso, e dos sobrantes do corte das mesmas, no segundo. As plantas da primeira coorte encontravam-se em locais em que as variáveis relacionadas com o fogo tinham uma forte presença, nomeadamente: abundância de carvão e cinza, solo com pH e teor de potássio elevados. A distribuição agregada destas plantas dentro dos povoamentos parecia denotar a existência de relações de facilitação e/ou o aproveitamento de locais com elevada concentração de recursos. Todos estes fatores poderão ter contribuído para o desenvolvimento e para a persistência desta primeira coorte. Por outro lado, plantas da segunda coorte eram muito mais escassas, estavam situadas em microlocais onde a perturbação causada pelas operações de corte era bastante evidente e que pouco diferiam dos locais sem regeneração. Isso demonstra uma eventual limitação da segunda coorte pela quantidade de propágulos disponível e a importância da perturbação causada pelo corte no recrutamento de plantas. A diversidade de tamanhos das plantas observadas, ter-se-á ficado a dever, não só aos dois momentos de recrutamento, mas também à competição assimétrica entre plantas jovens e as árvores adultas.

Em último lugar, procurou-se estudar o efeito que o aquecimento da folhada, existente no solo, poderia ter no desenvolvimento inicial de *E. globulus.* Foi feito um ensaio laboratorial, onde foram semeadas sementes de *E. globulus* sobre folhadas de quatro espécies diferentes. Essas folhadas haviam sido previamente aquecidas a temperaturas de 100–600ºC e arrefecidas antes do contacto com as sementes, o controlo foi efetuado com folhadas não aquecidas. As folhadas eram das seguintes espécies: *E. globulus*, *Acacia dealbata*, *P. pinaster*, e *Quercus suber*. Todas as folhadas foram analisadas quimicamente através de técnica de ressonância magnética para detetar os grupos funcionais de carbono que continham. O cultivo decorreu em condições ótimas de humidade, luz e temperatura. Verificou-se que o aquecimento alterou a composição química de todas as folhadas. Ocorreu uma diminuição, para menos de metade, dos teores de carbono alquil, carbono *O*-alquil, e carbono metoxil + *N*-alquil. Os teores de carbono aromático mais do que quintuplicaram. As folhadas não

aquecidas ou moderadamente aquecidas inibiram a germinação e o crescimento, com a inibição máxima a ser causada pelas folhadas de *Quercus* e *Eucalyptus*. As folhadas severamente queimadas tiveram efeitos neutros ou estimulantes do desenvolvimento de *E. globulus.* Estas respostas das plantas estavam associadas com as concentrações dos vários tipos de carbono que dominavam em cada tipo de folhada. Demostrou-se que o aquecimento e a queima de folhada durante os incêndios são potencialmente críticos para o desenvolvimento da regeneração seminal de *E. globulus* após os mesmos.

Conclui-se que a regeneração seminal de natural *E. globulus* depende primariamente da existência de fontes de sementes e é condicionada por variáveis que operam a diferentes escalas. O fogo é um fator de grande importância para a regeneração seminal natural desta espécie, criando oportunidades para a sua ocorrência, desenvolvimento e estabelecimento. A naturalização da espécie em Portugal é confirmada como um processo em curso e altamente difundido pelo território de Portugal continental.

Palavras-chave: *Eucalyptus globulus* Labill.; regeneração seminal; fogo; povoamento florestal; naturalização.

Symbols and abbreviations

Abbreviation	Meaning
^{13}C	carbon-13 (isotope)
A.	*Acacia*
ADRP	Secretaria de Estado do Desenvolvimento Rural e das Pescas (Portuguese State Secretariat of Rural Development and Fisheries)
AED	analysis of ecological data
a.s.l.	above sea level
AEET	Asociación Española de Ecología Terrestre
AEMET	Agencia Estatal de Meteorología (Spanish Agency of Meteorology)
AFN	Autoridade Florestal Nacional (Portuguese National Autority for Forests)
AIC	Akaike's information criterium
al.	*alli*
ANOSIM	ANalysis Of SIMilarity
ANOVA	ANalysis Of VAriance
app.	appendix
approx.	approximately
AUC	area under the ROC curve
BP	before present
BRT	boosted regression trees
C	carbon or control
Ca	calcium
CA	cluster analysis
ca.	*circa*
CABI	The Centre for Agriculture and Bioscience International
Cal-IPC	California Invasive Plant Inventory. California Invasive Plant Council
CEABN	Centro de Ecologia Aplicada "Prof. Baeta Neves"
CELPA	Associação da Indústria Papeleira (Portuguese Association of Pulp and Paper Industry)
cf.	*conferatur*
Ch.	chapter
CI	confidence interval
cm	centimetre
CM1	Casal do Malta 1
CM2	Casal do Malta 2
CMR	Consellería do Medio Rural (Council of Rural Areas Galicia, Spain)
CNR	Conselho Nacional de Reflorestação (Portuguese National Council for Reafforestation)

Abbreviation	Meaning
COS	Carta de Ocupação do solo (Portuguese landuse map)
CPMAS	cross polarization magic angle spinning
CRC	Chemical Rubber Company (publisher)
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CV	Currelos Valdeias
D.C.	district of Columbia (Washington)
DBH	diameter at breast height
df	degrees of freedom
DGAPFF	Departamento de Gestão de Áreas Públicas e de Proteção Florestal (Department for Management of Public Areas and Forest Protection)
DGF	Direção-Geral de Florestas (Portuguese National Forestry Services)
DGRF	Direção-Geral dos Recursos Florestais (Portuguese National Forestry Services)
DSR	daily severity rating
DSVPF	Direcção de Serviços de Valorização do Património Florestal
DOI	digital object identifier
E	East
E.	*Eucalyptus*
e.g.	*exempli gratia*
ed.	editor
EFI	European Forest Institute
eq.	equation
ESRI	Environmental Systems Resource Institute
et al.	*et alli*
F1	the first generation of offspring
F	Fisher's statistic
FLAD	Fundação Luso-Americana
FAO	Food and Agriculture Organization of the United Nations
FCT	Fundação para Ciência e Tecnologia (Portuguese National Foundation for Science and Technology)
Fig.	figure
FWI	Canadian forest fire weather index
g	gram
GIS	geographic information system
GLM	generalized linear model or general linear model
GPS	geographical positioning system
GTR	general technical report
h	height or hours
ha	hectares

Abbreviation	Meaning
Hz	hertz
IBM	International Business Machines (company)
i. e.	*id est*
ICNF	Instituto para a Conservação da Natureza e das Florestas (Portuguese National Authority for Conservation and Forestry)
IFN	Inventário Florestal Nacional (Portuguese National Forest Inventory)
IFN4	4º Inventário Florestal Nacional (fourth Portuguese National Forest Inventory, also called the third revision of NFI)
IFN5	5º Inventário Florestal Nacional (fifth Portuguese National Forest Inventory)
IFN6	6º Inventário Florestal Nacional (sixth Portuguese National Forest Inventory)
IGeoE	Instituto Geográfico do Exército (Geographical Institute of the Portuguese Army)
IGP	Instituto Geográfico Português (Portuguese Institute of Geography)
INAmb	Instituto Nacional do Ambiente (Portuguese National Institute of the Environment)
IITF	International Institute of Tropical Forestry
INIA	Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Spanish Food and Agriculture Institute of Research and Technology)
ISA	Instituto Superior de Agronomia (School of Agriculture of the University of Lisbon, Portugal
ISBN	International standard book number
ISO	International Organization for Standardization
ISRIC	International Soil Reference and Information Centre
ISTA	International Seed Testing Association
IUFRO	International Union of Forest Research Organizations
IUSS	The International Union of Soil Sciences
JRC	Joint Research Centre of the European Commission
K	potassium
kg	kilogram
km	kilometre
km^2	square kilometre
L	litter species
LD	last disturbance
ln	natural logarithm
LPN	Liga para a Proteção da Natureza (Portuguese League for Protection of Nature)
m	metre or mass
m^2	square metre
max.	maximum
MCPFE	Ministerial Conference on the Protection of Forests in Europe
MDS	multidimensional scaling

Abbreviation	Meaning
med	median
MED	molarity of an ethanol droplet test
MEDECOS	International Mediterranean Ecosystems Conference
mg	milligram
Mg	magnesium
min.	minutes or minimum
mL	millilitre
mm	millimetre
Mn	manganese
ms	millisecond
MS	mean of squares
My	million years
N	North
n	statistical sample size
N	North or nitrogen
n. d.	no date
NE	Northeast
NFI	Portuguese National Forest Inventory
NFI4	fourth Portuguese National Forest Inventory (also called the third revision of NFI)
NFI5	fifth Portuguese National Forest Inventory
NIRD	near infrared detection
NMR	nuclear magnetic resonance
no.	number
n. s.	statistically not significant
NW	Northwest
O	oxygen
º	degrees
ºC	Celsius degrees
P	statistical probability
P	phosphorous
P.	*Pinus*
PCA	principal component analysis
pers. comm.	personal communication
pers. obs.	personal observation
pH	potential of hydrogen
ppm	parts per million
PSW	Pacific Southwest (research station)
Q	Cochran Q test statistic

Abbreviation	Meaning
Q.	*Quercus*
Q-Q	quartile *vs*. quartile
Q1	first quartile
Q2	second quartile or median
Q3	third quartile
R	R software
r^2	correlation coefficient
RMRS	Rocky Mountain Research Station
ROC	receiver operating characteristic curve
S	South or sulfur or target species (seed or seedling)
S.A.	sociedade anónima (public limited company)
SA	Santo António
SD	standard deviation
Se	seedling
SE	Southeast
secs.	seconds
SEDRF	Secretaria de Estado do Desenvolvimento Rural e das Florestas (Portuguese State Secretariat of Rural Development and Forests)
SIAM	Project "Climate change in Portugal: scenarios, impacts, and adaptation measures"
SIcat	site index category
sp.	one species from the aforementioned genus
spp.	*species pluralis* (several species from the aforementioned genus)
SS	sum of squares
SSa	short sapling
SW	Southwest
T	temperature
tab.	table
TM	trade mark
TRANZFOR	Transferring Research between European Union and Australia-New Zealand on Forestry and Climate Change
TSa	tall sapling
T_{50}	median lethal temperature
UNECE	United Nations Economic Commission for Europe
UniNA	Universitá Federico II di Napoli (Napolitan University of Federico II, Italy)
USA	United States of North America
USDA	United States Food and Agriculture Department
UTas	University of Tasmania, Australia

Abbreviation	Meaning
v	volume
VIF	variance inflation factor
vs.	*versus*
W	West
WDPT	water drop penetration time test
α	statistical significance level
Φ_2	phi coefficient
χ^2	chi-square test statistic
χ^2_r	Friedman's test statistic
ψ	water potential
ψ_m	soil matrix water potential
%	percent
’	minutes
”	seconds
<	minor than
=	equal to
>	major than
±	more or less
≤	minor than or equal to
≥	major than or equal to

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Section I - General Introduction

General introduction

Exotic plant species occur all over the world (van Kleunen *et al.*, 2015) and may cause important ecological and social impacts, which can either be positive or negative (Lambdon *et al.*, 2008; Andreu *et al.*, 2009; Vilà *et al.*, 2010; Simberloff *et al.*, 2013). Such impacts substantially increase when these species reach naturalization, because they become able to self-perpetuate their populations, regardless of any human effort (Richardson *et al.*, 2000). The proportion of exotic species that are naturalized ranges from 9.3% to 73.8% in the various regions of the world (Richardson and Pyšek, 2012). For instance, vascular plant floras in European countries total 5,789 exotic species, 64.8% of which are naturalized there (Lambdon *et al.*, 2008).

The naturalization process is affected by numerous factors, including environmental changes (Dale *et al.*, 2001). Namely, climate and land-use are changing in many regions of the world (Santos *et al.*, 2002; Silva *et al.*, 2009; van Lierop *et al.*, 2015; FAO, 2016) and these changes affect biocoenoses both directly and indirectly. Amongst the indirect effects are those mediated by altered fire regimes (Dale *et al.*, 2001; van Lierop *et al.*, 2015; FAO, 2016; Oliveira *et al.*, 2017). Once those changes occur, the community composition will depend on which species are fit enough to the new conditions, *i. e.*, those that survive and are, or become, able to naturally reproduce in the altered ecosystems. These environmental changes may be opportunities for some exotic species to become naturalized and/or outcompete other species (Vitousek *et al.*, 1997). Therefore, understanding the species naturalization process is of utmost importance, in order to control the impacts of exotic species, especially when environmental conditions are changing.

1. Exotic species

The impacts of exotic species on biocoenoses and on human society are increasing rapidly (Vilà *et al.*, 2010; Simberloff *et al.*, 2013). However, the knowledge about those species is still limited in subjects as their distribution in the arrival territories and the extent of their integration within the ecosystems there (Richardson and Pyšek, 2012; van Lierop *et al.*, 2015).

Humans have been transporting living organisms between different geographical regions for millennia. This transportation, which has resulted in species introductions, has been boosted by several events in the History of mankind (*e. g.* diffusion of agriculture, development of transports, and intensification of international trade), often closely connected with human interest on some species (del Monte and Aguado, 2003; Pyšek and Jarošík, 2005; Almeida and Freitas, 2006). Human needs for food, shelter, fuel, clothing, to name a few, determined which plants were cultivated (Pyšek *et al.*, 2003; Richardson and Rejmánek, 2011). Contrastingly, other species have been introduced accidentally and, thus, not chosen (Lambdon *et al.*, 2008). The geographical origin was not important for choosing plant species, as long as they were able to survive and to produce satisfactorily under management, in the arrival territory (Pyšek *et al.*,

2003). However, the physiological adaptation of an exotic species to cultivation conditions does not always reflect an ecological adaptation to the local natural conditions (da Silva *et al.*, 2011). Therefore, many exotic plants, whether cultivated or not, do not necessarily have the natural capacity to survive and reproduce in their arrival territories.

Upon arrival in their new territories, species may follow a path of integration in the receiving communities with varying degrees of success, as they overcome successive barriers (Fig. 1). Firstly, they might perish or be able to survive without human protection, under the local

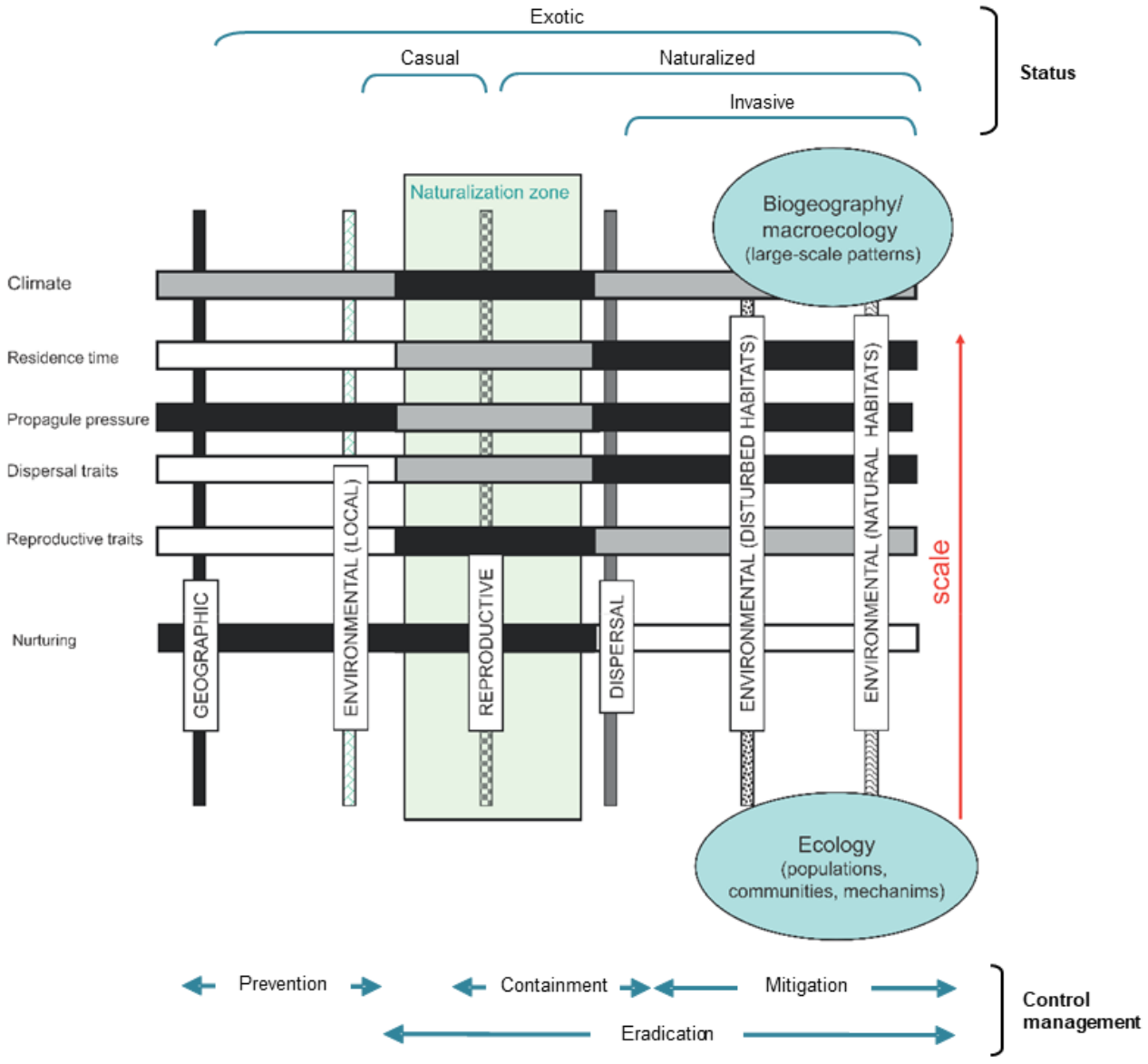


Fig. 1 - Introduction-naturalization-invasion continuum. The factors that determine the progression along the continuum are listed according to their importance on different space scales, from the largest scale to the smallest. Shade intensity of horizontal bars represents the importance of those factors along the continuum. Vertical bars represent barriers to be overcome by exotic species for progression along the continuum (adapted from Blackburn *et al.*, 2011; Richardson and Pyšek, 2012).

conditions they meet. Secondly, those which survive may or may not be able to reproduce. This reproduction may or may not be effective for the maintenance of self-sustaining populations. The populations which break reproduction barriers and attain self-sustainability are naturalized (Box 1, Fig. 1). Finally, self-sustained populations may remain in a restricted area or cross dispersal barriers and spread into other ecosystems. Those which spread are called invasive populations (Box 1, Fig. 1). All these steps are phases of the so called 'introduction-naturalization-invasion continuum' (Richardson and Pyšek, 2012). The progress along this continuum is a population process, which depends on the species' traits, the characteristics of the receiving ecosystem, and the introduction history (Catford *et al.*, 2009; Richardson and Pyšek, 2012). The relative importance of each factor varies as populations make headway (Catford *et al.*, 2009; Richardson and Pyšek, 2012). The number of species that overcome the successive barriers decreases along the continuum (Williamson and Fitter, 1996).

Box 1 – Key concepts concerning plant *taxa* occupying either their original geographical areas or other areas.

Native plants	Plant *taxa* that were naturally originated in a given area or that have arrived there by natural means, coming from another area in which they are native (Pyšek *et al.*, 2004).
Exotic plants	Plant *taxa* in a given area whose presence there is due to intentional or unintentional human transportation from their native ranges, or which have arrived there without human help, coming from an area in which they are alien (Pyšek *et al.*, 2004).
Casual plants	Exotic plant *taxa* that exist and may even reproduce occasionally in a given area, but eventually become extinguished there, because they not form self-replacing populations, they rather rely on repeated introductions for their persistence (Richardson *et al.*, 2000; Pyšek *et al.*, 2004).
Naturalized plants	Exotic plant *taxa* that reproduce consistently and sustain populations over many life cycles, regardless human intervention, recruiting from seeds or ramets capable of independent growth; they often recruit offspring freely, usually close to adult plants, and do not necessarily invade other ecosystems (Richardson *et al.*, 2000; Pyšek *et al.*, 2004).
Invasive plants	Naturalized plant *taxa* that produce reproductive offspring, often in very large numbers, at considerable distances from parent plants (scale: > 100 m; < 50 years for *taxa* spreading by seeds and other propagules) (Richardson *et al.*, 2000; Pyšek *et al.*, 2004).
Weeds*	Plant *taxa* in a given area with populations growing entirely or predominantly in situations markedly disturbed by humans but without being deliberately cultivated (Baker, 1974). Plant *taxa* that exist in sites where they are not wanted by humans and which usually have detectable economic or environmental effects (Richardson *et al.*, 2000).

***** In contrast to the other concepts, this one is essentially anthropocentric.

1.1. Naturalization

Naturalization is a cornerstone for the integration of exotic species in receiving communities, as it enables these species to persist in the new ecosystems, regardless of human intervention. However, research on exotic species progress along the continuum has mostly focused on the invasion process and its immediate drivers, often disregarding naturalization. The latter is rarely studied explicitly and with real data (Pyšek *et al.*, 2008). Some authors misuse the terms naturalization and invasion, making the communication about the subject more difficult (Richardson *et al*., 2000; Blackburn *et al*., 2011). Furthermore, many

conceptual syntheses even fail to recognize naturalization as a precondition to invasion (Richardson and Pyšek, 2012). Thus, naturalization deserves to be studied for keeping exotic species under human control and the use of precise terminology is very important (Box 1).

The naturalization process, which occurs at the population level, relies on demography, and, thus, requires time (several generations) and happens on local to regional scales (Blackburn *et al.*, 2011). This process is mainly mediated by ecological and biogeographical factors (Williamson, 2006). Although some of these factors are deterministic, the majority are probabilistic factors (Richardson and Pyšek, 2012). Some plant traits and biotic interactions are known to increase the chances of plant naturalization. Among them are: the capacity of individuals to produce seeds independently (van Kleunen *et al.*, 2008); the rapid profuse seedling emergence (van Kleunen and Johnson, 2007); the small genome size, as far as it influences life-history strategies of plants (Grotkopp *et al.*, 2004; Kubešová *et al.*, 2010); the symbioses with mycorrhizal fungi (Richardson *et al.*, 2000); and the pollination modes similar to those of native species (Pyšek *et al.*, 2011). The most influential abiotic factors favouring naturalization are: a close climatic match between the original and the arrival territories; a large native range (Diez *et al.*, 2009; Richardson and Pyšek, 2012); an availability of similar habitats in the original and arrival territories (Hejda *et al.*, 2009; Essl *et al.*, 2011). Human causes of naturalization are mainly related to introduction history and cultivation (Mack, 2000; Mack and Lonsdale, 2001). Propagule pressure is not as relevant for naturalization as it is for other stages of the continuum, according to some authors (*e. g.* Richardson and Pyšek, 2012; Catford *et al.*, 2009). However, others highlight its relevance stating that increased reproduction is pivotal in naturalization (Simberloff, 2009). Disturbance, whether abiotic or anthropic, also influences naturalization (Lozon and MacIsaac, 1997). Furthermore, interactions among the aforementioned factors also play an important role in plant naturalization (Vitousek *et al.*, 1997; Dale *et al.*, 2001; Richardson and Pyšek, 2012), making this a more complex process to understand and deal with. In addition, as naturalization patterns differ over space and time, the role of ecological factors operating on different scales needs to be integrated, and biogeography should be taken into account, in order to understand naturalization drivers (Hierro *et al.*, 2005; Richardson and Pyšek, 2012). This knowledge may provide an objective basis for identifying, monitoring, and managing exotic populations whose naturalization may raise ecological problems.

1.2. Human influence on plant naturalization

The influence of humans on naturalization is determinant and often occurs beyond the transportation/introduction step. Human activity has been responsible for the naturalization of 13,168 vascular plant species worldwide (3.9% of extant global flora) (van Kleunen *et al.*, 2015). In 2005, exotic species were used in 29% of afforestation and in 36% of reforestation programmes at a global level, while the corresponding figures for Europe were 19% and 3% (FAO, 2010). Amongst the naturalized species in Europe, 63% were cultivated, 2% were introduced for forestry purposes, and nearly 30% inhabit forests and woodlands (Lambdon *et al.*, 2008).

1.2.1. Cultivation

Cultivation is the most common reason for intentional plant introductions and is also responsible for many accidental introductions, through contaminated and transported seedlots, tools or soils (Lambdon *et al.*, 2008). Cultivation actions change environmental conditions. They remove previous vegetation and replace it by selected target species; and they keep the ideal conditions for the target species by management, until harvest. The special conditions created in cultivated areas are aimed at improving the production of cultivated species (Richardson and Rejmánek, 2011). Concomitantly, these conditions can either promote or prevent those plants from reproducing, and their offspring to naturally establish (Richardson and Rejmánek, 2011).

Some features and processes enable the exotic species to succeed in the new ranges where they are cultivated. The first process is the selection of the species to cultivate. Forestry species are often selected according to their: tolerance to a broad range of conditions (Richardson and Rejmánek, 2011; Dodet and Collet, 2012); rapid growth (Richardson, 1998; Richardson and Rejmánek, 2011; Dodet and Collet, 2012); ability to grow with reduced human intervention (Dodet and Collet, 2012); high survival rate (Dodet and Collet, 2012); and precocious and prolific seed production (Richardson and Rejmánek, 2011; Dodet and Collet, 2012). Furthermore, plant species for cultivation are selected for their high performance and their ability to become community-dominant (at least temporarily) under the conditions of the target territory (Dodet and Collet, 2012). Secondly, a good match between the conditions in the exotic territory and the ecological requirements of the introduced species is often observed (Thuiller *et al.*, 2006). Actually, the use of an environmental match as a criterion for selecting which species to cultivate is becoming quite sophisticated (Rejmánek and Richardson, 2011; Dodet and Collet, 2012). Moreover, exotic species are sometimes cultivated in sites where native species perform poorly (Dodet and Collet, 2012), such as degraded areas, marginal lands, or sites close to the distribution limits of some natives (Richardson, 1998). If the environmental conditions are favourable for the exotics in such sites, these species will have a competitive advantage comparatively to the natives. Thirdly, if crops are harvested after reproduction, continuous cultivation leads to a sustained propagule pressure in the new range, which may enhance the probability of natural regeneration to occur and to thrive (Pyšek *et al.*, 2009; Simberloff, 2009; Wilson *et al.*, 2009). Forestry species are usually grown in large plantations, allowing for the accumulation of massive propagule banks (Richardson and Rejmánek, 2011). Plantation extent has a strong positive correlation with naturalization of cultivated woody species (Thuiller *et al.*, 2006). In addition, harvesting can directly promote the release of seeds and, consequently, seedlings of some species are common thereafter (Skolmen and Ledig, 2000). Fourthly, the cultivation of exotic species generally enhances their probability of naturalization. It provides crucial protection for their founder populations against environmental stochasticity, when these are small and more vulnerable (Mack, 2000; Lockwood *et al.*, 2005). Moreover, this cultivation sustains populations for a long time period, from which new wild foci can be established (Mack, 2000; Wilson *et al.*, 2009). Finally, anthropic disturbance is often an important factor fostering exotic plant establishment (Lozon and

MacIsaac, 1997), and forestry species are frequently grown in highly disturbed areas (Richardson and Rejmánek, 2011), to which management operations add extra disturbance.

Conversely, other cultivation-related factors hamper the flourishing of plant species in their arrival territories. If propagule production is reduced or prevented, either by early harvesting or by the use of sterile plants, the existence of any further generation is prevented or hindered (Richardson *et al.*, 2004). Moreover, many cultivated species occupy extensive regions but fail to establish wild populations there, despite the likelihood of a massive propagule pressure (Simberloff, 2009). Physiological adaptation to cultivation conditions does not always reflect ecological adaptation to the local natural conditions, which would imply the capacity of those species to naturally establish through natural regeneration from seeds in the target territory (da Silva *et al.*, 2011). Another contributing factor for this failure may be the selection for the desired traits (*e. g.* high biomass production), which sometimes comes at the expense of the reproductive output, dispersal ability and/or competitiveness (Simberloff, 2009). Additionally, several management actions, targeting the improvement of the cultivated individuals, may impair their offspring. Indiscriminate understorey removal, usually made for reducing either competition or fire risk (Pereira, 2007; Soares *et al.*, 2007; Moreira *et al.*, 2009; Alves *et al.*, 2012), kills or severely injures the shoot of understorey plants, including exotic juveniles. In line with this, thinning effects may be even more harmful for wildlings, since this operation is species specific. Although tillage improves soil conditions for root development, it can also injure the roots of any plant and destroy the smaller individuals, including the exotics' offspring (Catry *et al.*, 2010). Finally, logging can be detrimental to remnant plants, when soil is compacted and substantial amounts of biomass are removed, because water and nutrient availability in soil are reduced (Gonçalves *et al.*, 2004) and root development becomes more difficult. Moreover, if logging occurs after wildling establishment, severe mortality can happen amongst the smallest plants as a direct effect of site disturbance (McIver and Starr, 2000).

2. Fire

2.1. Fire as an ecological factor

Fire in ecosystems is as old as terrestrial plants are. Plants produce two of the elements required for fire occurrence: oxygen and fuel (Fig. 2). The Earth's atmosphere only had abundant oxygen when photosynthetic organisms arose by evolution, and it only had appropriate fuel when plants colonized land. The earliest evidence of fire on ecosystems dates from the early Silurian (440 My BP) and consists of charcoal remains of the earliest stomata-bearing plants (Glasspool *et al.*, 2004). Nevertheless, the interest of ecologists in fire as an ecological factor was only evident since the 1940s and substantially increased in the 1960s (Gill, 1975).

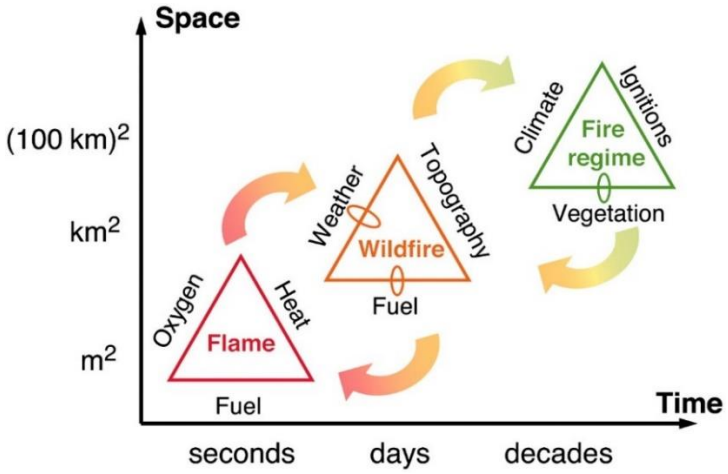


Fig. 2 - Controls on fire at different scales. Small loops represent the feedbacks that fire has on the controls themselves. Arrows represent the feedbacks between processes on different scales (from Moritz *et al.*, 2010)

Fire is an abiotic ecological factor with some particularities. It depends upon both abiotic factors (mainly weather/climate) and biotic factors (fuel/vegetation) (Gill, 1975) (Fig. 2). Its presence is intermittent in ecosystems. Although each episode lasts for a very short time period, its effects are long-lasting. Contrarily to other disturbance types, the nature of fire is both physical and chemical, because combustion consists of an array of chemical reactions that release large amounts of heat (Fox; and Fox, 1987; Pyne *et al.*, 1996). Thus, various features must be taken into account to characterize fire as an ecological factor.

Fire regime is a concept that encompasses several characteristics of fire (Box 2), which facilitates the understanding of the role that fire plays in ecosystems (Gill and Allan, 2008; Krebs *et al.*, 2010). The characteristics of fire classically included in fire regime are: intensity, season, extent, type, and frequency (Box 2) (Gill, 1975; Gill and Allan, 2008). The different components of fire regime interact with each other. Intensity is influenced by weather, topography, and fuel (chemistry, load, spatial distribution) (DeBano *et al.*, 1998). Fire frequency influences fuel availability for the subsequent fire and, thus, the intensity of that fire. Fire season is determined by climate: dry weather turns potential fuel into readily flammable fuel and lightning enables natural ignitions (Whelan, 1995). Fire season may influence the fuel flammability and, consequently, fire extent and intensity (Gill and Allan, 2008). In addition, fire extent and patchiness depend on landscape heterogeneity, which in turn, is determined by topography, vegetation heterogeneity, moisture content of fuels, and past fires in the area (Raison, 1979; Whelan, 1995; Fernandes, 2006). Fire type depends on the fuel distribution in the ecosystem, which ultimately is determined by the existing plant species and the age structures of their populations. The potential fire frequency depends on fuel productivity in the ecosystem and on the ignition frequency (Whelan, 1995). Thus, the fire regime of an ecosystem is a result of the combination of multiple drivers, including climate and vegetation, and fire effects feedback on vegetation distribution (Moritz *et al.*, 2005) (Fig. 2).

Box 2 – Key concepts concerning fire

Fire behaviour	The reaction of fire to the combination of fuel, weather, and topography (DeBano *et al*., 1998).
Fire regime	Combination of features that characterize fire occurrence patterns in a given geographical area throughout a historical time period, *i.e.*, when, where, and which fires occur in a given spatiotemporal window (Krebs *et al*., 2010).
Fire intensity	The amount of heat produced per unit of fuel consumed per unit of time, or energy per unit of area, which is produced by combustion and transferred to the environment (Pyne *et al.*, 1996).
Fire season	The time period along the year when ignitions coincide with low fuel moisture, allowing fire to occur, usually the driest time of the year (Bond and Keeley, 2005).
Fire extent or size	The area over which a fire burns, it may be either continuous or patchy (Fox and Fox, 1987).
Fire type	Classification of fire behaviour according to type and distribution of fuel and rate of spread: surface fire (burns litter and small plants, with a variable rate of spread); ground fire (burns deep in soil organic matter, with slow spread); and crown fire (burns through crowns of trees and shrubs, with rapid spread) (DeBano *et al*., 1998).
Fire interval	The time lag between two successive fire events in a given area (DeBano *et al*., 1998).
Fire period	The average time between successive fires in a given area. It is a property of all the fires of the area, considering a long time (Fox and Fox, 1987).
Fire frequency	The actual number of fires that occur in a given area, during a given time (DeBano *et al*., 1998).
Fire severity	A measure of the fire impact on ecosystems (Bond and Keeley, 2005). The degree to which a site has been altered by fire or the magnitude of disruption caused by fire on successional processes (DeBano *et al*., 1998).

Fire effects on plants occur on several scales (Fig. 2) and can be direct and/or indirect. The former are due to the heating or combustion of living structures, while the latter are mediated by fire-induced changes in soil and water (Neary *et al.*, 2008). Biological tissues can be either injured or destroyed by fire. Extensive injuries can kill organisms. Population demographic structure and spatial distribution can be affected, by mortality and changes in reproduction opportunities. As the impact fire is not the same in the different species, community composition is also affected. Fire triggers secondary succession in ecosystems (Ashton, 1981). Fire can even affect the distribution of biomes on Earth (Bond and Keeley, 2005). Nevertheless, little is known about the interactions among these scales and how emergent properties of ecosystems are generated (Moritz *et al.*, 2005).

The effects of fire on ecosystems derive, not only from discrete events, but also from the fire regime (Box 2). Firstly, the timing of fire is very important. It concerns both fire interval and fire season. More specifically, the way cyclic patterns of fire are matched (or not) with life cycles and phenological cycles of plants is critical for the persistence of the respective species, in recurrently burnt areas. If fire events have a seasonal pattern and/or another type of fairly regular frequency, some life-cycle lengths and phenological cycles may be more advantageous than others (Malanson, 1987; Gill and Allan, 2008). The same may occur with different regenerative strategies (vegetative or seminal) (Gill and Allan, 2008; Pausas and Keeley, 2014). Fire frequency is selective for life-history traits (Malanson, 1987), as age at first reproduction, number of reproductive events, and senescence. Fire season affects the timing of growth and

reproduction during the year (Malanson, 1987). Secondly, fire extent and patchiness are relevant when fire-affected organisms are not able to regenerate and the species presence in the burnt area depends on the external sources of propagules. Those species have to recolonize the burnt area from its edges to its centre or from islands of vegetation left unburnt. The dispersal abilities of plant species are very important, especially if the fire was extensive and left few green patches (Gill and Allan, 2008). Hence, fire extent is selective for dispersal (Malanson, 1987). Finally, fire intensity is selective for protective and escape mechanisms (Malanson, 1987), so species can keep their critical organs alive in order to regenerate after fire. In this context, buds and seeds are the most valuable organs, as plant regeneration depends on them. Fire is therefore a selective agent and different fire regimes select for different combinations of plant traits.

2.2. Plant adaptations to fire regimes

Several plant traits and strategies are common in fire-prone ecosystems. These traits are often related to the protection of critical tissues and life stages, as well as to the post-fire regeneration. The most common structures are: thick heat-insulating bark; plant architecture that keeps aerial apical buds away from heat; latent buds (buried or bark-protected); carbohydrate storage structures (*e. g.* lignotubers); serotinous fruits or cones; dehydrated or hard seeds; and persistent seed bank (Trabaud, 1987; Whelan, 1995; DeBano *et al.*, 1998). In what concerns post-fire regeneration processes, the most common are sexual reproduction and resprouting from epicormic or subterranean buds. After fire, some species depend exclusively on one of these regeneration modes (obligate seeders and obligate resprouters), while others have both (facultative seeders) (Pausas and Keeley, 2014). Resprouting is a general response to damage on the aerial parts of plants. When apical buds are destroyed and stop producing the hormones responsible for apical dominance, dormant buds are released from hormonal constraints and start producing new shoots (Pyne *et al.*, 1996). In addition, some plant species are able to use cues produced by fire as triggers of certain stages of sexual reproduction, namely: flowering; seed release and dispersal; and germination (Keeley and Fortheringham, 2000). That being so, they are able to synchronize massive recruitment events with post-fire conditions. Another strategy that exists in some species is flammability. These species have biochemical and structural traits that enhance flammability, and benefit from post-fire environment for recruitment (Bond and Midgley, 1995). Hence, when fire occurs, it burns the individuals of the most flammable species and the surrounding vegetation, creating the conditions for offspring of the former and eliminating the competitor species (Bond and Midgley, 1995).

The different combinations of these traits are syndromes, which consubstantiate strategies of tolerance, resistance, or escape to the stress induced by fire (Box 3). They allow the plant species which existed in a site before fire to persist there subsequently, conferring resilience to the ecosystem in the face of the fire (Box 3). While some species have several of those traits, some are mutually exclusive, thus, no species combines all of them (Trabaud,

1987). Moreover, the combinations of traits differ from one species to another, enabling the occupation of different niches and avoiding intraspecific competition (Malanson, 1987). The interpretation of the adaptive significance of these traits should take into account both the life cycle of each species and the fire regime the respective population is submitted to, in a given site (Trabaud, 1987). It is important to highlight that adaptation is a property of the species rather than a property of the traits. Moreover, it is not a matter of whether or not the species are adapted to fire, but rather if the species are adapted to the particular fire regimes of the sites they inhabit (Gill, 1975).

Notably, some of the aforementioned plant traits might have resulted from the selective action of ecological factors other than fire. These traits may have adaptive value for more than one disturbance type, thus, some of those adaptations to other disturbances might function as pre-adaptations for a determined fire regime. Most of the plant strategies or syndromes observed in the fire-prone environments involve trade-offs between traits of resource gathering and traits of regeneration (Malanson, 1987). When species are transported beyond their native range, they may be either adapted or non-adapted to the fire regime they encounter in the arrival territory. Moreover, whenever the fire regime in this territory changes, exotic species may be more or less adapted to the new regime than the natives. The former case eventually happens, when changes in the exotic territory turn it very similar to the environment in which the exotic species have evolved.

Box 3 – Key concepts concerning the behaviour of biological systems in the face of disturbance

Resistance	The ability of a biological system to reduce the amount of damage which can be caused by disturbance (Kover and Schaal, 2002; Leimu and Koricheva, 2006). The ability of a biological system to prevent the displacement of its own internal balance by disturbance stress (Begon *et al.*, 2009). It is frequently achieved through the existence of protective traits (structural or chemical) that reduce the contact of the stressful agent with the inner parts of the biosystem (DeBano *et al.*, 1998; Kover and Schaal, 2002; Leimu and Koricheva, 2006)
Tolerance	The ability a biological system to withstand disturbance (DeBano *et al.*, 1998), without significant adverse effect, thus keeping its normal functions (Fitter and Hay, 2002). It is the way of reducing the impact of disturbance on fitness (Kover and Schaal, 2002; Leimu and Koricheva, 2006). It is often achieved by the possession of alternative biochemical composition or metabolic pathways (Whelan, 1995; DeBano *et al.*, 1998; Fitter and Hay, 2002).
Resilience	The degree, manner, and pace in which a biological system recovers its properties after a disturbance (Westman, 1986). The speed with which a biological system returns to its former state, after it has been perturbed and displaced from that state (Begon *et al.*, 2009).
Escape	The ability of a biological system to avoid stress caused by disturbance, either in space or time (Fitter and Hay, 2002). It is often achieved by matching an inactive phase of the life cycle with the stressful period (Fitter and Hay, 2002). Some plants also can escape in spatial terms, because their life forms have the most sensitive structures distant from disturbing factors, while others do it by long-distance dispersal of their propagules (Whelan, 1995).

2.3. Fire and humans

Although fire timing and behaviour are highly dependent on natural conditions such as weather, topography, and fuel; anthropic factors may also determine when, where, and how fire burns (Flannigan *et al.*, 2009). Humans are able to ignite fuel and control fire by their own means. Ever since mankind learnt how to produce and use fire during the Palaeolithic Age, substantial changes have occurred in fire regimes of regions inhabited or frequented by humans (Mount, 1969; Naveh, 1975; Pausas and Keeley, 2009; Archibald *et al.*, 2012). Major changes in human societies have brought changes to fire regimes, namely in those transitioning from hunting and gathering societies to agricultural-grazing societies and then to industrial societies (Pausas and Keeley, 2009). Currently, the majority of fires happening on a global level are caused by humans (Flannigan *et al.*, 2009). Nevertheless, natural factors are still important.

2.4. Fire in the Mediterranean Basin

The Mediterranean Basin has a Mediterranean-type climate, which is characterized by: cool rainy winters, mild springs and autumns, and hot dry summers (Naveh, 1975). These conditions are favourable to plant growth during most of the year. The high primary productivity of Mediterranean ecosystems results in large amounts of fuel. This fuel becomes very flammable due to the summer drought. In addition, this region has been densely populated by people who have been using fire for several millennia (Naveh, 1975; Pausas *et al.*, 2008). Thus, fires have been occurring quite frequently in this region for a very long period of time. Working as a selective agent, fire has contributed not only to the genetic diversity of species, but also to the composition and structure of Mediterranean communities and niche differentiation (Naveh, 1975). More recently, industrialization led many people to move from the countryside to the city. The consequent reduction of agriculture and livestock grazing, coupled with the establishment of tree plantations, led to an accumulation of fuel in many areas of Mediterranean Europe (Pausas and Keeley, 2009). The development of urban areas which spreads into wildland areas has also increased accidental and arson ignitions (Pausas and Keeley, 2009). Nowadays, the vast majority of fires that occur in European countries are caused by humans (Pereira *et al.*, 2006; JRC, 2017). The high amounts of fuel and frequent ignitions are resulting in large, catastrophic wildfires in Mediterranean Europe (Pausas, 2004; Pausas and Keeley, 2009). This change in the fire regime is occurring very quickly, preventing species from adapting to the new conditions. Hence, this change can have a major impact on plant communities (Díaz-Delgado *et al.*, 2002; Pausas *et al.*, 2008).

The Mediterranean Basin has one of the highest fire frequencies in Europe and it is the European region with the largest proportion of burnt forest (Fig. 3) (FAO, 2010). From 1980 to 2016, Portugal, Spain, Italy, France, and Greece accounted for nearly 4×10^6 wildfires, 38% of which occurred in Portugal, the smallest of the aforementioned countries (JRC, 2017). The annual burnt area in these countries has a large interannual variation (JRC, 2017), which is somehow related to the interannual variation of precipitation, a characteristic of Mediterranean climate (Pausas, 2004).

2.4.1. The case of Portugal's mainland

Despite the large interannual variation of burnt area, evident trends have been observed in Portugal's mainland. The yearly burnt area mean has clearly increased in this country between the 1980–89 and 2000–09 decades (Pereira *et al.*, 2006; JRC, 2017); whereas the 2000–09 decade and the current one have similar yearly means up to now (DGAPPF, 2017; JRC, 2017). During the period 1998–2007, Portugal attained the highest proportion of yearly burnt forest in Europe, surpassing 0.9% in all the regions of its mainland (Fig. 3) (FAO, 2010). Moreover, some large wildfires (> 100 ha) have been responsible for a significant portion of that burnt area (Pereira *et al.*, 2006; Tedim *et al.*, 2013). Despite the reafforestation effort that has been made, wildfires have been responsible for the decrease in forest area in this country, since the 1990s, in contrast to the other Mediterranean European countries (Oliveira *et al.*, 2017). Economically, wildfires have caused very significant losses in Portugal. For instance, yearly financial losses caused by wildfires ranged 20–80% of the yearly richness produced by Portuguese forests, in the 2000–2004 period (Presidência do Conselho de Ministros, 2015). Therefore, fire regime in Portugal is changing and as such is causing serious ecological and economic issues.

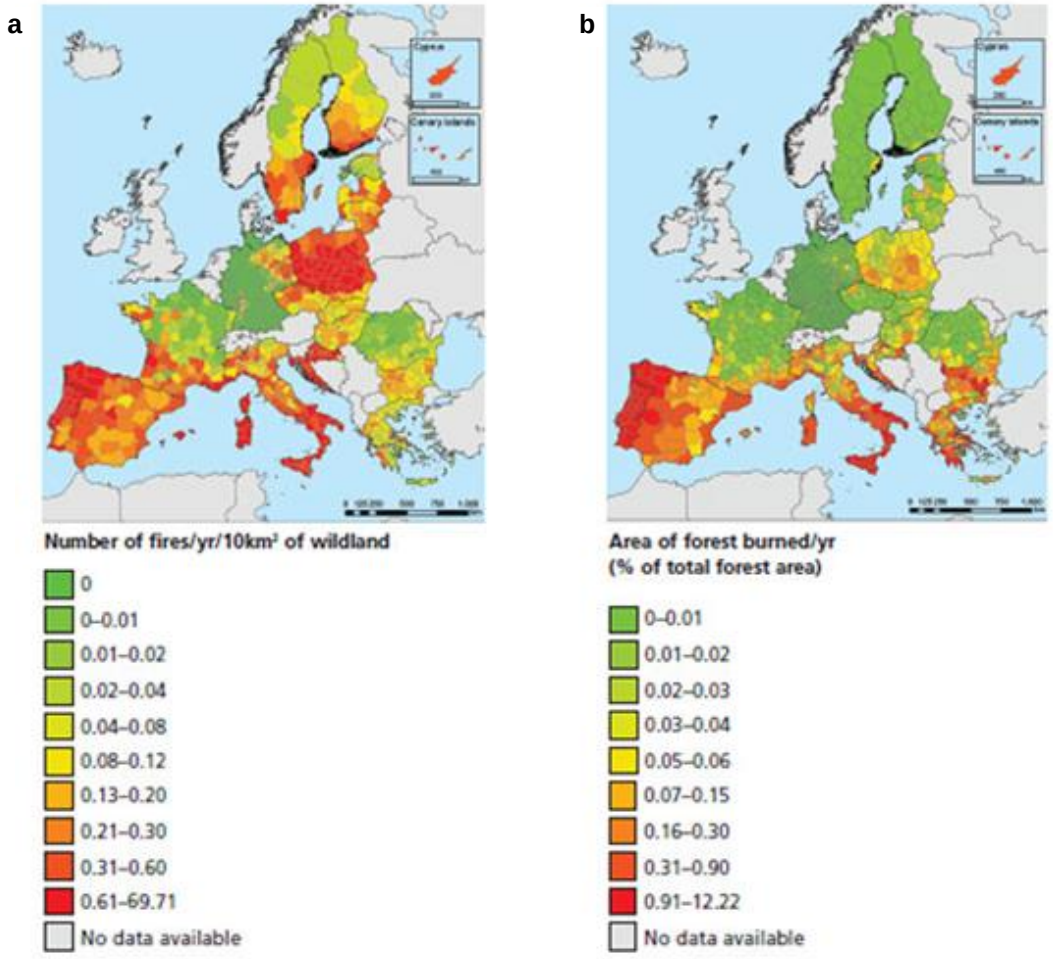


Fig. 3 – Geographical distribution of forest fires in Europe, in the period 1998-2007. **a.** Average forest fire density. **b.** Average burnt forest fraction (from FAO, 2010).

The most common forest types in mainland Portugal are stands dominated by either *Pinus pinaster* (native), *Eucalyptus globulus* (exotic), or *Quercus suber* (native) (ICNF, 2013). The geographical distributions of *P. pinaster* and *E. globulus* are largely overlapping (ICNF, 2013). Forests dominated by either of them include some of the most fire-prone forest types in Portugal (Silva et al., 2009), however, these two species have different regeneration strategies (Kirkpatrick, 1975b; Nicolle, 2006; Fernandes and Rigolot, 2007). These species are often involved in post-fire land-use transitions in Portugal, some related to land abandonment and others related to active management (Silva *et al.*, 2011; Oliveira *et al.*, 2017). Recently, the two species have interchanged their position in the abundance rank in Portugal(DGF, 2001; ICNF, 2013), and wildfires have contributed for this interchange (Silva *et al.*, 2011; Oliveira *et al.*, 2017).

2.5. Fire and climate changes

The current climate change scenario induced by human activity is having profound effects on forest ecosystems (Boisvenue and Running, 2006; Kirilenko and Sedjo, 2007; Lindner *et al.*, 2010). As plants are unable to step away from the place where they were recruited, they are extremely affected by changes in both CO_2 levels and regimes of precipitation and temperature (Boisvenue and Running, 2006; Kirilenko and Sedjo, 2007; Lindner *et al.*, 2010). Moreover, climate changes are causing alterations in fire regimes. The favourable climatic conditions for fire occurrence, such as drought and high temperatures, are changing with regards to timing, frequency, duration, and intensity, in many regions around the world (Kirilenko and Sedjo, 2007; Flannigan *et al.*, 2009). Predictions indicate a global increase in fire occurrence and burnt area with large spatial variability, as a result of climatic changes that are ongoing (Flannigan *et al.*, 2009). As far as forestry is concerned, the climate change-induced modifications on the frequency and intensity of wildfires and other extreme events may outweigh the direct effects of elevated temperatures and CO_2 (Dale *et al.*, 2001; Kirilenko and Sedjo, 2007). Furthermore, fire regimes may respond immediately to climate changes, and their effects on vegetation may be greater and faster than the direct effects of climate changes on species distribution (Dale *et al.*, 2001). Thus, the changes in climate and fire regimes are particularly important for plant species distribution and, consequently, to the composition of terrestrial communities, including forests.

2.5.1. Mediterranean Basin and Portugal

The climate of the Mediterranean Basin is predicted to change in the upcoming decades. On the one hand, mean temperatures are expected to rise, especially in the summer (4–5ºC) (Lindner *et al.*, 2010). While, on the other hand, annual precipitation rainfall is expected to decrease to 80% of its current value, with a drastic decrease to 50% in the summer (Lindner *et al.*, 2010). Thus, the risk of fire occurrence will generally increase. This increase will have several consequences, namely: higher frequency of years with fire risk; longer fire season; more extreme events (highest daily fire weather index (FWI); more days with FWI >45; more

persistent episodes of FWI >45) (Moriondo *et al.*, 2006). These climate and fire predictions are in line with the changes that were observed in eastern Spain during the 20th century (Pausas, 2004).

A broad study concerning climate changes in Portugal, by Santos *et al.* (2002), generally supports the aforementioned predictions for the Mediterranean Basin. The changes that are likely to occur for the Portuguese mainland are: (1) an intense increase in maximum summer temperature anomaly; (2) a significant increase in the frequency of daily maximum temperatures above 25ºC, including spring and autumn; (3) a large increase in the frequency of daily maximum temperatures above 35ºC and in the duration of hot spells; and (4) reduced precipitation in spring, summer, and autumn; (5) and an extended drought season from May to October. In addition, the authors' predictions for future fire regime indicate: (1) a significant increase in fire severity in that territory, with the enlargement of temporal variations of daily severity ratio (DSR); and (2) a relatively large increase in DSR from mid-May to, at least, mid-October, which will prolong the fire season. Their calculations of fire return intervals, from the 1990s, indicated that land cover changes related to degradation of ecosystems and ecological regression were already in progress at that time. These ideas by Santos et (2002) were confirmed by Oliveira *et al.* (2017).

3. *Eucalyptus globulus* Labill.

3.1. Taxonomic note

The term 'eucalypt' is commonly used to name approximately 900 species from three closely-related genera: *Eucalyptus, Corymbia*, and *Angophora* (Slee *et al.*, 2006). The first of these genera comprises the vast majority of those species.

The current study is about one species of the genus *Eucalyptus.* The researcher has decided to follow the classification presented by Brooker (2000), as the taxonomical framework for this species:

Species: *Eucalyptus globulus* Labill., 1800
Subseries: Euglobulares Blakely, 1934
Series: Globulares Blakely, 1934
Subsection: Euryotae Brooker, 2000
Section: Maidenaria Pryor & Johnson, 1971
Subgenus: Symphyomyrtus Schauer, 1844
Genus: *Eucalyptus* L'Her., 1879

Kirkpatrick (1974) proposed to convert four formerly described species into subspecies of a single species (*E. globulus*). Those former species were *E. globulus* Labill., *E. pseudoglobulus* Naudin ex Maiden, *E. maidenii* F. Muell., and *E. bicostata* Maiden, Blakely & Simmonds. This proposal was based on the occurrence of geographically separated core populations of the four taxa, which were differentiated primarily on reproductive traits. Although,

some authors still follow Kirkpatrick's classification, the researcher opted for Brooker's classification. With regards to the focus group of plants, the latter classification is a more recent perspective and corresponds to the classic classification. Thus, it was decided that the precedence rule of botanical nomenclature would be followed.

3.2. Geography, history, and forestry

3.2.1. From Australia to overseas

Eucalyptus globulus Labill. is native to SE Australia (Tasmania, Bass Strait islands, and adjacent coastal area of Victoria) (Kirkpatrick, 1975b) (Fig. 4). It was discovered by the Europeans in 1792, and described by Labillardiére in 1800. Within a short period of time, it was present all over the world (Potts *et al*., 2004). The first eucalypt seed supply that arrived in Europe, around 1804, belonged to this species (Penfold and Willis, 1961). In fact, as it was the first eucalypt species to become widely known, it became synonymous with 'eucalypt' for the general public at some point in time (Jacobs, 1979). In 2008, it was probably the most cultivated hardwood species in the world, occupying 2.3 x 10^6 ha (Simberloff and Rejmánek, 2011). This species is used for several environmental purposes (*e. g.* windbreaks), and to produce raw materials for different industries in the introduction territories (Jacobs, 1979). Its wood is used for producing pulp, railway sleepers, construction, and other purposes (Jacobs, 1979). Its leaves are the primary source of eucalypt essential oil, cineol (Jacobs, 1979). Its use for reafforestation purposes outside Australia had its first impetus in the second half of the 19th century, in the Mediterranean Basin (Radich, 1994). This use was mainly promoted by Prosper

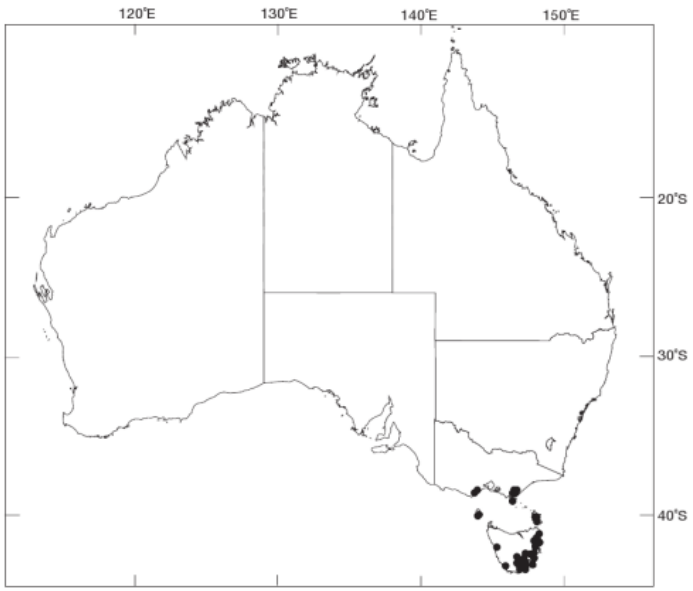


Fig. 4 – Geographical distribution of *E. globulus* Labill. within its native range (black dots in SE Australia, Tasmania, and adjacent islands) (from Boland *et al.*, 2006).

Ramel, and encouraged by Ferdinand von Mueller (von Mueller, 1879-1884; Radich, 1994). Interest in the species has been on the rise ever since, especially due to its excellent productivity and fibre characteristics, which allow high-yield production of high-quality pulp (Goes, 1977; Volker *et al.*, 1994). The recent exponential expansion of this species worldwide is precisely due to the needs of the pulp and paper markets (Potts *et al*., 2004). Nowadays, this species is naturalized in several regions of the world and has been classified as an invader in areas such as northern Spain, California (USA), and the south of Australia (Kirkpatrick, 1977; Sanz-Elorza *et al.*, 2004; Larcombe *et al.*, 2013; Wolf and DiTomaso, 2017).

3.2.2. Arrival and expansion in Portugal

Although *Eucalyptus globulus* was introduced in Portugal in the 19th century, some authors do not agree with the exact year. Almeida (1918, *apud* Goes, 1962) wrote that eucalypts were introduced in 1929, but species' names were not detailed. Pimentel (1876) explicitly stated that *E. globulus* was introduced in Portugal in 1852. Coutinho (1886-87) supported that idea by stating that this species had been cultivated in Portugal since *ca.* 1886. This species was first cultivated by plant collectors, but was soon deemed suitable for afforestation (Radich, 1994). In the 1860s, afforestation with this species was occurring in several different regions of mainland Portugal (Pimentel, 1876). Coutinho (1886-87) stated in his forestry manual that it was already being 'cultivated on a large scale' for wood production, thus predicting a promising future. The uses and expansion of this species in Portugal followed a similar trend to other countries. Although, the first pulp factory was built in 1926 (Radich, 1994), this industry only gained momentum after political stimuli in the 1950s (Alves *et al.*, 2007). Since the mid-20th century, the huge development of the pulp industry coincided with the expansion of the *E. globulus* planted area in Portugal (Radich, 1994; Alves *et al.*, 2007). Nowadays, its wood is almost exclusively used for pulp and paper production (DGRF, 2006; Presidência do Conselho de Ministros, 2015). In 2005/06, forests having *E. globulus* as their most abundant species were one of the three largest forest types (in area) in the Portuguese mainland (740 x 10^3 ha), amounting to 23% of forests there (AFN, 2010). In Spain, this species' expansion followed a similar pattern, occupying 325 x 10^3 ha, majorly in the north and northwest parts of the country (Federico *et al.*, 2008). Indeed, the western Iberian Peninsula is currently the region with the largest area of *E. globulus* in the world.

3.2.3. From cultivation to naturalization – the independence from humans, in Portugal

During the expansion of the *E. globulus* area in Portugal, plantations were established all over the mainland, but their productivity levels widely varied in the different regions (Tomé, 2000). Due to the economic importance this species has achieved, the current distribution of its plantations in the mainland has a closer correspondence to its ecophysiological requirements, enabling improvements in productivity (Alves *et al.*, 2012). The concentration of *E. globulus* stands in the best ecological regions for this species is a strategy that has been nationally

reinforced by the two most recent versions of the Portuguese 'National strategy for forests' (DGRF, 2006; Presidência do Conselho de Ministros, 2015).

In Portugal, nearly all *E. globulus* stands have artificial origins, the vast majority of which have been planted (Goes, 1977). These stands are managed in a coppice system whose rotations last between 10–12 years (Turnbull and Pryor, 1984; Soares *et al.*, 2007). Productivity at the best quality sites in Portugal can attain 30 m^3 ha^{-1} $year^{-1}$ (Tomé, 2000). The most limiting abiotic factors to the development of *E. globulus* individuals in Portugal are temperatures below 0ºC and drought (Almeida *et al.*, 1994; Ribeiro and Tomé, 2000; Alves *et al.*, 2012).

Eucalyptus globulus has a large genetic diversity, both on a wide geographical scale and on local scale, which can be used for selection aimed at afforestation (Potts *et al.*, 2004). The Portuguese landrace has a broad provenance origin (Potts *et al*., 2004). This landrace results not only from genetic drift associated with the introduction process (Borralho *et al.*, 2007), but also from subsequent natural selection (Potts *et al*., 2004), and artificial selection (Lopez *et al.*, 2001; Potts *et al.*, 2004). Formal breeding programmes began in Portugal in 1966 (Potts *et al*., 2004). The high genetic diversity of the Portuguese landrace is comparable to that of native landraces (Borralho *et al*., 2007). For instance, its performance, in terms of survivorship, growth, or frost tolerance, ranks across the full distribution of native localities (Almeida *et al.*, 1995; Lopez *et al.*, 2001).

The first reference to *E. globulus* naturalization in Portugal dates back to 1943 (Almeida and Freitas, 2006). Coutinho (1886/87) states that the species was able to produce fertile seeds in Portugal. Goes (1962) notes that, after coppicing, *E. globulus* wildlings were common in sandy-soil plantations, in central and northern Portugal, where rainfall is above 600 mm. He further reports the creation of some new plantations through the transplantation of those seedlings, especially in years in which spring was rainy. As he considered that these cases of success were merely circumstantial, he advised against this procedure and direct sowing. Nevertheless, rural inhabitants have been collecting *E. globulus* seeds for sowing and have successfully managed natural regeneration (transplantation, thinning), in several Portuguese regions, for decades. Despite all the evidence that naturalization could be occurring in mainland Portugal, the phenomenon was neglected by the scientific community for years, as it was considered of little use. Recently, there have been further reports of the occurrence of wildlings, not only inside stands, but also in their vicinity (Marchante *et al.*, 2008; Silva and Marchante, 2012).

These facts seem to corroborate the ideas of several authors. If an exotic species has a long residence time, combined with large cultivated areas in a region, the propagule pressure may become large enough to enable frequent natural regeneration, when other environmental conditions are not limiting (Pyšek *et al*., 2009; Simberloff, 2009; Wilson *et al*., 2009). Cultivation protects plants from environmental stochasticity (Mack, 2000). Artificial selection for forestry purposes often chooses plant material which is able to succeed in the field with little or no human support (Richardson and Rejmánek, 2011; Dodet and Collet, 2012). Therefore, introduction history and cultivation are likely drivers of spontaneous seminal regeneration of *E. globulus* in Portugal.

3.3. Reproduction

3.3.1. From flower to seed

As *E. globulus* is an economically important species, some facts about its reproduction are well known (Goes, 1977; Turnbull and Pryor, 1984; ISTA, 2003; Krugman and Whitesell, 2008). This species can regenerate both vegetatively and sexually (Nicolle, 2006; Kirkpatrick, 1975a; Kirkpatrick, 1975b). However, the production of offspring relies on seeds alone. Individuals start to produce seeds when they are 4–7 years old (Kirkpatrick, 1975b; Turnbull and Pryor, 1984; Jordan *et al.*, 1999). The development from flower bud emergence to ripe fruit takes between 1.3–2 years (Goes, 1977). In Portugal, *E. globulus* trees are in bloom between October and July, and their fruits dehisce between October and March (Goes, 1977). The fruits are woody capsules which usually bear 5–50 fertile seeds, besides chaff (Goes, 1977), but capsules from mother trees of orchards may bear up to 100 fertile seeds (L. Leal, personal communication). Fertile seeds tend to be at the bottom of capsules, while chaff tends to be at the top (Boland *et al.*, 1980). Together they completely fill capsule locules when they are ripe (Boland *et al*., 1980).

Eucalyptus globulus fertile seeds of are irregularly shaped and tooth-edged, and their coat is reticulated and black or dark-brown (Boland *et al.*, 1980; Krugman and Whitesell, 2008). Their shape is due to the growth of many seeds inside the limited space of locules. Moreover, these seeds are small (length: 1.25–2.5 mm; width: 1.0–1.75 mm) (Krugman and Whitesell, 2008) and light (mean mass: 1.2–3.2 mg) (Kirkpatrick, 1975a). Seed size variability is associated to fruit size (Kirkpatrick, 1975a). *Eucalyptus globulus* seeds are not adapted to any specific type of dispersal (Calviño-Cancela and Rubido-Bará, 2013).

3.3.2. Seed dispersal

Eucalyptus globulus capsules may dehisce either before or after their abscission, so seeds can be released from a tree's canopy either on their own (Cremer, 1965b) or inside fruits (Calviño-Cancela and Rubido-Bará, 2013). The main disperser of free seeds seems to be the wind (Cremer, 1977). However, it usually does not transport these seeds very far, as eucalypt seeds have no flight-adapted structures (Booth, 2017). Free individual seeds of this species would travel no farther than the horizontal distance equivalent to half of their initial height in the canopy, under winds of 10 km h^{-1} (Cremer, 1977). Surveys in the surrounding of *E. globulus* stands corroborate this finding, as the distance between wildlings and the putative mother trees was, in the vast majority of cases, no longer than 15 m (Calviño-Cancela and Rubido-Bará, 2013; Larcombe et al., 2013; Fernandes *et al.*, 2016). However, the very same surveys have occasionally detected wildlings at much longer distances (up to 175 m). Seed dispersal at those distances could be caused by gales or other dispersal mechanisms, such as water streams or wildfire updrafts (Jacobs, 1955; Kirkpatrick, 1975b; Williams and Potts, 1996; Gill, 1997; Booth, 2017).

3.3.3. Germination

Seeds of *Eucalyptus* species tend to germinate as soon as they have the appropriate environmental conditions, which usually happens within a one-year period in nature (Jacobs, 1955; Penfold and Willis, 1961). Germination is highly dependent on water availability: substrate water-potential below -0.05 MPa greatly reduces germination capacity and, below -0.25 MPa, it completely prevents germination (López *et al.*, 2000). The optimal temperature range for germination of *E. globulus* seeds is 24.8–25.5ºC (Boland *et al.*, 1980; ISTA, 2003; Rix *et al.*, 2011). In laboratory, under optimal conditions, more than 95% of these seeds germinate within 4–15 days (Boland *et al.*, 1980; ISTA, 2003; Calviño-Cancela and Rubido-Bará, 2013). Primary dormancy of seeds is only known in a few *Eucalyptus* species (Florence, 1996), and *E. globulus* is not one of them (Nair, 2006). Nevertheless, Whelan (1995) reminds us that the absence of dormancy under laboratorial conditions does not mean the same happens in nature. Indeed, Turnbull and Pryor (1984) recommend cold-moist stratification to achieve faster and more uniform germination of *E. globulus* seedlots. Nair *et al.* (2007) also found that germination delays observed in nurseries may be related to secondary dormancy, induced by soak and dry-back treatments, which can be broken in the dark. Actually, in nature, seeds can take up to four months to germinate, depending on the season they hit the soil (Calviño-Cancela and Rubido-Bará, 2013). In addition, germination might be hampered in soils having phytotoxic substances. For instance, *E. globulus* leaf litter has several of those substances (del Moral and Muller, 1969; Molina *et al.*, 1991; Souto *et al.*, 1994; Babu and Kandasamy, 1997), which may persist for months in both litter and soil (Souto *et al.*, 1994). Therefore, although *E. globulus* seeds have no primary dormancy, their germination in the field spreads over a long period of time.

Information regarding *E. globulus* demography is less abundant than information about its reproduction. The strategy of eucalypt reproduction generally consists of the production of very many seeds, which are simply released and reach the soil (Booth, 2017). Large *E. globulus* trees can produce 280–350 g of seeds per crop, which may correspond to $11.2 \times 10^3 – 14 \times 10^3$ seeds, according to estimates of 4.0×10^5 seeds kg^{-1} (Goes, 1977). However, seed production varies both in space and time. Seed production largely differs among individual eucalypts (Penfold and Willis, 1961). For instance, *E. globulus* individuals from stand borders tend to produce more flowers and fruits than those located deep inside the stands (Barbour *et al.*, 2008). This interindividual variability contributes to spatial heterogeneity of seedling recruitment. In addition, seed production has interannual variability. Mast years of *E. globulus* occur in 3–5 year cycles (Turnbull and Pryor, 1984). This species produces serotinous capsules (Tng *et al.*, 2012), which might be a valuable seed reservoir under otherwise seed scarcity conditions (Lamont *et al.*, 1991). Although patterns and mechanisms of seed release from serotinous fruits/cones are well-documented for many woody species and are often related to ageing and fire (Lamont *et al.*, 1991), there is no information regarding this species in particular. Seed longevity depends on environmental conditions. *Eucalyptus globulus* seedlots keep 81% of their original germinability, after a 10-year storage, under mild (18–22ºC) and dry conditions (Gunn, 2001). However, safe-site availability also influences the spatial distribution of eucalypt

recruitment (Battaglia and Reid, 1993; Bailey *et al.*, 2012). As temperature and moisture conditions are constantly changing in the field, free *Eucalyptus* seeds do not stay viable in soil for more than 6–12 months (Florence, 1996). Moreover, seed predators, mostly ants, are able to severely restrain *Eucalyptus* plant recruitment in Australia (Wellington and Noble, 1985b; Andersen, 1987; Stoneman, 1994; Yates *et al.*, 1995). Several ant species and one small-rodent species were recently observed transporting and eating *E. globulus* seeds to the detriment of morphologically similar native seeds, in Portuguese forests (Deus *et al.*, 2018). However, the magnitude of this predation is still unknown. The temporal variability of seed availability resultant from all these factors may affect birth rate along time. In the end, *E. globulus* seedlings emerge from less than 5% of the seeds that reach the soil in several habitats, and the emergence probability varies from one habitat to another and among sowing seasons (data from NW Spain) (Calviño-Cancela and Rubido-Bará, 2013). Therefore, from the large amounts of seeds produced by adult populations of *E. globulus*, only a small fraction germinate, and the germination is uneven in both space andtime.

3.4. Growth and survival

3.4.1. Seedlings and their environment

Eucalyptus seedlings are very sensitive to several environmental factors (Florence, 1996). Safe-site availability also influences establishment inside forests (Battaglia and Reid, 1993; Bailey *et al.*, 2012). Under favourable field conditions, 7% of seeds released by adult *E. globulus* may become established plants; but under normal field conditions 0.1% is a common outcome for *Eucalyptus* species (Jacobs, 1955).

Drought can be the cause of high mortality rates in young *Eucalyptus* cohorts, especially in their first summer (Jacobs, 1955; Stoneman *et al.*, 1994). This mortality was even observed in *E. globulus* in Galicia (Spain), where the summer is mild (mean temperature: 23.5ºC) and slightly moist (minimum monthly precipitation: 20 mm). In Galicia, mortality in summer was higher among the youngest seedlings which emerged in the previous spring or in that summer, than among the eldest seedlings which emerged in the previous autumn or winter (Calviño-Cancela and Rubido-Bará, 2013). Water scarcity hampers the growth of *E. globulus* individuals. Pita and Pardos (2001) observed that a soil water potential (ψ_m) lower than -1.0 MPa reduces plant growth. Osório *et al.* (1998) also observed reduced growth in plants with pre-dawn water potential at -0.970 MPa, or less. Drought also hinders *E. globulus* plant establishment when ψ_m is -1.52 MPa or lower (González-Muñoz *et al.*, 2011). *Eucalyptus globulus* does not tolerate severe drought, especially if soils are shallow (Turnbull and Pryor, 1984). In its native range, annual rainfall varies from 500-2600 mm (Jovanovic and Booth, 2002). The recommended annual rainfall for commercial plantations is 600–1500 mm (Jovanovic and Booth, 2002). In regions where the rainfall is close to the lower limit, appropriate water supply must be warranted. This can be achieved either by uniform precipitation over the year or by facilitated water supply to plants, whether by topographic or edaphic conditions

(Kirkpatrick, 1975b; Jacobs, 1979). Notably, drought-resistant genotypes existent in Portugal and Spain resist drier conditions than those from the native range (Cromer, 1975 *apud* Turnbull and Pryor, 1984).

Conversely, water excess may also limit the occurrence of *E. globulus.* The species has failed to subsist in excessively humid areas, and it has been dropped from cultivation in montane sites of tropical regions whose mean annual rainfall is higher than 2000 mm (Jacobs, 1979). This species has also not been observed growing naturally in ill-drained soils in Australia. Nevertheless, it has been grown from seedlings in ill-drained areas in Italy (Kirkpatrick, 1975b). In addition spontaneous saplings have also been observed in some ill-drained spots, inside plantations, in central Portugal (personal observation). Flooding is known to induce reduced growth, leaf epinasty and the development of adventitious roots in *E. globulus* (Gomes and Kozlowski, 1980).

In its native range, the species occurs in sites where summers vary from mild to warm (mean maxima of warmest month: 13–25ºC); and winters vary from cool to mild (mean minima of the coldest month: -1–8ºC) (Jovanovic and Booth 2002). Temperature is also crucial for plant development. The optimal temperature range for the development of normal *E. globulus* seedlings is 21.2–24.8°C; while cardinal temperatures are 9.1–10.5°C, at the lower limit, and 40.3–41.4°C, at the upper limit. Winter frost is an important menace to *Eucalyptus* seedlings, just as summer drought is (Stoneman, 1994). Boland *et al.* (2006) refer that the species does not tolerate over 50 days of frost a year at high altitudes. While frost is particularly damaging to *E. globulus* seedlings and sprouts under the age of 1–2 years, larger plants are moderately resistant to light frosts (Jacobs, 1979). The median lethal temperature (LT_{50}) ranges between -5.6ºC and -5ºC for unhardened *E. globulus* juveniles (Kirkpatrick, 1975a; Almeida *et al.*, 1994), and between -8.47ºC and -7.8ºC if plants are hardened (Almeida *et al.*, 1994; Moraga *et al.*, 2006). Plants tolerate extracellular frost (Almeida, 1993), while they resist intracellular freezing through supercooling mechanisms, which involve high sugar concentrations inside cells (Almeida, 1993; Moraga *et al.*, 2006). Frost resistance increases with maturity, juvenile foliage being less resistant than mature foliage (Chen *et al.*, 1987 *apud* Skolmen and Ledig, 1990). In fact, mature trees are more able to survive to lower temperatures than juveniles are (Turnbull and Pryor, 1984). In addition, cold tolerance differs among provenances of this species (Almeida, 1993). These differences are related to the severity of frost at the different provenance regions (Kirkpatrick, 1975a; Turnbull and Pryor, 1984).

Browsing is a major factor in *Eucalyptus* seedling survival and growth (Wilkinson *et al.*, 1993; Florence, 1996). The majority of introductions of *Eucalyptus* spp. outside Australia used seeds, which reduced the chances of insect co-transportation into the introduction territories (Pryor, 1976). As an exotic species, *E. globulus* stood pest free in Portugal, for nearly 100 years. Neves (1950) reported what seemed to be the first pest attack to *E. globulus* in Portugal, performed by *Melolontha papposa*. Nowadays, this insect species and several others attack *E. globulus* juveniles in Portugal (*e. g. Gonipterus scutelattus* and *Ctenarytaina eucalypti*), nearly all of which were accidentally introduced from Australia (Branco, 2007).

Contrastingly, the species is safe from vertebrate browsers in Portugal, because it is unpalatable to livestock and its natural browsers from Australia do not exist in Portugal (Turnbull and Pryor, 1984). European rabbits browse this species in Australia and Chile, affecting seedling survival (Turnbull and Pryor, 1984; McArthur and Appleton, 2004; Becerra and Bustamante, 2008), however no reports of such behaviour exist in Portugal. Therefore, *E. globulus* growth and survival seem to be less affected by herbivory in Portugal than in Australia.

3.4.2. Establishment

The longer the juvenile eucalypts grow under favourable conditions, the larger their chances to survive adversities will be (Florence, 1996). This improvement in survival capacity was observed by Calviño-Cancela and Rubido-Bará (2013) in *E. globulus* seedlings under one year old. In addition, if these plants live long enough to develop lignotuber, they will become very resilient to shoot damage (Kirkpatrick, 1975a; Nicolle, 2006). Lignotuber has dormant buds and stores reserves, enabling the vegetative regeneration of shoot when it is damaged or destroyed (Kirkpatrick, 1975a; Nicolle, 2006). Moreover, eucalypts which already have a well-developed lignotuber are more tolerant to competition. They are able to persist in forest understorey for several years, until competition release occurs and they become fully developed trees (Florence, 1996). Finally, the spatial heterogeneity of environmental conditions may affect the persistence of several *Eucalyptus* species, even within sites. In fact, conditions of each individual's microsite are determinant for its survival (Bailey *et al.*, 2012).

3.5. Disturbance

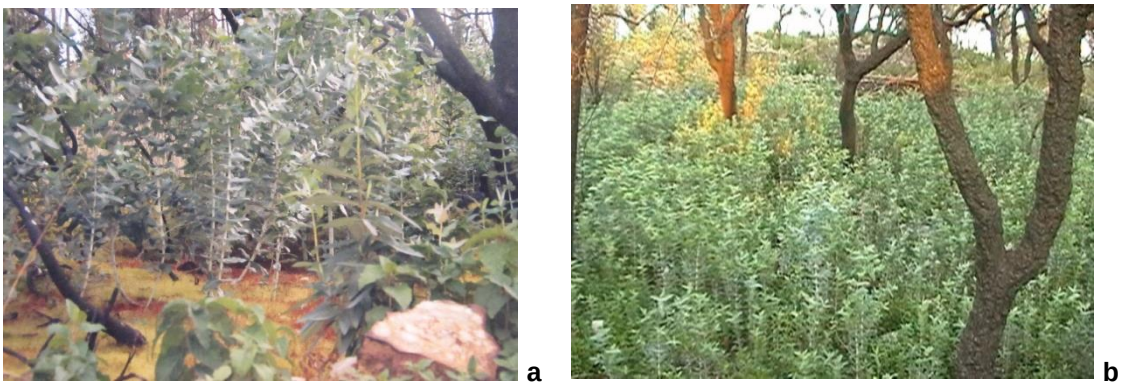
3.5.1. Mechanical disturbance

Undisturbed forest floor is generally a harsh substrate for the early development of *Eucalyptus* seedlings (Florence, 1996; Pennington *et al.*, 2001). *Eucalyptus globulus* seedlings tend to exist in sites where both vegetation and soil were disturbed (Virtue and Melland, 2003). This trend is also observed in other *Eucalyptus* species and is probably due to two aspects. Firstly, the absence of strong competitors in those sites is positive for *Eucalyptus* juveniles because they are very sensitive to competition (Jacobs, 1955; Florence, 1996). Namely, *E. globulus* usually regenerates from seeds only in the absence of overstorey, under full sunlight (Stoneman, 1994). Secondly, the mechanical disturbance of soil, derived from ploughing, reduces its bulk density and improves its aeration and water infiltration (Madeira *et al*., 1989; Florence, 1996). Moreover, this disturbance enhances the access of soil flora to organic compounds, which leads to the accelerated mineralization of nutrients (Florence, 1996). As a result, root development of *E. globulus* juveniles is facilitated (Madeira *et al*., 1989). Pennington and Churchill (2001) have observed larger germination and establishment rates in several species of *Eucalyptus*, including *E. globulus*, in tilled soils over untilled ones. These authors induced that such a result was a consequence of favourable conditions created by tillage, at the microsite level. Results from a field trial with *E. globulus*, in Portugal (Fernandes *et al.*, 2017),

are in agreement with that idea. In that trial, a smaller number of germinants and survivors were observed in forests and shrublands that had been left undisturbed, than in sites where understorey vegetation had been removed and/or soil scarified.

3.5.2. Fire

As an ecological factor, fire may be either favourable or unfavourable to eucalypts and has played an important role in the natural history of *Eucalyptus* genus (Pryor, 1976; Florence, 1996; Crisp *et al.*, 2011). As a result, many *Eucalyptus* species have traits with adaptive value in environments where fire is frequent, including some traits related to regeneration from seeds (Wilkinson *et al.*, 1993; Florence, 1996). 'Wheatfield' regeneration of some *Eucalyptus* species, which is common in burnt areas in Australia, may overpass 20,000 seedlings ha^{-1} and can even reach up to 900 seedlings m^{-2} in hollows where seeds were dragged into (Pryor, 1976; Florence, 1996; Ashton, 2000). These flushes of generation were also observed in *E. globulus* inside some burnt forests, in Portugal (personal observation, Fig. 5).



a **b**

Fig. 5 – Extreme densities *E. globulus* wildlings in two different stand types, as observed in the winter following the wildfire of September 2003, in Monchique (SW Portugal). **a.** *E. globulus* dominated stand with *Arbutus unedo* L.. **b.** *Quercus suber* L. dominated stand. (Photos are a courtesy of A. M. Águas)

Eucalypt forests are potentially very flammable for several reasons. Eucalypts produce high amounts of litter which accumulates on forest floors, in a loosely packed way (Florence, 1996). When fires are intense, fire spotting may occur, through the release of fire brands from burning trees (Florence, 1996). In addition, leaves of species as *E. globulus* are rich in essential oils and waxes (Jacobs, 1979; Li *et al.*, 1997), and the decomposition of their litter is slow (Ribeiro *et al.*, 2002). In this species, the bark of the stem has shedding strips and slabs, except in the base where slabs are persistent (Slee *et al.*, 2006). *Eucalyptus globulus* forests may be very flammable indeed, especially if fuel distribution enables fire spread (Silva *et al.*, 2009; Fernandes *et al.*, 2011). Nonetheless, most eucalypts are very tolerant to fire, as individuals, due to their capacity to resprout from basal or epicormic buds (Gill, 1997). This capacity has evolved in the *Eucalyptus* genus in the early Paleogen (62/60 My BP) (Crisp *et al.*, 2011). Moreover, fire can facilitate sexual reproduction of eucalypts (Florence, 1996). In post-fire environments, *E. globulus* is strategically a facultative resprouter (Catry *et al.*, 2013; Kirpatrick,

1975b, Nicolle, 2006). Some authors even argue that the persistence of *Eucalyptus* spp. in sites and the occurrence of fire in those sites are interdependent (Mount, 1964; Mount, 1969; Chambers and Attiwill, 1994). Kirkpatrick (1975b) also considers that perpetuation of *E. globulus* in sites is deeply influenced by fire regime. Nonetheless, other authors highlight that any interdependence between eucalypts and fire can only occur if life-cycle, fire regime and other environmental conditions are matched (Gill, 1997).

Fire is a facilitator of *Eucalyptus* regeneration from seeds for several reasons. On the one hand, it induces capsule dehiscence and, thereby, intensifies seed release (Cremer, 1965a; Pryor, 1976; O'Dowd and Gill, 1984; Wellington and Noble, 1985b; Florence, 1996), which allows seed predators satiation (O'Dowd and Gill, 1984; Wellington and Noble, 1985b; Gill, 1997). On the other hand, it also reduces the number of seed predators (Whelan, 1995). Both these effects result in more seeds available to undergo germination. Fire destroys and injures plants, thus eliminating or temporarily reducing the capacity of pre-fire plants to compete with regeneration from seeds (Wellington and Noble, 1985a; Whelan, 1995; Gill, 1997). As a result, light availability is increased (Jacobs, 1955; Kirkpatrick, 1975b; Gill, 1997) and water availability may also be increased (Gill, 1997) for survivors or newborn plants. Moreover, the combustion of organic matter changes soil´s chemical properties of. Phytotoxic substances may be destroyed or modified (Pryor, 1976; Stoneman, 1994; Florence, 1996), allowing for the development of plants that otherwise would be prevented or, at least, hampered. Nutrients are mineralized by combustion and, as a consequence, most become temporarily more available for plants (Knoepp *et al.*, 2005). The main mineral nutrients of *E. globulus* aerial biomass are Ca, K and N (Brañas *et al.*, 2000), while Ca and K dominate in *Eucalyptus* spp. ashes (Raison, 1979; Khanna *et al.*, 1994). Further changes in nutrient dynamics of soil are caused by the fire-induced changes in soil microbiota (Raison, 1979; Busse and DeBano, 2008). In 1960, Pryor, created the concept of 'ash-bed effect' (Pryor, 1963; Wilkinson *et al.*, 1993) to describe the growth enhancement observed in plants standing in soils which were previously heated above 150ºC (Willis, 1999). The ash-bed effect can be a direct result from the soil´s heating and the chemical composition of ashes; as well as consequence of the heat and the ash deposition on soil chemistry and community (Raison, 1979). This growth stimulation occurs during the early development of *Eucalyptus* individuals and enhances the likelihood their establishment (Cremer and Mount, 1965; Wilkinson *et al.*, 1993; Chambers and Attiwill, 1994; Ashton and Spalding, 2001). Thus, fire can facilitate *Eucalyptus* spp. seed release, germination and growth.

However, there is a reverse side of fire effects. Tissues are directly killed by fire when the temperature of living cells is raised to a lethal level (DeBano *et al*., 1998). The most critical structures for post-fire regeneration are sexual propagules and meristematic tissues.

The sexual regeneration capacity of *Eucalyptus* populations is immediately reduced when seeds or fruits are severely damaged by fire (Gill, 1997). The threshold of lethal temperatures for seeds of several Myrtaceae species is 90-100ºC (Judd, 1993). At least 90% of seeds are killed within that temperature range, and mortality steadily increases with temperature and exposure time, reaching 100% mortality when seeds are at 100ºC for 10 seconds (Judd,

1993). Fire reaches much higher temperatures, so free seeds are very susceptible. However, *Eucalyptus* seeds can be protected from heating. As *Eucalyptus* capsules are usually located below the leaves in the canopies, so that heat flux from burning leaves to capsules is minimized when leaves burn (Mount, 1969). In addition, *E. globulus* capsules are solitary, obconical to globoid, and relatively large (15–25 x 20–30 mm) (Goes, 1977). Studies regarding the insulation capacity of these capsules determined that they keep seeds viable inside when external air is 440ºC for up to four minutes (green capsules) (Webb, 1966 *apud* Ashton, 1981), or is 200ºC for up to two minutes (capsules collected a few days before) (Silva *et al.*, 2016). The varying results might have been due to capsule water content or to capsule size (unreported by these studies), which are related to their insulating capacity (Judd, 1994). Despite these differences, results from both studies are compatible with an effective protection of seed from heat inside fruits during wildfires, given the temperatures and residence times of fire in individual burning canopies, as estimated by Ashton (1986 *apud* Judd, 1993).

In addition, deaths or serious injuries caused by fire in plants of all ages affect sexual regeneration of *Eucalyptus* populations, in the long term, and their vegetative regeneration, in the short term (Gill, 1997). Young *E. globulus* are quite sensitive to fire. In even-aged stands under five, plant mortality can reach 100% (Silva *et al.*, 2007). Older *Eucalyptus* individuals are more resistant to fire because they have thicker bark, which can be effective in protecting critical vegetative tissues (Ashton, 1981). In addition, their canopies are farther from the soil, thus, out of the reach of surface fires (Ashton, 1981). In order to regenerate vegetatively after fire, eucalypts have numerous deep epicormic traces, which are well protected under bark; and many species also develop lignotubers after seedling stage, benefiting buds from soil protection (Burrows, 2002; Clarke *et al.*, 2013). *Eucalyptus globulus* has both epicormic and lignotuber buds for resprouting after fire (Kirkpatrick, 1975a; Nicolle, 2006). In what concerns sexual regeneration, the deaths of pre-fire plants may either postpone the subsequent reproduction events of a population or lead to local extinction of the species. Therefore, although fire may reduce or prevent *E. globulus* regeneration capacity, it needs to be pointed out that the species has several mechanisms of fire resistance and escape.

Aside from the general effects fire has on the natural regeneration of *E. globulus*, the several fire regime aspects are also important.

Fire timing is determinant for the existence of post-fire *Eucalyptus* seminal regeneration. As *Eucalypts* do not have fire-induced flowering, pre-fire seed stock is required (Florence, 1996). Trees had to reach reproductive age, and had to bear mature seeds in the canopies or had to have deposited them in safe sites prior to fire occurrence (Florence, 1996). In terms of fire timing, two components are important: frequency and seasonality. Firstly, plant species life cycles must be matched with fire frequency, if those species are meant to persist in fire-affected areas. Frequent fires may prevent *E. globulus* populations from recovering their reproductive capacity, because adults take seven years to produce seeds after crown destruction, and post-fire recruits cannot produce seeds before reaching 4–7 years (Kirkpatrick, 1975b; Turnbull and Pryor, 1984; Jordan *et al.*, 1999). However, the lack of fire for periods longer than the maximum

lifespan of these trees, might also result in the disappearance of the populations, because free seeds do not last for long in nature and seedlings need to be released from competition to establish (Kirkpatrick, 1975b). Thus, the permanence of *E. globulus* in sites is ensured if periods without stand-replacing fires are shorter than individuals' lifespan and longer that the time required for them to attain sexual maturity (Kirkpatrick, 1975b). In Portugal, fire frequency is likely compatible with this range (Pereira *et al.,* 2006). Moreover, as *E. globulus* is a cultivated species there, long fire intervals would not be limiting for seed availability. Secondly, species phenology and fire seasonality must be synchronized. In Portugal, fires occur mainly during summer, when drought is more intense and temperatures are higher. *Eucalyptus globulus* has ripe fruits by the end of summer (Goes, 1977) and also produces serotinous fruits (Tng *et al.*, 2012). Although the summer is an unfavourable season for the growth of this species, due to the severe drought; autumn is usually favourable because of its mild temperatures and rain (Goes, 1977). Consequently, a s hort time after a fire, it is likely that numerous *E. globulus* seeds find favourable weather conditions to germinate in Portuguese forests.

Fire intensity is also relevant for the natural regeneration process. Low intensity fires are not able to induce some of the aforementioned beneficial effects, in the required magnitude to enable successful regeneration (Wilkinson *et al.*, 1993; Florence, 1996). Conversely, very intense fires may completely destroy both propagules and plants, totally precluding regeneration. The amount of fuel is a determining factor for fire intensity (DeBano *et al.*, 1998). *Eucalyptus* forests accumulate large amounts of litter as they age (Mount, 1964; Florence, 1996). Coppiced stands of *E. globulus* also accumulate logging residues, rotation upon rotation (Silva *et al.*, 2007). Dry *Eucalyptus* debris is very flammable and its accumulation represents an important fuel source which feeds wildfires (Mount, 1964; Florence, 1996; Gill, 1997). Thus, the oldest forests and the highest-rotation stands tend to have more intense fires, which cause high mortalities and produce a good seedbed for regeneration (Mount, 1964; Silva *et al.*, 2007). Moreover, fire severity is spatially heterogeneous within each burnt area, because fire behaviour depends on meteorological conditions, topography, and fuel distribution and type (Fernandes, 2006). Bailey *et al.* (2012) have found that this heterogeneity influences *Eucalyptus* spp. distribution, because both regeneration and persistence niches of *Eucalyptus* juveniles include characteristics specifically associated to fire, such as canopy gaps and ashes on soil.

The overall balance of fire effects on the natural regeneration from seeds is clearly positive to many *Eucalyptus* species. This fact is profusely referred in both scientific and technical literature about *Eucalyptus* forests. This balance should also be positive to *E. globulus* in particular. Larcombe *et al*. (2013) have found higher densities of *E. globulus* wildlings at the borders of burnt plantations than in similar unburnt conditions, in Australia. Nevertheless, little is known about the conditions that either favour or limit this regeneration in post-fire context, and the mechanisms underlying it. As far as we know, only a very recent study exists about post-fire reproductive behaviour of this species outside its native range (Calviño-Cancela and van Etten, 2018).

4. Research needs

Eucalyptus globulus is one of approximately 900 eucalypt species (Slee *et al.*, 2006). The majority of eucalypts are native to Australia and they exist in nearly all terrestrial environments there, from tropical rainforests to alpine lands or savannahs (Wardell-Johnson *et al.*, 1997). Many eucalypts are planted worldwide, outside their native ranges. During the second half of the 20th century, *E. globulus* was mainly planted in the Iberian Peninsula. Notably, the interest of Australian foresters on this species is quite recent. Australian plantations of this species have substantially increased in the beginning of the 21st century. The scientific research done on this eucalypt species in particular seems to have mainly followed the interest raised by industry. As a result, most of the available scientific knowledge about this species is related to economic production purposes, including forestry, breeding, and genetic improvement. Information about the species' ecology and ontogeny is much less abundant. Although this species occupies a much vaster area outside the native range than inside it, only a few recent studies exist about its autoecology overseas (Calviño-Cancela and Rubido-Bará, 2013; Fernandes *et al.*, 2016; Fernandes *et al.*, 2017; Calviño-Cancela and van Etten, 2018; Fernandes *et al.*, 2018).

Moreover, eucalypt plantations raise social controversies in several introduction territories (*e. g.* USA, Brazil, Portugal, and Spain), concerning species weed and invasive potentials, wildfires, soil impoverishment, phytotoxicity, among other issues (INAmb, 1990; Rejmánek and Richardson, 2011; De Vechi and Júnior, 2018). However, quantitative studies about these issues are disproportionally scarce for the exotic eucalypts around the world and *E. globulus* is no exception. The flammability and the post-fire erosion of forests dominated by this species is documented (*e. g.* Fernandes *et al.*, 2011; Coelho *et al.*, 1995). Its post-fire vegetative regeneration was also studied (Catry *et al.*, 2013). Information about invasive potential of *E. globulus* has been recently produced for Portugal (Fernandes *et al.*, 2016; Fernandes *et al.*, 2017; Fernandes *et al.*, 2018) and NW Spain (Calviño-Cancela and Rubido-Bará, 2013; Calviño-Cancela and van Etten, 2018). Nonetheless, the naturalization process, which is prior to invasion, has barely been studied.

Naturalization is the crucial step along the 'introduction-naturalization-invasion continuum' that enables exotic species to become independent from humans. Indeed, it is at the root of some problems that easily escape human control, like weedery, fuel build-up, or plant invasions. Nevertheless, scientific research about *E. globulus* has almost skipped this step. Systematic information about the distribution and frequency of spontaneous seminal regeneration in introduction territories of *E. globulus* is scarce, beyond local scale (Fernandes *et al.*, 2018). Information exists about some interspecific interactions that influence reproduction and establishment in those territories, such as: pollination (Feás *et al.*, 2010; Calviño-Cancela and Neumann, 2015); seed predation (Deus *et al.*, 2018); seedling browsing (Becerra and Bustamante, 2008); and mycorrhizae (Marchante *et al.*, 2001; Díez, 2005). In addition, despite the importance of fire in eucalypt ecology, information about post-fire regeneration from seeds is scarce for this species. Few specific studies exist on this issue and only one is field based

(Reyes and Casal, 1998, 2001; Silva *et al.*, 2016; Calviño-Cancela and van Etten, 2018). Little is known about the mechanisms that underlie this post-fire regeneration (Reyes and Casal, 1998, 2001; Silva *et al.*, 2016). Nevertheless, stands of this species are frequently affected by fire, especially in regions where climate is drier than in its native territory, as the Mediterranean-climate regions. Global climate changes are making climate in these regions drier (Santos *et al.*, 2002), leading to increased fire frequency and intensity. Forests dominated by *E. globulus* are one of the most fire-susceptible forest types in Portugal (Silva *et al.*, 2009). Land abandonment is increasing in Portugal, and is particularly evident after fire. This species is often involved in post-fire land-use transitions (Silva *et al.*, 2011). In those transitions, the increase of this species representativeness is more common than its decrease, and the former is frequently associated with processes of land abandonment (Silva *et al.*, 2011). Natural regeneration from seeds is likely to be relevant to those transitions. However, little information exists about this issue.

Considering that naturalization is a population process that requires several generations to be completed (Blackburn *et al.*, 2011), the main object of naturalization studies should be the populations. However, biological systems have modular and hierarchical organization (Campbell, 1996; Chave, 2013). Each level of this organization has novel properties that do not exist in lower levels. These emergent properties result from interactions of components/modules of lower levels (Campbell, 1996; Chave, 2013). Moreover, biological processes cross several levels of organization, as well as spatial and temporal scales, having causes and effects on several scales that may differ from those at which processes act (Campbell, 1996; Chave, 2013). Relating phenomena across scales is a crucial issue in biology research (Levin, 1992). Studies at single levels, as those mentioned in the former paragraph, are very important to understand the components and the mechanisms related to naturalization occurring at each level. Nevertheless, integration of the various levels is required to get a broader perspective of biological processes, including those affecting exotic species (Campbell, 1996; Brown *et al.*, 2008). Cross-scale studies complement other studies focused on single scales of space, time, or complexity; making them more meaningful (Levin, 1992). Multiscale approaches have proved to be very useful in identifying the different factors that influence plant population dynamics on several scales and in recognizing the scale dependence of those factors (Urbieta, 2008; Brown *et al.*, 2008). Unfortunately, this type of approach has rarely been used for analysing either native or exotic plant populations. Furthermore, little is known about the effects of wildfires across scales and their consequences on the emergent properties of ecosystems (Moritz *et al.*, 2005). Therefore, naturalization should be studied through the combination of analyses on different scales, in order to be better understood.

In conclusion, it would seem difficult to dispute the need to study *E. globulus* natural regeneration from seeds in mainland Portugal, given the wide distribution of this exotic species there, the flammability of its forests, the frequent land abandonment, and the predicted climate changes. Moreover, a multiscale approach should be used to grasp how the different ecological factors influence this process at the different biosystem organization levels (from biogeography down to molecules).

5. Thesis objectives

The **general objectives** of this thesis are to:

- Contribute to understand the naturalization process of *E. globulus* in Portugal's mainland.
- Evaluate the influence of fire on natural regeneration of *E. globulus* from seeds.

In order to achieve these objectives, a multiscale approach was chosen: ranging from an overview, on the whole Portuguese mainland, down to detailed characterization of microsites. A biogeographical perspective was also included. In addition, some mechanisms underlying naturalization were also analysed. *Eucalyptus globulus* stands were selected as study sites for several parts of the research presented in this thesis for three reasons. Firstly, these stands are the areas where most individuals of this species are located inside the Portuguese territory. Secondly, stand management could influence the process of natural regeneration from seeds. Finally, these stands are amongst the most fire-prone forest types in this territory.

The **specific objectives** of the thesis are as follows:

- to briefly describe the distribution of *E. globulus* regeneration in the Portuguese mainland.
- to survey the occurrence and abundance of natural *E. globulus* regeneration from seeds on different spatial scales.
- to assess drivers of natural *E. globulus* regeneration from seeds on different spatial scales.
- to analyse fire influence on the occurrence, abundance, development, and establishment of *E. globulus* wildlings on different spatial scales.
- to identify mechanisms triggered by fire that influence early development *of E. globulus* individuals from seeds.

6. Thesis outline and rationale

This thesis consists of three sections. Section one is this general introduction. Section two contains the thesis core (Fig. 6). It starts with a description of the general distribution of *E. globulus* regeneration in the Portuguese mainland. Then, it follows with three chapters that assess factors deemed to affect the process of natural regeneration of *E. globulus* from seeds, on different space scales. This section ends with another chapter that analyses one of the mechanisms associated with regeneration from seeds, in fire context. Chapter 1 summarizes the exploratory work data, as well the study led by F. Catry, in which the researcher had a

participatory role (published as a research article: Catry *et al.*, 2018; App.1). Chapters 2 to 5 are the thesis core, where the research work led by the researcher is described and discussed. These four chapters are presented as autonomous units, following the standard structure of research articles, corresponding to three published articles (chapters 2, 3, and 5) and a submitted manuscript (chapter 4). In the third section, results from chapters 2–5 are summarized and discussed together with other results from the exploratory work and the articles where the researcher was a co-author. Also in this section, conclusions are presented, as well as forest management implications and issues for future research.

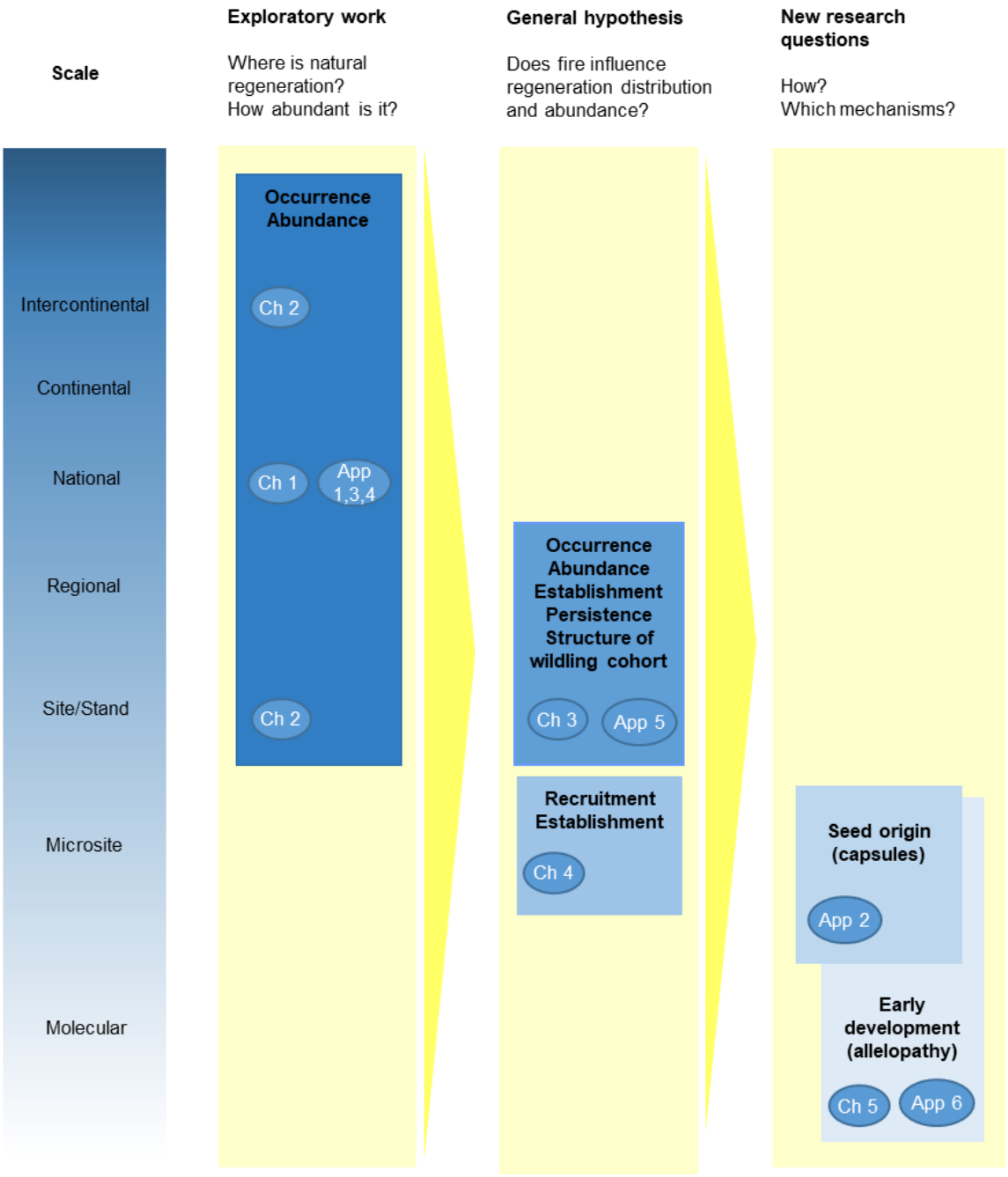


Fig. 6 – Outline of the research presented in this thesis and in its appendices.

This thesis also has several appendices. There are two published research articles led by other investigators, in which the researcher was a co-author (App. 1–2), and four posters that were presented at congresses (App. 3–6). The first of these articles was led by F. Catry and presents the distribution of natural regeneration from seeds in roadsides across Portugal's mainland (App. 1). The second one was led by P. Santos and deals with the origin of seeds that germinate in burnt areas (App. 2). The posters concern analyses of regeneration data from Portuguese National Inventories (App. 3–4) and preliminary results of studies that were published as articles later on (App. 5 corresponds to chapter 3; App. 6 corresponds to chapter 5). The poster in App. 3 was supervised by F. Rego and all the others were led by the researcher.

The rationale of section two, as well as the specific contents of its chapters, are described below.

Chapter 1 – Overview of the distribution and abundance of *E. globulus* Labill. regeneration in Portugal's mainland

This chapter aims to provide a wide spatial context of the *E. globulus* regeneration. It consisted of a brief description of the distribution of *E. globulus* natural regeneration in Portugal's mainland as a whole. This description is based on two sources: (1) data from the 4th and the 5th Portuguese National Forest Inventories (NFI4 and NFI5) (*cf.* Apps. 3-4); and (2) results from an extensive roadside survey (App. 1). The exploratory work, which was based on the NFIs, was produced under the supervision of F. Rego. Meanwhile, the work based on roadside surveys was initially planned to be exploratory work, but turned out to be a more solid work led by F. Catry, in which the researcher participated. As NFI data screening and roadside surveys produced complementary results, on the same spatial scale, it was decided to combine and summarize them in this chapter.

Chapter 2 – Understanding the naturalization of *E. globulus* Labill. in Portugal: a comparison with Australian plantations

During the exploratory work, it became evident that the geographical distribution of *E. globulus* regeneration from seeds, at a national level, is associated to stands dominated by this species. Hence, it was decided to study the most common type of these stands, the pure *E. globulus* plantations, in further detail. This chapter aims to study plantation-scale factors that may influence the regeneration from seeds and the naturalization phenomenon. It departs from the assessment of plantation-scale drivers of *E. globulus* wildling establishment in Portugal. It then compares Portuguese plantations with Australian plantations (Australian data from Larcombe *et al.,* (2013)) to provide a biogeographical understanding of the naturalization process, as recommended by Hierro *et al.,* (2005) and Richardson and Pyšek, (2012). The Portuguese plantations were located in central Portugal and the Australian were located in seven different regions in the southern part of Australia.

Chapter 3 – Natural establishment of *E. globulus* Labill. in burnt stands in Portugal

Stands dominated by *E. globulus* or *Pinus pinaster* are amongst the most abundant and flammable forest types in Portugal (Silva et al., 2009, IFN, 2013). Moreover, the exploratory work identified *P. pinaster* dominated stands as the stand type where *E. globulus* regeneration occurred more frequently, just after the *E. globulus* dominated stands (chapter 1). Therefore, this third chapter aims at assessing local factors that could affect the occurrence, density and structure of the post-fire wildling cohort in three different types of burnt stands: pure *E. globulus* stands, pure *P. pinaster* stands, and mixed stands with both species. Assessed factors were site characteristics, stand type, and post-fire management. Study sites were located in central and northern Portugal and were burnt by wildfires 5–7 years prior to sampling.

Chapter 4 – Microsite drivers of natural seed regeneration of *E. globulus* Labill. in burnt plantations

Eucalyptus globulus natural regeneration from seeds is not uniformly distributed within stands. An important part of variability occurs on small spatial scales within sites (Fernandes *et al*., 2018). The intensity of a fire is variable even across a single stand (Raison, 1979, Fernandes, 2006), enhancing variability of the post-fire environment. The *Eucalyptus* genus has evolved in environments where fire worked as a selective agent (Pryor, 1976; Gill, 1997). It was hypothesized if factors related to fire affect *E. globulus* regeneration from seeds and its distribution in burnt sites. Hence, this chapter aims to assess microsite factors that are associated with the occurrence of this regeneration in burnt plantations, namely those factors related to recruitment, establishment, and growth of regeneration. Study sites were *E. globulus* plantations, located in central and northern Portugal that were burnt approximately two years prior to sampling.

Chapter 5 – Fire effects on litter chemistry and early development of *E. globulus* Labill.

Inside burnt stands, wildlings are more common at microsites where fire residues are more abundant (Ch. 4). It was hypothesized that changes in chemical interactions between coexisting species may be at stake. Hence, this chapter aims to analyse the allelopathic potential of heat-treated leaf litter of several species on the early development of *E. globulus*, in order to better understand the potential of *E. globulus* to persist in self-dominated forests or to colonize heterospecific communities after fire. Leaf litters were submitted to six heat treatments, chemically analysed, and used as substrate for germination and seedling growth of *E. globulus.* Seeds and litter of *E. globulus*, as well as litter of *Acacia dealbata* Link, *Pinus pinaster* Aiton, and *Quercus suber* L., were collected in SW Portugal. These species spontaneously coexist in Portugal and/or Australia.

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Section II

Chapter 1

Overview of the distribution and abundance of *Eucalyptus globulus* Labill. regeneration in Portugal's mainland

This chapter concerns the preliminary work that was done to assess the broad scale distribution of *E. globulus* regeneration in Portugal's mainland. As this work does not belong to the thesis corpus but was important for creating the thesis, it is briefly synthetized here.

This work led to the publishing of one research article, the presentation of two posters in two different congresses, and one oral presentation in one of those congresses. Ana Águas was co-author of the article (led by F. Catry) and of one poster (supervised by F. Rego). She was the leading author of the other poster and made the oral presentation. The article and the posters are presented in the appendix section. The published references of this chapter are the article and the abstracts of the congress participations, as follows:

- Catry, F.X., Moreira, F., Deus, E. Silva, J. S., Águas A. 2015. Assessing the extent and the environmental drivers of *Eucalyptus globulus* wildling establishment in Portugal: results from a countrywide survey. Biological Invasions, 17(11), 3163-3181. DOI: 10.1007/s10530-015-0943-y
- Águas, A., Nunes, L., Rego, F.C., 2017, Utilização dos inventários florestais para estimar a probabilidade de regeneração de espécies arbóreas - o caso de *Eucalyptus globulus* em Portugal. In: 8º Congresso Florestal Nacional, 11-14 Outubro, Viana do Castelo, Portugal, pp: 103.
- Rego, F.C., Águas, A., Nunes, L., 2017, Assessing regeneration of *Eucalyptus globulus* Labill. with the Portuguese National Forest Inventory. In: XIV MEDECOS International Conference & XIII Asociación Española de Ecología Terrestre (AEET) Meeting – Human-Driven Scenarios for Evolutionary and Ecological Changes, 31 January-04 February, Seville, Spain, pp: 212. Part of DOI: 10.7818/MEDECOSandAEETCongress.2017

1.1. Introduction

Eucalyptus globulus is a eucalypt species which is native to SE Australia and Tasmania (Kirkpatrick, 1975b, a). It was introduced in many temperate regions worldwide, where it is now cultivated. One of those regions was Portugal´s mainland. The species was introduced for ornamental purposes in the mid-19th century (Radich, 1994). About one hundred years later, the planted area of this species was expanded throughout the entire Portuguese mainland (Radich, 1994), corresponding to 26% of its forest nowadays, according to recent estimates (ICNF, 2013). However, plantations experienced different degrees of success in the different parts of that territory (Tomé, 2000). As a result, the most recent plantations are being established mostly in areas that meet the environmental requirements of this species (DGRF, 2006; Alves *et al.*, 2012; Presidência do Conselho de Ministros, 2015) (Fig. 1.1a). Tomé *et al.* (1998) created a model which divided the Portuguese mainland into several regions, according to their ecological suitability for *E. globulus* growth. This division was based on information concerning climate and soil. The model was then subjected to successive updates (Ribeiro and Tomé, 2000; Tomé and Ribeiro, 2000; Tomé *et al.*, 2001; Tomé *et al.*, 2006). This model is a useful tool to ascertain the adequate sites, for establishing or keeping productive *E. globulus* plantations, from those sites which cannot sustain profitable plantations of this species and should be occupied by other forest species or even other land uses (Fig. 1.1b).

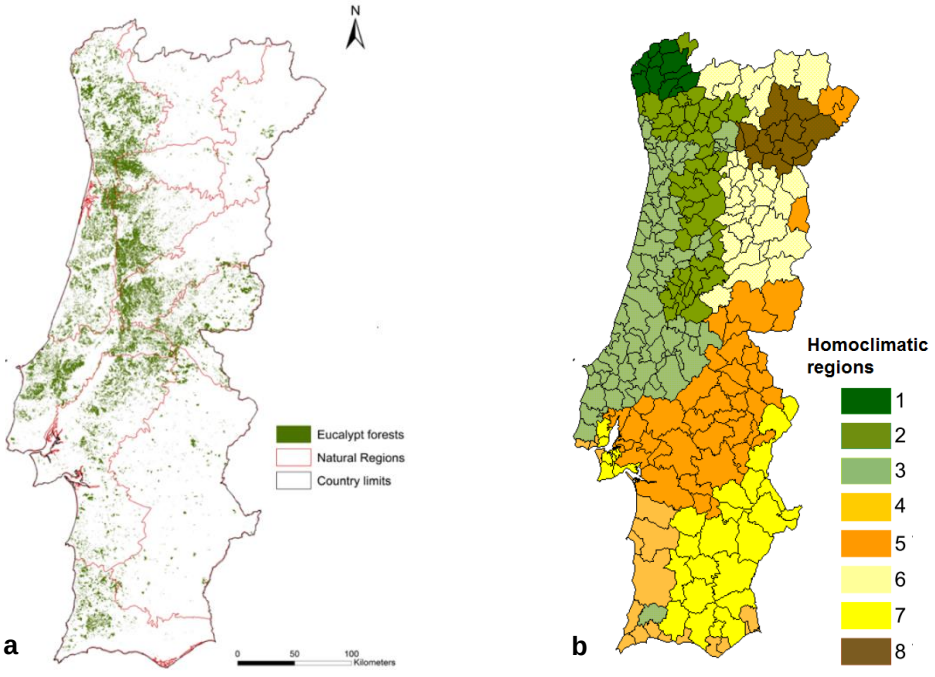

Fig. 1.1 - **a.** Distribution of *Eucalyptus globulus* stands in 12 natural regions in mainland Portugal (Catry *et al.*, 2015). **b.** Homoclimatic regions, according to their suitability for *E. globulus* (potential growth of the species decreases from region 1 down to region 8) (Tomé *et al.*, 2001).

This species regenerates both vegetatively and from seeds. Vegetative regeneration is achieved when dormant buds are activated due to the occurrence of damages in the plant shoot

(Pyne *et al.*, 1996; Clarke *et al.*, 2013). These buds lie protected underground in lignotuber and beneath the shoot bark (Clarke *et al.*, 2013). Plants of this species only use vegetative regeneration for replacing damaged parts of their bodies, they cannot produce ramets with capacity to grow independently under natural conditions. This regeneration is very vigorous and it is the base for the forestry exploitation of this species in Portugal, where plantations are coppiced in rotations of 10–12 years (Turnbull and Pryor, 1984; Soares *et al.*, 2007).

Meanwhile, sexual regeneration is achieved by the annual production of flowers and seed-bearing fruits. The fruit and seed sets do not seem to be limited by pollination in the western Iberian Peninsula, as the flowers are often visited by bees and birds (Feás *et al.*, 2010; Calviño-Cancela and Neumann, 2015). Fruits are loculicide capsules with no special features to enhance seed dispersal (Cremer, 1965). Dehiscence of capsules occurs between August and April, in Portugal (Goes, 1977). Seeds are small (length: 1.25–2.5 mm; width: 1.0–1.75 mm) (Bonner and Karrfalt, 2008). Large *E. globulus* trees can produce 280–350 g of seeds per year which may correspond to 11.2×10^3–14×10^3 seeds, considering the estimate of 4×10^5 seeds kg^{-1} (Goes, 1977). Eucalypt seeds also have no specialized structures for any particular type of dispersal (Booth, 2017). When capsules are dry enough, they just open the valves and shed the seeds, which are then moved by gravity and wind onto the ground (Cremer, 1977; Booth, 2017). Hence, seeds fall close to parent trees, at horizontal distances no longer than half of the tree height, when winds are 10 km h^{-1} (Cremer, 1977). Once eucalypt seeds are released, they last less than one year in the field for several reasons (Jacobs, 1955; Penfold and Willis, 1961; Florence, 1996). First of all, they are small and susceptible to pathogenic soil fungi (Stoneman, 1994). Secondly, they can also be predated. Some seed predators prefer *E. globulus* seeds over other morphologically similar seeds from native species in Portugal (Deus *et al.*, 2018). Most importantly, *E. globulus* seeds appear to lack primary dormancy (Nair, 2006), and, in some seasons, they find appropriate conditions to germinate in the field, in the western Iberian Peninsula (Calviño-Cancela and Rubido-Bara, 2013). Moreover, aside from the seeds that are shed during the normal dehiscence period, others are stored in canopies inside serotinous capsules (Tng *et al.*, 2012), for several years (pers. observ.). Thus, this exotic species is able to produce offspring via sexual reproduction in Portugal. In fact, this species was first referenced as naturalized in Portugal, in 1943 (Almeida and Freitas, 2006).

Despite the aforementioned facts, the Portuguese *E. globulus* stands have almost exclusively artificial origin. In other words, their plants are produced in nurseries either from seeds (seedlings) or from cuttings (clones). Curiously, the perspectives that different agents have had about the reproductive capacity and the naturalization of this species in Portugal have been contradictory. Countryside people have been collecting seeds for direct sowing, and managing wildlings (transplantation and thinning), for decades, in several regions of Portugal (Goes, 1977 and pers. obs.). Nevertheless, foresters have advised against the use of this reproductive capacity for forestry purposes, arguing that wildling occurrence is sporadic and only happened under very specific conditions (Goes, 1962). The scientific community, in turn, disregarded this issue. The scientific reports that mention the occurrence of natural

regeneration are quite recent and refer a potential for invasion (Marchante *et al.*, 2008; Silva and Marchante, 2012; Marchante *et al.*, 2014). Quantitative studies which are even much more recent confirm the existence of regeneration in Galicia and Portugal with restricted invasion capacity, which is dependent on the habitat type in the vicinity of plantations and is affected by disturbance (Calviño-Cancela and Rubido-Bará, 2013; Fernandes *et al.*, 2016; Fernandes *et al.*, 2017; Calviño-Cancela *et al.*, 2018; Fernandes *et al.*, 2018). However, no study was conducted to assess the territorial extent of *E. globulus* regeneration in the whole Portuguese mainland, prior to the work presented in this chapter.

Given this knowledge gap, the work summarized in this chapter was aimed at assessing the geographical distribution of *E. globulus* natural regeneration, in Portugal's mainland as a whole. Two separate approaches were chosen to achieve this goal: screening successive Portuguese National Forest Inventories (NFI); and extensively surveying roadsides in the boundaries of *E. globulus* plantations across mainland Portugal. The first approach was intended to provide systematic information of regeneration inside forests and a follow-up of this regeneration, while the second could provide information about regeneration in the vicinity of *E. globulus* plantations. For detailed information about the two approaches, please see: (1) appendices 3 and 4, for the work based on NFI data; and (2) appendix 1, for the work based on the roadside survey. The work based on NFI data was supervised by F. Rego, while the one based on the wildling survey was led by F. Catry.

1.2. Material and methods

1.2.1. Study area

In a broad sense, the study area of this exploratory work was the Portuguese mainland in its entirety. More specifically, the data from the NFI concern all forests that exist in that territory, whereas the roadside survey only dealt with the roadsides next to *E. globulus* stands.

1.2.2. Screening NFI databases

Field work of the 4th NFI (NFI4) occurred in 1997/98 (DGF, 2001), whereas the one of the 5th NFI (NFI5) occurred in 2005/06 (AFN, 2010). The NFI4 had a total of 2,222 sampling plots stratified by forest types. The set of sampling points was obtained, by stratified random selection of points, from a grid overlaid on aerial photographs of the whole mainland (DGF, 1999). The NFI5 had a total of 6,897 sampling plots which were systematically distributed across all the forests in same territory, according to a 2 x 2 km grid (AFN, 2009). These were the first Portuguese NFIs that collected data on the forests' vertical structure, as the previous ones did not have this information. For each NFI plot, the plants were assigned to classes, according to their respective height (h). The height classes were as follows: $h < 0.5$ m; $0.5 \leq h < 1$ m; $1 \leq h < 2$ m; $2 \leq h < 4$ m; $4 \leq h$ 8 m; $8 \leq h$ 16 m; and $h \geq 16$m (DGF, 1999; AFN, 2009). Then, for each height class, the plant cover of the three most abundant woody species was registered. No specific information was available for the direct identification of regeneration in

either NFIs. Thus, the height classes were used to detect *E. globulus* regeneration. This way, the *E. globulus* plants with h < 1m were considered as regeneration. For plots where those plants were detected, further information was drawn from the NFI databases, namely: dominant species name (independently of the height); and codominant species name (only for species represented by individuals with h ≥ 1 m); stand dominant height; and stand basal area.

1.2.3. Surveying roadsides

Roadsides close to the borders of *E. globulus* stands were visually surveyed for detecting *E. globulus* wildlings, from December 2013 to May 2014. Surveys were visually made by one observer who was inside a car, at a constant speed of 30–40 km h^{-1}. Transects consisted of strips of non-cultivated land, between *E. globulus* stands and the road asphalt, with a maximum width of 10 m and maximum length of 100 m, located at every 1000 m, with topography that allowed a good visual detection. The sampling was prepared overlaying a road map (all asphalt roads but the highways), a forest map (all *E. globulus* stands), and a map of the Portuguese natural regions (APA, 2013). Approximately 3000 possible transects were distributed across mainland Portugal, using a stratification procedure, based on its twelve natural regions. Adjustments to this planning were done on a later date, according to the conditions found in the field. A total of 3111 transects were sampled in the field. Their geographical distribution is presented in Fig. 1.5a. The likely error of this survey method was assessed in twenty transects. A complementary sampling was made in the office, using 'Google™ Street View' images of the same transects (photos taken between May 2009 and October 2010). A final database of wildling abundance was produced combining the two samplings, replacing the field wildling count of each transect by the respective office count, whenever the latter was superior. This procedure resulted in the replacement of 16% of the field counts. The wildling abundance was converted from counts to densities (no. wildlings ha^{-1}), using the estimated areas of the respective transects.

The wildling densities were used to map the distribution patterns across the mainland, using the inverse distance weighting method to make spatial interpolation, in ArcGIS software (ESRI, 2012).

With regards to exploring the main drivers of wildling abundance, wildling densities of transects were assigned into seven classes and were analysed together with environmental data (climate, topography, and soil type). The environmental information was drawn from geographical points, in digital maps, corresponding to the coordinates of the geographic centre of each transect. Then, boosted regression trees were used to analyse the data (De'ath, 2007). Two models were produced using packages from R software. One model did not take into account the auto correlation in its residuals. While the other considered this autocorrelation using the residual autocovariate approach (Crase *et al.*, 2012).

1.3. Results

1.3.1. NFI4 and NFI5

The screening of the NFI4 and NFI5 databases did not allow to distinguish neither vegetative from sexual regeneration nor natural from artificial regeneration. Thus, the results presented here concern all types of regeneration. Nonetheless, the used screening method should be strongly biased towards sexual regeneration and, in some extent, to its natural version, for several reasons. Firstly, the vegetative regeneration from epicormic buds had almost no chances of being detected, because it mainly occurs after fire and predominantly in plants taller than 1 m. Secondly, the vegetative regeneration from stumps is very common in coppiced stands, but it is usually so vigorous that it attains heights over 1 m in a couple of months after logging (rotations: 10–12 years). Finally, the vast majority of *E. globulus* stands in Portugal are planted with seedlings from nurseries. These plants are a few months old when they are transplanted and transplantation occurs at favourable growing seasons, thus they need to be much less time in the field until they attain 1 m in height, comparatively to the germinants recruited *in situ*.

Regeneration occurred in forest stands of every natural region of mainland Portugal (Figs. 1.3a and 1.4a). However, important differences were evident in the frequency of occurrence among regions.

The proportion of Portuguese forest stands with *E. globulus* regeneration did not show evident changes between the NFI4 and the NFI5 (Figs. 1.2, 1.3b, and 1.4b). This result was consistent for all stand types and for the forests as a whole.

In regards to stand types by their dominant species, the stands dominated by *E. globulus* were the ones where regeneration of this species was more frequently found (Fig. 1.2). These were followed by pinewoods of *Pinus pinaster.* Regeneration of *E. globulus* was residual in all the other stand types. This regeneration occurred in about 30% of all *E. globulus* stands, was coarsely one tenth in pinewoods and one hundredth in other stand types.

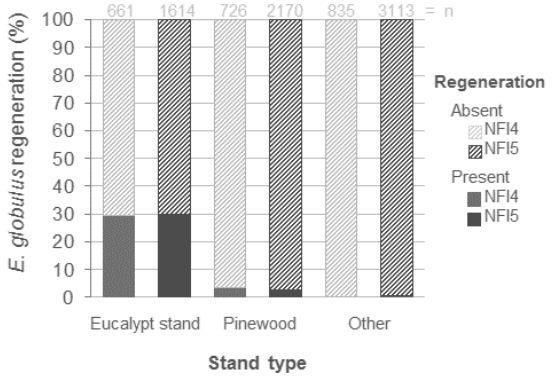

Fig. 1.2 – Occurrence of *Eucalyptus globulus* regeneration in different of forest stands, according to data drawn from the 4th and the 5th Portuguese National Forest Inventories (NFI4 and NFI 5, respectively). In this graph, eucalypt stands are dominated by *E. globulus* and pinewoods by *Pinus pinaster.*

Stands dominated by *E. globulus* having conspecific regeneration were mainly located in central and NW Portugal, and in a restricted cluster in SW Portugal (Fig. 1.3a). Amongst these stands, the conspecific regeneration was 1.5 to 2-fold more frequent in the pure ones than in the mixed (Fig. 1.3b).

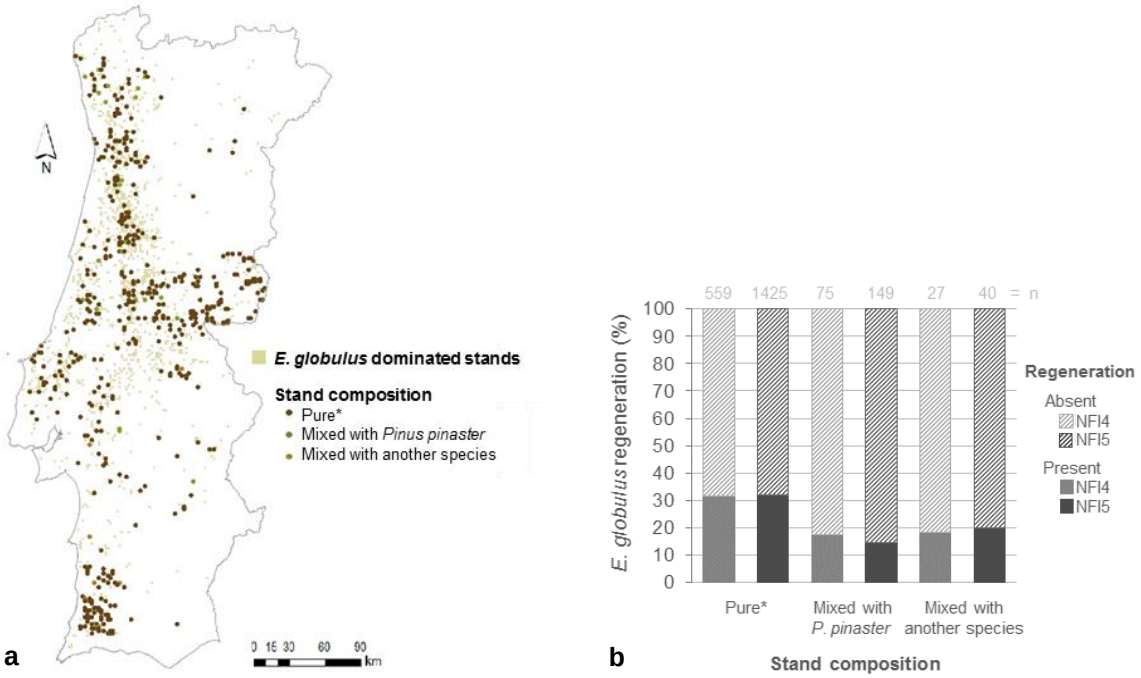

Fig. 1.3 - *Eucalyptus globulus* regeneration in stands dominated by the same species, according to data drawn from the Portuguese National forest Inventories (NFI). **a.** Geographical distribution (data from the 5th NFI). **b.** Distribution according to the stand type (data from the 4th and 5th NFIs, NFI4 and NFI5, respectively). * Pure stands – stands whose *E. globulus* has at least 75% of the total cover.

Most pinewoods of *Pinus pinaster* with *E. globulus* regeneration were located in central Portugal (Fig. 1.4a). Amongst these stands, the regeneration occurred more frequently in those where *E. globulus* was the second most frequent overstorey species. Regeneration occurred in those pinewoods at least 4-fold more frequently than in any other pinewood type.

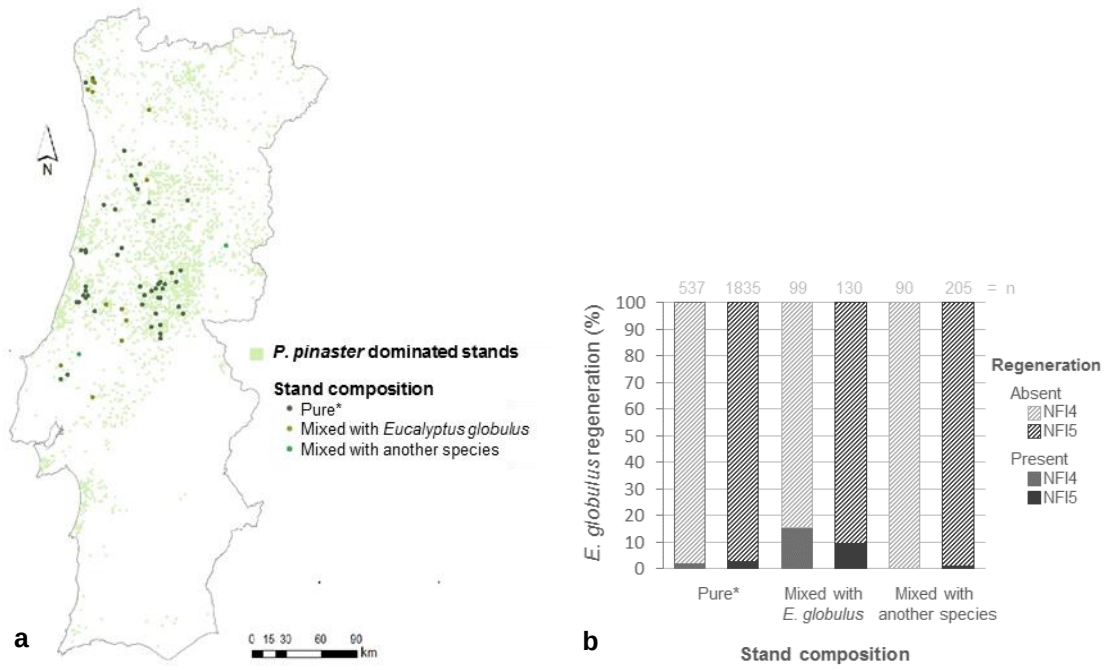

Fig. 1.4 - *Eucalyptus globulus* regeneration in stands dominated by *Pinus pinaster*, according to data drawn from the Portuguese National forest Inventories (NFI). **a.** Geographical distribution (data from the 5th NFI). **b.** Distribution according to the stand type (data from the 4th and 5th NFIs, NFI4 and NFI 5, respectively). * Pure stands – stands where *P. pinaster* had at least 75% of the total cover.

1.3.2. Roadside survey

The mean likely error of estimating wildling densities in roadsides from a moving car was estimated in *ca.* 30%. Main errors consisted of underestimation of wildling density in transects where wildlings were very abundant. This result justified the complimentary survey using 'Google™ Street View', the combination of the results of both surveys, and the establishment of density classes for the data analyses. Error associated to results of presence/absence was small, because: (1) regeneration is very conspicuous due to the light-blue colour of juvenile leaves; (2) wildlings are easy to distinguish from trees or stumps regenerating vegetatively by their very different habits; and (3) roadsides were selected for their good visibility characteristics

Wildlings of *E. globulus* occurred at roadsides in all natural regions of the Portuguese mainland. However, their distribution was not even across these regions. Wildling occurrence was very frequent at roadsides in central and NW Portugal, frequent in a separate cluster in SW Portugal, and much rarer in the other regions (Fig. 1.5a). Individual transects had wildling densities ranging from 0 to 10,000 wildlings ha^{-1}. Roadside transects with densities higher than 200 wildlings ha^{-1} were prevalent in western Portugal, more specifically along a strip no wider than 100 km, between the Lima River and the mountain-range complex of Aire and Candeeiros. In addition, some restricted areas with such densities occur between these mountains and the Tagus River, and also in SW Portugal.

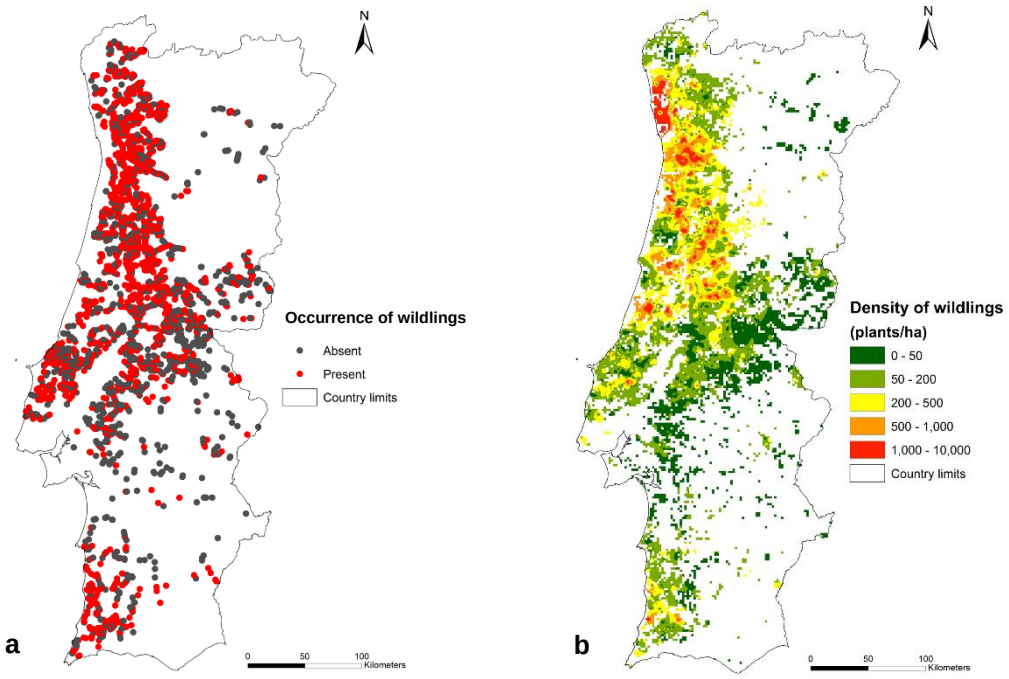

Fig. 1.5 - Geographical distribution of *Eucalyptus globulus* regeneration in continental Portugal (from Catry et al., 2015). **a.** Occurrence of wildlings in roadsides (*n* = 3111 transects). **b.** Density of wildlings in areas with major *E. globulus* plantations. (Map **b** was produced by spatial interpolation of densities observed at 3111 roadside transects, thus the representation of areas beyond roadsides is just a simplification for visualization purposes).

The most relevant environmental variables affecting wildling abundance in roadsides were, by decreasing order of importance: precipitation; distance from the sea; elevation; slope; number of frost days; and soil type (Fig. 1.6). The maximum abundance occurred in areas where

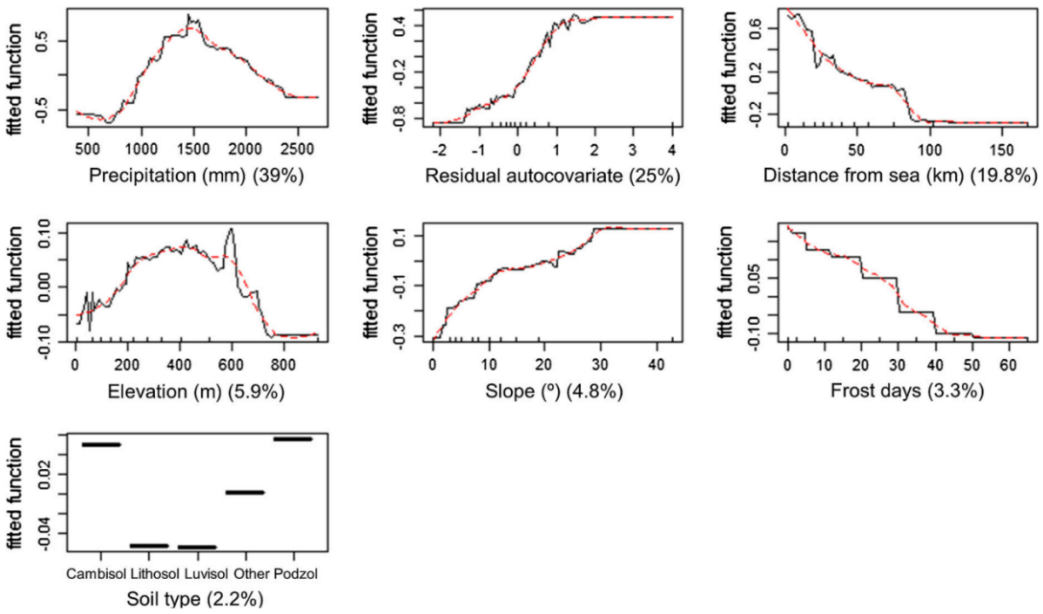

Fig. 1.6 - Partial dependence plots for the variables influencing the abundance of *Eucalyptus globulus* in mainland Portugal (from Catry et al., 2015). A smoothed version of the fitted function is also shown (dashed line). The relative importance of the variables is shown as %. Ticks across the bottom of each plot show the distribution of deciles for each predictor variable

annual precipitation ranged from 1300 to 1700 mm. Wildling abundance steadily decreased with distance from the sea. Roadsides at altitudes between 200 and 600 m had the highest wildling abundances, as did those nearby the steepest slopes (30–40º). Wildling abundance in roadsides decreased with the yearly number of frost days. In addition, it differed across soil types, being the highest in podzols and cambisols, the lowest in lithosols and luvisols and intermediate in the other soil types.

1.4. Discussion

1.4.1. Occurrence of wildlings

Two different methods were used to assess the geographical distribution of *E. globulus*: screening NFI databases and direct survey of roadsides. The two methods allowed to obtain information from all regions of mainland Portugal, but from different types of ecosystems: all kinds of forest stands *vs.* roadsides in the vicinity of *E. globulus* stands. Thus, the produced information is complementary, portraying the reality of forests and some of their vicinities.

Importantly, the wide-scale distribution of regeneration obtained by the two methods was very similar, despite the difference in the sampling universes, and the limitations of either method. Results from both methods showed that regeneration of *E. globulus* occurs in every natural region of Portugal, but the frequency of occurrence is not spatially uniform. The regions where this occurrence is more frequent are those where stands dominated by *E. globulus* are also more frequent, which happen to be the same regions recognized as having medium to good quality for *E. globulus* growth (Ribeiro and Tomé, 2000; Tomé *et al.*, 2001) (Figs. 1.1, 1.3-a, 1.4-a, and 1.5-a). Thus, regeneration of *E. globulus*, including the natural regeneration from seeds, has a vast geographical distribution in Portugal and seems to be associated to regionally favourable environmental conditions for *E. globulus* growth.

The proportion of stands having *E. globulus* regeneration differed, across the several stand types of Portuguese forest, as follows: pure *E. globulus* stands (31.7% in NFI4 and 31.9% in NFI5); mixed stands of *E. globulus* and *P. pinaster* (eucalypt dominant: 17.3% in NFI4 and 14.8% in NFI5; pine dominant: 15.2% in NFI4 and 9.2% in NFI5); pure *P. pinaster* stands (1.7% in NFI4 and 2.7% in NFI5); and the other forests (0.2% in NFI4 and 0.4% in NFI5). These proportion differences were probably related to whether parent trees were present and how abundant they were. The origin of lignotuberous, epicormic, or stump resprouts must have been the *E. globulus* ‘parent’ plants or stumps located in the very same place where this regeneration was. On the contrary, the origin of seedlings and saplings might have been either endogenous and/or exogenous; because seeds could have been naturally dispersed or artificially sown and seedlings planted. However, the dispersal capacity of *E. globulus* seeds from parent trees is very limited (Cremer, 1977). Notably, when considering 10–12 year rotation periods, artificial procedures (planting, sowing, and coppicing) could not possibly be the only causes of the high prevalence of regeneration (in 15–32% of *E. globulus*-dominated stands). Moreover, basal resprouting only occurs under natural conditions, if the shoot is seriously damaged, by browsing

or fire. However, this species is not browsed in Portugal. Finally, at least with regards to NFI5 data, burnt individuals would have resprouts taller than 1 m, when they were sampled, because most fires occur in summer and this field work occurred between December and June (AFN, 2010). Thus, it may be said that natural regeneration from seeds was indeed present in many forests dominated by *E. globulus*, and the local propagule pressure was a relevant factor for this presence. Indeed, the results from the roadside survey confirm this idea.

Wildlings were detected in 60% of roadside transects next to stands dominated by *E. globulus*, while 30% was the overall proportion of regeneration occurrence inside stands of this type in NFI databases (Figs. 1.2, 1.5 a). This large difference could be attributed to two main facts. Firstly, wildlings taller than 1 m could be distinguished from other plants in the roadside survey but not in NFI databases, considered as regeneration in the former but not in the latter. Secondly, NFI databases only have data for the three most abundant (cover) species of each height class, preventing the detection of regeneration where its density was low. It is also worth to mention that roadside survey was less likely to wrongly include vegetative regeneration than the NFI screening. Thus, the presence of *E. globulus* regeneration inside 30% of stands dominated by the same species appears to be an underestimate. Nevertheless, results from both approaches are high enough to recognize that *E. globulus* wildlings are frequently associated to stands dominated by this species. Moreover, considering that these stands are 26% of Portuguese forests (ICNF, 2013), this natural regeneration from seeds is also frequent in mainland Portugal. Remarkably, these results go against the ideas which have prevailed in technical and scientific communities.

The occurrence of *E. globulus* regeneration is uncommon in stand types where *E. globulus* is not one of the two most represented species (Figs. 1.2, 1.4b). The absence of this regeneration might be due to unfavourable environmental conditions or to the lack of parent trees both inside and in the vicinity of these stands. Conversely, the presence may be due to either exogenous sources (seed dispersal, sowing, or planting) or endogenous sources, because the presence of a few *E. globulus* trees next to NFI sampling plots cannot be discarded with NFI data.

The occurrence of *E. globulus* regeneration in pinewoods was mostly concentrated in central Portugal (Fig. 1.4a). This result was probably due to the combination of several factors in this region. Firstly, climate and soil conditions are good for *E. globulus* growth (Fig. 1.1b) (Ribeiro and Tomé, 2000; Tomé *et al.*, 2001). Secondly, fire has been frequent in this region in the most recent decades (Pereira, 2006). Thirdly, mixed stands with both species and pure stands of either species are very common there. Finally, the mean area of rural estate is very small in this region. In other words, the geographical distribution *E. globulus* and *P. pinaster* is rather mingled in this region, facilitating the recruitment of *E. globulus* in any of these stands. Moreover, more than one half of all forests dominated by *P. pinaster* in Portugal are concentrated in this region (AFN, 2010). Thus, central Portugal was the most likely region for the occurrence of *E. globulus* regeneration inside pinewoods. Notably, the pinewood type where *E. globulus* regeneration was more frequently present was the one which had *E. globulus* as the

second most represented overstorey species. Once again, the presence of parent trees *in situ* seems to have been determinant for the occurrence of regeneration.

In terms of dynamics, no substantial changes in the spatial distribution of *E. globulus* regeneration were found, when NFI4 and NFI5 data were compared. No changes were detected in the geographical distribution (data not shown) nor in the proportion of stands affected, overall and by stand type (Figs. 1.2, 1.3b, 1.4b). These results may have one of two meanings. Either the considered time period (from 1997/98 to 2005/06) was too short for observing such changes or a near 'steady state' condition was observed.

1.4.2. Density of wildlings

Densities above 200 wildling ha^{-1} were almost restricted to the regions which belong to the three top quality classes for *E. globulus* growth (Figs. 1.1b and 1.5b). However, such high densities did not occur in each region in their entirety. Moreover, no important differences stood out among these regions, in terms of wildling density at roadsides. The main environmental drivers of wildling density were, by decreasing order of importance: mean annual precipitation; distance from the sea; elevation; slope; yearly number of frost days; and soil type. Generally speaking, roadside wildling abundance fitted the descriptions existing in literature for the environmental envelope of the species (limit and optimal values).

The mean annual precipitation was by far the most influential environmental variable on roadside wildling density. Wildlings were scarce in sites where annual precipitations were below 700–800 mm. The densities of wildlings increased in sites having precipitation above this limit and up to 1500 mm $year^{-1}$, and they decreased in rainier sites. This distribution has a total overlap with the annual precipitation range in the native territory of this species, which is 500–2600 mm (Jovanovic and Booth, 2002). However, the wildling density was strongly limited in the lowest part of the range, in mainland Portugal. This was an expected result because this species only tolerates precipitation below 600 mm $year^{-1}$, if it is evenly distributed along the year (Kirkpatrick, 1975b). In Portugal, sites with such precipitation levels have a Mediterranean climate with several months of drought, which would be hard for *E. globulus* to tolerate, especially at the seedling stage (Stoneman, 1994; Calviño-Cancela and Rubido-Bará, 2013). The observed decrease of wildling densities at sites with annual precipitation above 1500 mm could be related to several factors. Sites with high levels of precipitation have an increased likelihood of fungal attacks (*e. g*. *Mycosphaerella*) (Keane *et al.*, 2000; Silva *et al.*, 2008) and waterlogging (Kirkpatrick, 1975b; Gomes and Kozlowski, 1980). In addition, those sites may have better adapted species that outcompete *E. globulus*, as eucalypt seedlings are very sensitive to competition (Jacobs, 1955; Florence, 1996). In fact, most *E. globulus* native territory site are within the 600–1100 mm $year^{-1}$ precipitation range (Kirkpatrick, 1975b), and recommendations for plantations of this species indicate that the precipitation range should be 600–1500 mm $year^{-1}$ (Jovanovic and Booth, 2002). Finally, Portuguese sites with precipitations above 1800 mm $year^{-1}$ are in mountain ranges in the North, at elevations often higher than 700 m, where minimal temperatures may be inappropriate for the species.

The distance from the sea was the second most important variable influencing wildling regeneration, which was more abundant near the coast than inland. Densities become minimal beyond 80 km from the sea. This variable is a surrogate of thermal range associated to continentality. The temperature range of this species is from mean monthly minima of -1–8ºC to mean monthly maxima of 13–25ºC, in the native territory; while recommendations for plantations indicate minima of -1–12ºC and maxima of 13–29ºC (Jovanovic and Booth, 2002). In fact, inland regions often have temperatures above the upper limit because the mildening effect of the ocean is small there. This is aggravated in the southern inland due to its lower latitude and much lower elevation. On the other hand, inland regions may also have temperatures below the aforementioned lower limit in winter, due to continentality. These temperatures are more extreme in NE Portugal, where a higher latitude and elevation reinforce the continentality coldening effect. The result concerning frost days is also related with this lower temperature limit. Wildling densities are very low in sites having more than 40–50 frost days per year, which correspond to NE Portugal and some high elevation sites in other regions. This result matches the maximum number of frost days of this species in its native range, which is 50 days at high elevations (Boland *et al.*, 2006). Together, these results demonstrate the temperature tolerance limits of the species.

The other environmental variables were much less influential to wildling density. The highest wildling densities were found at elevations between 200–600 m. This variable may be considered a proxy of local edaphoclimatic conditions. Highlands tend to have lower temperatures, as well as thinner and poorer soils, comparatively to lowlands. *Eucalyptus globulus* is a lowland species in its native territory, typically occurring from sea level up to 400–450 m, but also found in some sites up to 830 m (Williams and Potts, 1996; Boland *et al.*, 2006). However, in exotic territories with lower latitudes, it can be cultivated at higher elevations (Jacobs, 1979), evidencing the role of temperature for setting the altitudinal upper limit of *E. globulus.* In Portugal, lowlands predominate in the South and their climate is generally very hot and dry in the summer, for this species. The highlands are predominantly situated in central and northern inland, where low winter temperatures and frost are likely limiting factors for the species, as previously mentioned. Thus, both the lower and upper altitudinal limitations for wildling density are strongly related to temperature.

The fact that wildling densities are the highest at intermediate elevations, could explain why slope was retained in the model of wildling density drivers. This variable refers to the slope of the site (map resolution: 30 m), not to roadside slope (max. width 10 m). The increase of wildling density with increasing site slope is probably related to areas of intermediate elevation on hillsides. In Portugal, plains are mostly located in the South, in areas where climate is not very favourable for the species; plateaus are rare; and flat riverbanks are used for agriculture. Thus, wildlings are most likely located at sites with a relatively steep slope. The soil type was the least important variable that was identified as influencing wildling density. This result is in agreement with Jacobs (1979), who considers that soil factors are generally less important than climate in limiting development of *E. globulus*. Density of *E. globulus* wildlings differed amongst

different soils types. This species has been planted in many different types of soils worldwide (Jacobs, 1979). It develops best in deep, sandy clay soils, but it also grows in shallow humus soils, good loams, clay-loams, and clay soils, because they are well drained (Jacobs, 1979; Boland *et al.*, 2006). The principal limiting soil factors for this species are insufficient depth, poor drainage, salinity, strong alkalinity, and the presence of a high content of assimilable carbonates (Kirkpatrick, 1975b; Jacobs, 1979). The highest wildling densities were registered in cambisols and podzols. Cambisols are moderately developed soils, derived from a wide range of rocks, and having fine to medium texture (IUSS *et al.*, 2007). Cambisols with high base saturation in temperate regions are amongst the most fertile soils and are very appropriate for agriculture, while the more acidic ones are less fertile but still suitable for forestry (IUSS *et al.*, 2007). In fact, Portuguese regions with these soils had the largest number of roadsides with high wildling densities, except for those found in highlands or inland, where climate was likely unfavourable. Podzols are usually derived from siliceous parent material and have a bleached surface horizon over B horizon with illuvial humus and/or aluminium and iron (IUSS *et al.*, 2007). They are usually nutrient poor soils, but can be deep (IUSS *et al.*, 2007). In mild moist climates of eastern and southern Australia, they are able to support eucalypt forests, often with very tall trees (Boland *et al.*, 2006). Thus, they are suitable for *E. globulus* development. In opposition, the lowest wildling densities were observed in lithosols and luvisols. Lithosols are shallow soils with excessive drainage, composed of partially weathered rock fragments, and thus are limiting for tree development (IUSS *et al.*, 2007). Luvisols are a much more diversified soil group, whose soils are derived from a wide range of unconsolidated parent materials, under very different environmental conditions. They have a distinctive argic B horizon with accumulation of illuvial clay with a high base saturation (IUSS *et al.*, 2007). As this soil type is very diverse it is difficult to relate its characteristics with wildling density. For instance, calcareous luvisols in central Portugal have very low wildling densities, whereas other luvisols in SW Portugal bear high wildling densities. These high densities indicate that some luvisols are suitable for wildlings, or climate conditions provided by altitude and sea proximity override soil effect. Therefore, the several types of Portuguese soils differ in their capacity to hold high densities of wildlings and even good quality soils may lack wildling in sites where climate in not favourable.

1.5. Conclusions

The two methods used to assess the occurrence of *E. globulus* regeneration showed that it is a widespread phenomenon in mainland Portugal, with regional differences associated to the distribution of stands dominated by this species and to climate and soil conditions. Although the screening of NFI databases did not allow to distinguish sexual regeneration from vegetative, as well as artificial from natural regeneration, the researcher believes that its results are biased in favour of natural regeneration from seeds. In contrast, the roadside survey was quite accurate at detecting the presence of wildlings, provided they had h > 40 cm. In a way

worthy of being noted, the results of the latter confirm the geographical pattern of distribution obtained by the former.

Densities of *E. globulus* wildlings were only assessed by roadside survey, next to forests dominated by this species. These densities were affected by both climate and soil types, with the former having more influence than the latter. The highest wildling generally coincide with the most favourable environmental conditions for the growth of this species. As the model used to detect the main drivers of these densities only used site variables, the geographical pattern of wildling densities is potentially similar in eucalypt forests neighbouring sampled roadsides. Nevertheless, no extrapolations can be made, because forests and roadsides have biotic and management contexts that are substantially different.

The assessment of local phenomena, as regeneration from seeds, in wide territories is very resource-consuming and requires expeditious methods, at the expense of some accuracy. The two methods used had their own advantages and drawbacks. It is obvious that a specifically targeted survey is more effective. Conversely, the use of information from NFI can be more efficient and systematic and allows monitoring for long periods, with no added costs. The major drawback of NFI screening was the lack of distinction between the different types of regeneration, which is something that could be solved in the future, with little extra effort during NFI field work. Moreover, the use of information about the vertical structure of forests, both of past and future NFI databases, would be an effective tool for assessing the sexual regeneration of obligate seeder species, providing useful information for management of forests.

In conclusion, *E. globulus* regeneration from seeds is frequent in mainland Portugal and has a wide geographical distribution in this territory, which was never assessed on this scale before. This regeneration seems to be mainly associated to forests dominated by this species and, to a lesser extent, to pinewoods with many *E. globulus* individuals. These results denote a widespread capacity of *E. globulus* trees to produce offspring. Moreover, the observed densities in roadsides are very high in some regions showing not only the magnitude of the reproductive capacity of *E. globulus* forests, but also the species capacity to colonize areas very close to trees. Therefore, the results of this exploratory work compelled the researcher to do further studies on *E. globulus* regeneration from seeds in mainland Portugal, in order to better understand the conditions which affect its occurrence and densities.

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Chapter 2

Understanding the naturalization of *Eucalyptus globulus* Labill. in Portugal: a comparison with Australian plantations

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2.1. Introduction

Human-mediated transportation of organisms between different geographical regions is an ancient process. This movement has often been bolstered by human interests in particular species (Almeida and Freitas, 2001; Wilson *et al.*, 2009). At the global level, introduced species were used in 29% of afforestation and in 36% of reforestation programmes, between 2003-2007 (FAO, 2010). Human activity resulted in the naturalization (*sensu* Richardson *et al.* 2000) of at least 3.9% of all known vascular plants outside their original territories (van Kleunen *et al.*, 2015). The naturalization of these species is part of the invasion continuum, and although not all naturalized species become invasive, all invasive species became naturalized first (Richardson and Pyšek, 2012).

Progression along this continuum involves several phases (Williamson, 2006; Pyšek *et al.*, 2009) and Blackburn *et al.* (2011) highlight that naturalization is essentially a multi-generational population process. Drivers of naturalization are context dependent and may interact (Lozon and MacIsaac, 1997; Richardson and Pyšek, 2006; Essl *et al.*, 2011). Features related to introduction history, like residence time and propagule pressure, are strong determinants of naturalization potential (Lockwood *et al.*, 2005; Pyšek and Jarošík, 2005; Pyšek *et al.*, 2009; Simberloff, 2009). Other important factors include: the susceptibility of the recipient community to invasion, and environmental and anthropic factors (Catford *et al.*, 2009; Blackburn *et al.*, 2011; Procheş *et al.*, 2012; Richardson and Pyšek, 2012).

The particular conditions created in cultivated areas can either promote or prevent reproduction and seedling establishment from introduced plants (Richardson and Rejmánek, 2011). The purpose of the introduction, for example agriculture or forestry, often guides the selection of provenances for cultivation, aiming at high survival, growth, productivity, and/or reproductive output in the target habitat (Richardson, 1998; Thuiller *et al.*, 2006; Rejmánek and Richardson, 2011; Dodet and Collet, 2012). Cultivated forests can induce a high propagule pressure through the accumulation of large amounts of seed which may be released into the environment, especially after harvesting of reproductively mature plantations (Wilson *et al.*, 2009; Rejmánek and Richardson, 2011). Moreover, cultivation inherently protects and maintains founder populations from which new *foci* can be continuously recruited (Mack, 2000; Lockwood *et al.*, 2005; Wilson *et al.*, 2009). Concurrently, the disturbance associated with cultivation often fosters the establishment of exotic plants (Lozon and MacIsaac, 1997). However, management operations intended to improve crop conditions, for instance removing crop competitors or reducing fire risk, may kill or at least injure offspring of cultivated individuals (*e. g.* Águas *et al.*, 2014). Even harvesting can have detrimental side effects on recruited plants, depending on the harvesting techniques (McIver and Starr, 2000; Gonçalves *et al.*, 2004).

Pinus and *Eucalyptus* are the most important genera used as exotics in forestry (Rejmánek and Richardson, 2011). *Eucalyptus globulus* Labill. is probably the most widely cultivated eucalypt in temperate regions of the world (Jacobs, 1979; Rejmánek and Richardson,

2011). This species is native to SE Australia but it has been planted in many other world regions particularly in late 20th century to supply the pulp and paper industry (Potts *et al.*, 2004). In 2008, the global area of *E. globulus* was estimated to be around 2.3 x10^6 ha (Rejmánek and Richardson, 2011).

The naturalization of *E. globulus* outside its native range has been reported by several sources (*e. g.* Sanz-Elorza *et al.*, 2004; Cal-IPC, 2006). The drivers of wildling establishment close to plantations were studied in seven planting zones in Australia (mostly outside the native range) by Larcombe *et al.* (2013), and in Portugal by Catry *et al.* (2015) and by Fernandes *et al.* (2016). These studies found important regional-scale variables that influence wildling establishment, including temperature seasonality and rainfall. The only significant plantation-scale variable identified was the age of stems, in the Australian study. Another study by Águas et al (2014) assessed the role of some plantation-scale variables in Portugal but it was only restricted to burnt areas. This assessment was not targeted to professionally managed plantations and therefore included a wide range of *E. globulus*-dominated stand types. All three Portuguese studies (Águas *et al.*, 2014; Catry *et al.*, 2015; Fernandes *et al.*, 2016) considered that poor or lack of management may create more favourable conditions for wildling establishment. Therefore, it is expected that in industrial plantations where standard silvicultural practices involve more intensive management, wildling recruitment should be lower, and more comparable with similar plantations in other parts of the world. Larcombe *et al.* (2013) found that *E. globulus* in industrial plantations in Australia was a low-risk invader, given the overall low density of wildlings detected (8 plants ha^{-1}) within a 20 m strip along the stand edge. Fernandes *et al.* (2016) found higher densities in Portugal, within the first 15 m on either side of the border of managed plantations (102 plants ha^{-1} inside; 67 plants ha^{-1} outside). The density difference may be related not only to stand characteristics, but also to the very different ecological context of Portugal compared to Australia where the genus *Eucalyptus* is native. These biogeographical differences could arise through a reduced competitive environment (*e. g.* Xiao *et al.*, 2016) or through enemy release (*e. g.* herbivores or pathogens) in the introduced range (Keane and Crawley, 2002). However, despite the importance of a biogeographical approach to better understand the mechanisms that enable the higher success of some exotic species in their introduced ranges, studies using this approach are scarce (Hierro *et al.*, 2005; Parker *et al.*, 2013). Therefore, wider comparative studies are required for better understanding the ecology of plant naturalization and invasion. In the specific case of *E. globulus*, Portugal is an ideal territory for such an assessment since it has a long introduction history, it is one of the farthest regions from the native territory, and it has about 1/3 of the global area of plantations of this species (Rejmánek and Richardson, 2011; ICNF, 2013). The work by Fernandes *et al.* (2016) provides an interesting basis for such assessment but it was not designed with that purpose, and does not address important plantation-scale variables such as stem age, rotation or site index.

The present work aims at filling some of the aforementioned knowledge gaps, by studying the influence of plantation-scale drivers on the abundance of *E. globulus* wildlings in

Portugal. The study adopted a sampling methodology very similar to Larcombe *et al.* (2013) in Australia, allowing comparison of the results from the two antipodal regions. Therefore, this study specifically aims to: (1) assess the plantation-scale drivers of wildling establishment in a representative sample of *E. globulus* plantations in Portugal; (2) compare the results obtained in Portugal with data previously obtained in plantations from Australia, managed under a similar silvicultural regime; (3) use this comparison to provide insights into the naturalization process of one of the world's most widely cultivated tree species.

2.2. Material and methods

2.2.1. Sampling

The sampling was undertaken in the Lower Tagus valley in central Portugal (Fig. 2.1) a region representative of much of the area which is intensively managed for pulpwood production in central-southern Portugal. The latitude of sampling ranged from 39°32'02'' to 38°47'04'' (83 km) and longitude ranged from -7°46'52'' to -9°00'08'' (106 km). This region has relatively homogeneous climate, topography and soils. Most of the sampled plantations were located on Podzols (66%) or Cambisols (29%), and a few (5%) on Lithosols (Cardoso *et al.*, 1971). The studied region includes hilly areas and river flats with altitudes ranging between 30 and 307 m above sea level (according to GPS registrations during field work). Climate is Mediterranean with clear seasonality of temperature and precipitation, corresponding to type Csa (temperate with dry and hot summer) of the Köppen classification (AEMET and IM, 2011). The region has a considerable area occupied by *E. globulus* plantations. The inspection of land cover maps (IGP, 2010) within 10 km buffers centred on each sampling unit, showed that *E. globulus* plantations correspond to 32% of forest occupation and to 15% of the total surface in these buffers (Fig. 2.1). According to our knowledge of the area, this indicator of planting intensity can be considered representative of the whole sampled region.

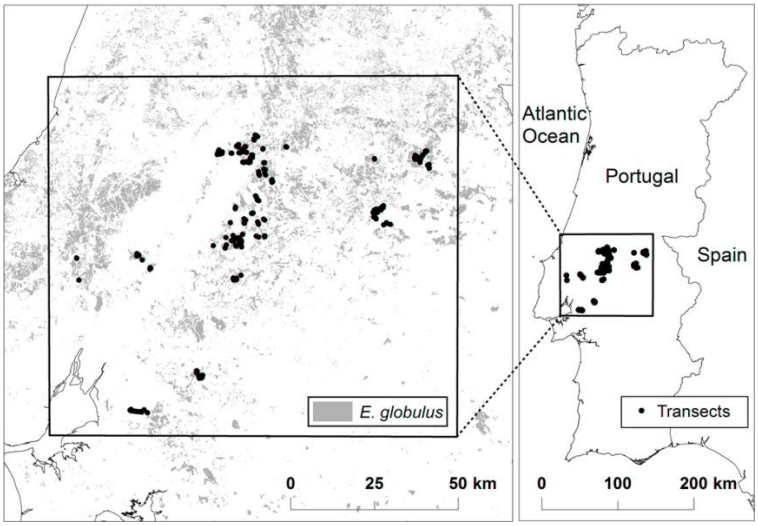

Fig. 2.1 - Location of sampled transects in Portugal. The *E. globulus* distribution map was based on land cover cartography (IGP, 2010)

All sampled *E. globulus* plantations were owed by Altri Florestal S.A. (hereafter Altri), one of the two major Portuguese pulp companies. Altri manages an overall *E. globulus* estate of about 63,500 ha, roughly corresponding to 8% of the total area of eucalypt-dominated plantations in Portugal and to 40% of the total surface managed by pulp companies (CELPA, 2015). Altri's plantations are usually coppiced every 10–14 years, over 2–3 rotations. Sampling was planned to include a balanced number of plantations from all rotations.

The field work protocol was based on Larcombe *et al.* (2013), in order to compare wildling densities between Portuguese and Australian plantations. Our sampling units were transects established along plantation borders, aimed at assessing the number of wildlings per surface unit. A plantation border was defined as a virtual line that crossed the boles of neighbour border trees. Border segments were eligible for sampling as long as they were: neighbouring a different land use; located at least 50 m away from another eucalypt plantation at reproductive stage; as well as homogeneous in terms of aspect and slope. More than one transect could be sampled along the border of each plantation. Given that high densities of wildlings were frequent, plant counts were performed using plots (sub-samples) set along each transect, and not along the whole transect as in Larcombe *et al.* (2013). Plots were squares (20 x 20 m) straddling the plantation border, divided in two halves. One half-plot (10 x 20 m) was established inside the plantation and the other half was established outside. The first plot of each transect was established 50 m after the starting point, and the remaining were established every 200 m farther along the transect. The geographical coordinates of plot centres, start point, and end point of each transect were registered with a handheld GPS. The numbers of plants inside and outside the plantation were recorded separately. Wildlings were distinguished from resprouts or planted individuals by checking their position and their size relatively to the planted trees, and by checking the presence of a lignotuber. Conservative criteria were used, so that only plants with strong evidence of being wildlings were retained. In total 514 plots were sampled along 213 transects established in 116 *E. globulus* plantations. The average number of transects per plantation was 1.84±0.12 (min. = 1, median = 1, max. = 8). The average number of plots per transect was 2.41±0.10 (min. = 1, median = 2, max. = 10).

2.2.2. Variables and datasets

Seven plantation-scale variables were used to model the abundance (density) of wildlings (Table 2.1) in the sampled region. These variables included information provided by Altri on: the developmental stage of the plantation (age, rotation), site index (see below), understory management and topography. Since tree age (corresponding to stump age in coppiced plantations) is correlated with the number of rotations, we only retained the latter for further analysis (variable 'rotation') and time-since-last coppice/planting was used as the age of tree stem (variable 'stem age'). Site index in *E. globulus* plantations is the height of dominant trees at 10 years. The original values (min.= 9.0 m, median = 18.8 m, max. = 22.5 m) were reclassified according to three categories (variable 'SIcat') with similar frequencies: low (< 18 m); medium (18 m $\leq$ SIcat < 20 m); and high (≥ 20 m). The topographic information

provided by Altri was originally obtained from 1:25,000 cartography produced by the Geographical Institute of the Portuguese Army (IGeoE). Altitude (variable 'altitude') was simply divided in two classes: low (≤ 100 m; a minimum of 30 m) and high (>100 m; a maximum of 307 m). Slope (variable 'slope') was divided in three classes: flat (0%); gentle (≤ 15%); and moderate (> 15%). This latter class included 12 transects with slope > 30%. Topographic aspect (variable 'aspect') was defined by two classes: wet (North and West) and dry (South, East). Flat slopes were discarded from this variable. Management information was considered relevant due to the potential disturbance caused to wildling establishment. Management information (years 2009–2014) was also checked with local managers and field observations. Two types of management operations (disturbances from an ecological point of view) were considered: mechanical cutting (tractor with rotary mower) and tillage (tractor with disc harrow). For analysis purposes only the last disturbance was retained for each transect, as it was considered the most relevant to explain wildling density. Therefore, management information was condensed in one single variable 'LD' – last disturbance (none, cut or till). The 'none' level includes transects in plantations where no management operations had been registered over the considered period. The average time-since-last intervention was 2.0 years for cutting and 0.7 years for tillage.

Table 2.1 - Explanatory variables, factor levels and corresponding number of transects, used in the BRT model. See text for detailed description

Variable	Description	Factor levels	Frequency (*n* transects)
Stem age	Age of tree stem (years)	–	–
Rotation	Plantation rotation	1 2 3	68 73 72
SIcat	Site index category	Low Medium High	57 86 70
Altitude	Altitude class	Low High	87 126
Slope	Slope class	Flat Gentle Moderate	63 92 58
Aspect	Aspect class	Dry Moist	82 68
LD	Last disturbance	None Cut Till	43 47 123

2.2.3. Data analysis

Collinearity among explanatory variables was checked by computing their VIF – variance inflation factors (function corvif from the R package AED; Zuur *et al.*, 2009). VIF values were all below 1.5, so all variables were retained in further procedures. Wildling density (plants ha^{-1}) was used as a dependent variable in a model developed using boosted regression

trees – BRT (Elith *et al.*, 2008) fitted with a Poisson distribution (R package dismo; Hijmans *et al.*, 2016). BRT modelling is a machine-learning technique that is able to automatically identify and model interactions, it is flexible in handling different types of data and predictors, and it has high predictive power compared to other methods (Elith *et al.*, 2008). BRT combine two basic algorithms: regression trees and boosting. Regression trees are built through successive binary splits of the response variable; each split results in two groups as homogeneous as possible, according to a simple rule, based on the most influential variable (De'ath and Fabricius, 2000). Boosting is a method that combines many simple models (in this case many regression trees) and it allows an improved predictive performance (Elith *et al.*, 2008). The relative importance of each explanatory variable is based on how often that variable is selected for splitting.

Given the recurrent disturbance of the outer half plot, owing to the existence of roads and fire-breaks close to the plantation border, modelling included only the inner half allowing to obtain more clear effects from the variables of interest. An initial model included the number of plots in each transect as a covariate to enable the detection of sampling bias. Modelling procedures followed the recommendations from Elith *et al.* (2008) and Elith and Leathwick (2016). The model for inside density was performed using: 2500 trees obtained from cross-validation; a bag fraction of 0.5 (proportion of data to be randomly selected at each step); a learning rate of 0.001 (the contribution of each tree to the growing model); a step size of 50 (initial number of trees); and a tree complexity of 5 (the number of interactions within a single tree). Model performance was assessed through the percentage of explained deviance. Differences in wildling density between the inner and the outer half plots were assessed using a Wilcoxon test.

In a separate analysis, data from Portuguese stands were pooled with those from plantations of seven planting zones (hereafter 'regions') in Australia, studied by Larcombe *et al.* (2013). Similarly to this latter work, wildling density was considered within the whole plot (inside and outside the plantation). In order to assess the relative influence of the 'country' factor, a generalized linear model (GLM) with a negative binomial error distribution and a log link (in order to account for overdispersion) was fitted to the wildling density data. Variables 'country' (two levels: Portugal and Australia), 'stem age' (continuous variable) and 'rotation' (two levels: first rotation and coppiced plantations), were used as explanatory variables from the joint dataset combining the results of the Portuguese and the Australian surveys. In this analysis we discarded burnt transects because this variable was unevenly distributed, being relevant only in one Australian region (Gippsland). This model was simplified using a backwards stepwise procedure where single non-significant terms were removed, and the subsequent simplified model was compared with the previous model using Chi-square tests. Higher order interaction terms were removed first, followed by lower order terms, starting with those with the highest *P*-value. Diagnostic plots of residuals *vs.* fitted values and QQ-plots were used to check respectively for heterocedasticity and residual normality.

Wildling density in Portugal was compared separately to each Australian region. This pairwise approach was used because variation in wildling density, as well as vast distances

between regions, meant an average wildling value for Australia could be misleading. Mann-Whitney tests were used to perform these comparisons, and the same procedure was used to test differences in stem age. All transects from each region were included to perform these pairwise comparisons. No *P*-value adjustment was introduced in the results of the multiple tests (*e. g.* Rothman, 1990). All analyses were performed using R software (R Core Team, 2015).

2.3. Results

2.3.1. Wildling establishment in Portugal

Overall 2604 wildlings were counted across the 213 transects. Wildling density varied widely, showing a strongly right-skewed distribution, with 38% of zeros. Mean wildling density was 128.4 ± 24.0 plants ha^{-1} (min. = 0; med. = 25; max. = 4000). Inside densities had a mean value of 180.8 ± 27.5 plants ha^{-1} (min. = 0; med. = 25; max. = 2950). Outside densities showed higher data dispersion with a mean value of 76.1±31.4 plants ha^{-1} (min. = 0; med. = 0; max. = 6375). According to the Wilcoxon test, inside wildling densities were significantly higher than those outside ($P < 0.001$). However higher densities were found further than 10 m from the plantation edge (data not shown); plants were from a range of different sizes, with about half of the individuals above 1.3 m height (data not shown).

The BRT model explained 43% of deviance and showed that the most influential variables explaining wildling density inside plantations were: ‘aspect’, ‘rotation’, ‘stem age’, and ‘SIcat’ (Fig. 2.2). These variables accounted for 80% of importance in the model. The remaining

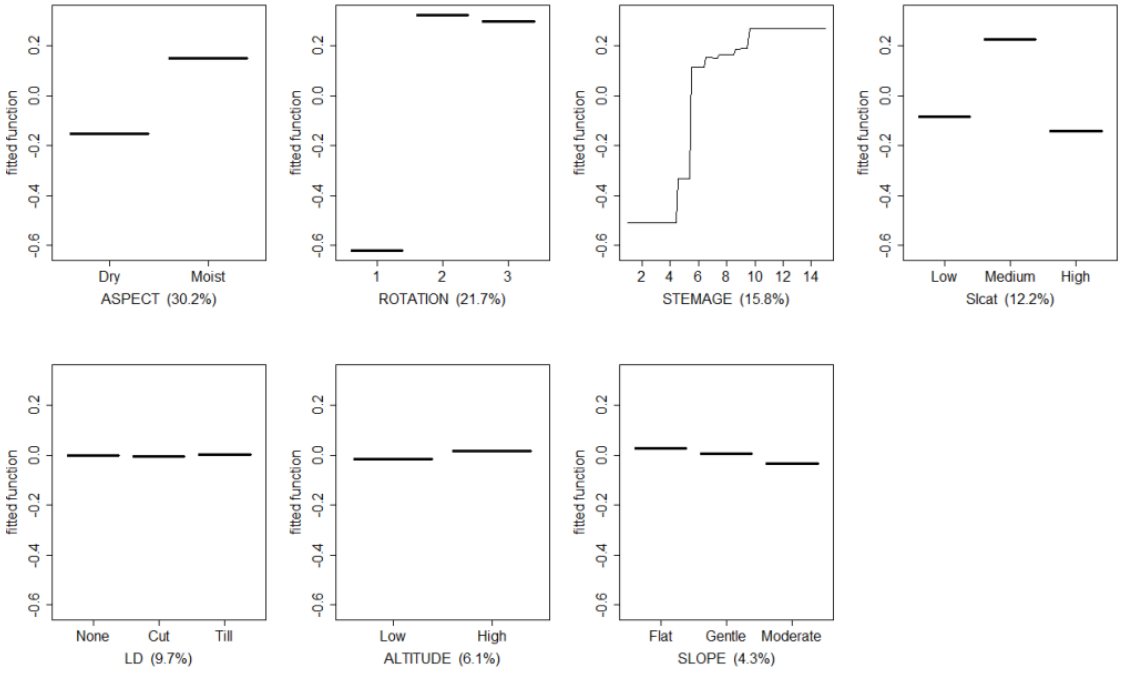

Fig. 2.2 - Partial dependence plots produced by the BRT model showing the relationships between the seven studied variables and wildling density. See Table 2.1 for variable description

three variables were all below 10% in importance. An initial checking run including the number of plots per transect, showed that this covariate had no particular effect on the model results (an importance of only 6.3% with no particular trend). The BRT model showed that wildlings are more abundant in moist aspects, in second and third rotations, in plantations with older tree stems and in intermediate site index categories. The results from the remaining variables were not conclusive, since the fitted function values were relatively close among factor levels.

2.3.2. A comparison with Australia

The characterization of the surveys performed in Portugal and in the seven Australian regions studied by Larcombe *et al.* (2013) is presented in Table 2.2. Although the length of surveyed plantation border was similar between Portugal and some Australian regions, the number of transects was in all cases much higher in the former. A considerable difference was also noticed in: the proportion of transects in coppiced plantations (much higher in Portugal); the mean age of stems (lower in Portugal; all $P < 0.001$); and the proportion of transects in replanted plantations. According to Altri registrations, plantations in Portugal were nearly all (> 95%) replanted in sites previously occupied by *E. globulus* plantations. It must be noticed that the concept of second rotation adopted in Larcombe *et al.* (2013) included both the coppiced plantations and the replanted plantations. Here, the concept of second and third rotation refers only to plantations that were coppiced once and twice, respectively. Plantations surveyed in only one Australian region (Gippsland) were considered to be within the local native range of *E. globulus*, and this was also the region with the highest number of transects in burnt plantations. The overall wildling density (total number of wildlings divided by the total sampled surface) in Portugal was 6.4 times higher than the highest overall density obtained in Australia (in the Manjimup region) and 14.9 times higher than in the Australian regions altogether. In a subset of unburnt first-rotation plantations this ratio was reduced to 3.1.

Table 2.2 – Characteristics of the survey in central Portugal compared with data from seven Australian regions. Australian data are according to Larcombe et al (2013)

Region	Surveyed plantation border (km)	No. transects	% transects in coppiced plantations	Mean stem age (years; ±SE)	% transects in replanted plantations	% transects in burnt plantations	Native range	Overall density [a] (plants ha^{-1})
Portugal	70.7	213	68.1	7.0±0.19	>95	2.8	No	126.7
Albany	56.1	31	12.9	10.1±0.51	12.9	0.0	No	11.3
Manjimup	21.5	33	24.2	10.8±0.58	48.5	0.0	No	19.8
Grampians	17.2	19	0.0	10.9±0.29	0.0	10.5	No	18.1
Penola	64.2	49	0.0	11.7±0.15	0.0	0.0	No	0.6
Portland	44.5	40	0.0	11.2±0.21	0.0	0.0	No	16.7
Gippsland	60.4	59	0.0	14.8±0.33	0.0	62.7	Yes	4.3
Tasmania	26.5	38	0.0	11.4±0.25	0.0	0.0	No	2.0

[a] Computed using the overall number of wildlings divided by the overall sampled area (400-m^2 square plots in Portugal; continuous survey along transects in Australia)

The generalized linear model (Table 2.3) shows the importance of 'country' in explaining wildling density, within the ensemble of the three explanatory variables. The model coefficients of 'country' and 'rotation' were similar and considerably higher than the coefficient of 'stem age'. This last variable also was less significant than the others in the model. The GLM showed that wildling density is higher in Portugal, in coppiced plantations and in plantations with older stems (Fig. 2.3). Interactions among variables were not significant and were not included in the final model. The model fitted with these three variables was able to explain 22.2% of deviance. When comparing the wildling densities found in Portugal against each Australian region separately, the Mann-Whitney tests showed that wildling densities in Portugal were significantly higher than in all Australian regions except Portland, but the detected differences varied widely in their significance (Fig. 2.4).

Table 2.3 – Generalized linear model fitted with *E. globulus* wildling densities in 437 transects established in industrial plantations from Portugal and Australia, using data from this study and from Larcombe *et al.*(2013)

Variable	**Coefficient (± SE)**	***P*-value**
Intercept	0.760 ± 0.534	1.55×10^{-1}
Country		
Portugal	1.466 ± 0.373	8.34×10^{-5}
Rotation		
coppiced	1.785 ± 0.317	1.78×10^{-8}
Stem age	0.142 ± 0.045	1.50×10^{-3}

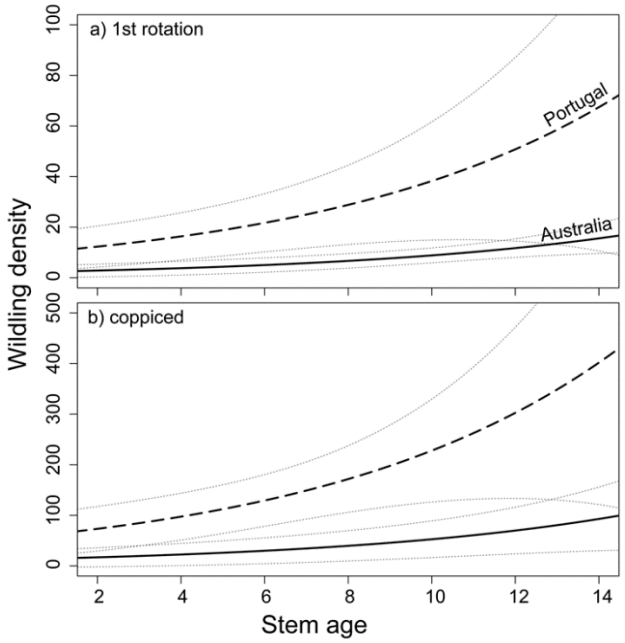

Fig. 2.3 – Relationships between stem age and wildling density (plants ha^{-1}) in **a)** first rotation unburnt plantations and in **b)** coppiced plantations, surveyed in Portugal and Australia, according to a GLM fitted using a database combining data from this study and from Larcombe *et al.* (2013). Dashed thin lines correspond to 95% confidence intervals. Note the different scales of the y-axis in each graph

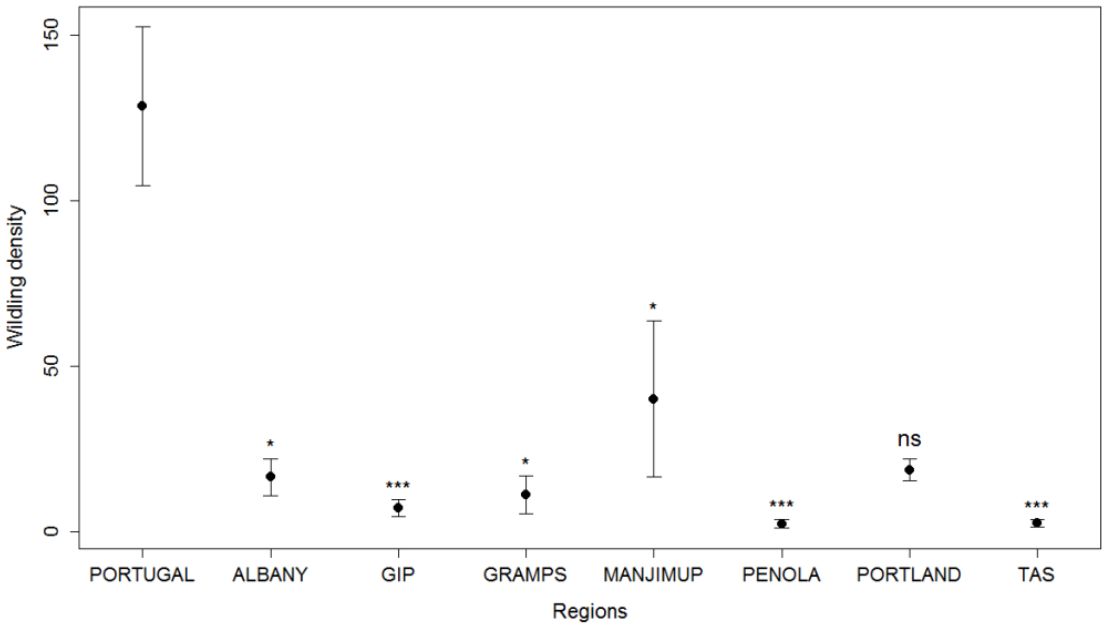

Fig. 2.4 – Wildling density (plants ha^{-1}; mean ± SE) in Portugal and in seven Australian regions using data from this study and from Larcombe *et al.* (2013). GIP stands for Gippsland, GRAMPS stands for Grampians and TAS stands for Tasmania. Asterisks represent the result of pairwise comparisons through Mann-Whitney tests, between Portugal and each Australian region (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns – non significant)

2.4. Discussion

2.4.1. Plantation-scale drivers of wildling establishment

We found that plantation-scale factors explained a significant component of the variation in wildling density along the borders of Portuguese *E. globulus* plantations. Topographical aspect was the most important driver of wildling density. Wildling density was higher on moist than on dry aspects. The wildling preference for moist/cool aspects might be related with edaphoclimatic constraints. The Portuguese study region is located on the transition zone between Atlantic and Mediterranean climates. Mean temperature in the vast majority of sampled sites in this region is close to or above the maximum found in the planting zones of *E. globulus* in Australia (Larcombe *et al.*, 2013). More than half of the studied area in Portugal has annual precipitation close to the lower limit for its natural distribution (Kirkpatrick, 1975; Jacobs, 1979; Turnbull and Pryor, 1984) and precipitation is clearly seasonal with a dry season of four months. This contrasts with the preference for reduced seasonality in precipitation (Larcombe *et al.*, 2013), especially if annual precipitation is close to its lower limit (Kirkpatrick, 1975). Therefore, drought (especially during summer) may limit wildling establishment in this region. This is in agreement with several references that identify either drought or frost as the main limiting factors for *E. globulus* development in different regions of Portugal (Almeida *et al.*, 1994; Ribeiro and Tomé, 2000; Alves *et al.*, 2012). In addition, drought can be aggravated in areas where soils have little water holding capacity, such as podzols that are the commonest soil type in the southern part of the sampled region. Actually, in regions where climate induces reduced water

availability, *E. globulus* persists only where mitigating factors exist such as southern aspects (in southern hemisphere), along streams (Kirkpatrick, 1975), and in deep moist soils (Jacobs, 1979). Moreover, González-Muñoz *et al.* (2011) showed that this species is more successful under moderate light and high water availability conditions, normally associated with deeper soils. In Portugal these conditions are more frequently found in northern and western aspects (Marques and Mora, 1992; Boer *et al.*, 1996).

There may be several reasons why wildlings were more abundant in coppiced than in first rotation plantations. Firstly, coppiced plants have well-developed root systems and are likely to reach maximum reproductive capacity sooner than seed-grown first rotation plants. Secondly, site preparation for plantation establishment involves ploughing, and may result in fewer residual seeds than in coppiced plantations where slash (canopy branches) carrying unopened capsules is left on the soil surface. Moreover, slash may act as a buffer against drought and predation, helping seeds that dehisce from the slash to germinate and establish (Fagg, 2001). In line with this, Skolmen and Ledig (1990) note that prolific germination occurs after logging *E. globulus*, resulting in abundant seedling recruitment. In addition, seed might be released from capsules on the ground over an extended period. For example, Santos *et al.* (2015) found that capsules on the ground, presumably aged 0-6 years, may contain significant proportions of viable seeds. Although we could not assess the effect of previously existing plantations, because nearly all plantations in the Portuguese study region had been replanted, these mechanisms might be equally valid in this case, contributing to explain the role of residence time in the naturalization of woody plants (Pysek *et al.*, 2009).

Wildlings were also more frequent in stands with older stems, as was found in Australian plantations (Larcombe *et al.*, 2013). This pattern could be related with the production and the release of seeds from trees. *Eucalyptus globulus* starts seed production in the wild approximately seven years after germination or crown destruction (Kirkpatrick, 1975), while, in fertilized plantations, trees can produce seeds at three-five years old (Turnbull and Pryor, 1984; Barbour *et al.*, 2008b). Our results match this age range.

Wildlings were more abundant in plantations with site index between 18 and 20 than in better or worse sites. It would appear as a logical preposition that wildlings would have more resources to thrive in the best sites. However, the sensitivity of *E. globulus* to asymmetrical intraspecific competition (Tomé *et al.*, 1994) may reverse this preposition. Indeed, under asymmetrical competition, the better suited the conditions are for *E. globulus* growth, the stronger this competition is and the earlier it begins (Tomé *et al.*, 1994). In cases of very strong and/or prolonged asymmetrical competition among eucalypts, the smaller plants may not be able to thrive (Florence, 1996). Hence, the low density of wildlings in high site index plantations may be a result of suppressive effects from the planted trees. There is also the possibility of a trade-off between growth and reproduction, with plants allocating more resources to growth in the highly competitive environments of eucalypt plantations located at the best sites (Suitor *et al.*, 2010). Meanwhile, in the plantations with the lowest site indexes, wildlings might have faced difficulties to thrive owing to more adverse abiotic conditions, like drought or nutrient availability.

The results for the remaining three studied variables were not conclusive, given the low importance attained in the variable selection process for building the regression trees. Nonetheless, it is worth mentioning the unexpected lack of importance of the management variable ('LD') and the very small differences between the different levels in the partial dependence plot. This result may have several explanations. Firstly, it may be partially explained by uncertainty in the management data. It is possible that not all recorded management operations have directly affected the relatively small area surveyed by each individual transect plot. For instance, the cutting and tillage operations were restricted to inter-rows, leaving the space between trees within the rows undisturbed and available for wildling establishment. In some cases, operations were not continuous (covering one inter-row in every two) potentially also contributing to the obtained results. Finally, as sampled sites are owned by a pulp company, the absence of cutting and tillage operations in some sites was likely due to low density of wildlings and other understorey vegetation, which would not require mechanical control. Although our results apparently contradict those found by Águas *et al.* (2014) in central and northern Portugal, the difference may be due to the different methods used to assess management, as well as to different sample characteristics. Additionally, the registration of management operations in Águas *et al.* (2014) was restricted to direct field evidence in areas that had been completely burnt and in many cases submitted to salvage logging operations and replanting. In such cases, the effect of tillage can be more effective in reducing wildling density, as it normally affects the whole surface. Moreover, the so-called unmanaged sites sampled by Águas *et al.* (2014) were more likely to be abandoned plantations as opposed to the ones sampled in the present study.

Nonetheless, disturbance factors definitely played a role in the resulting wildling density. In our study wildling densities were generally higher inside the plantation than outside, similarly to the results obtained by Larcombe *et al.* (2013) and by Fernandes *et al.* (2016). The type and the frequency of disturbance inside plantations can be significantly different from what happens outside. Plantations in the study region were usually delimited by dirt roads and firebreaks. Besides disturbance caused by traffic and trampling, these areas are exposed to maintenance operations, mainly for fire prevention. This contrasts with the less recurrent disturbance regime inside the plantations, therefore influencing the abundance of naturally regenerated eucalypt plants in either side of the plantation border. The existence of higher wildling densities in undisturbed areas beyond 10 m from the plantation edge (unpublished data), somehow confirms this explanation. Another possible reason contributing for this difference may be related with a potentially more intense seed rain inside than outside the plantation.

2.4.2. Establishment success compared to Australian plantations

The overall density of naturally regenerated plants found in the Portuguese *E. globulus* plantations was substantially higher than in the comparable Australian study by Larcombe *et al.* (2013). On the one hand, Portugal has a longer history of *E. globulus* domestication and plantation establishment than Australia (Doughty, 2000). On the other hand. eucalypt species

have evolved over millions of years on the Australian continent (Thornhill *et al.*, 2015). The comparison between the two studies thus involves different biogeographic contexts and different introduction histories in each specific region, including different sources of germplasm, planting intensity and residence time. While the observed difference is consistent with expectations of naturalization and invasion syndromes such as release from competition (Xiao *et al.*, 2016) or enemies (Keane and Crawley, 2002), there are numerous other factors which are confounded in the comparison between the countries, and decoupling these effects is a major challenge.

Part of the observed 'country' differences could be explained by differences in plantation-scale factors related with introduction history, including the proportion of coppiced stands and the proportion of replanted stands. Both these factors are associated with a longer residence time and a higher propagule pressure, therefore potentially contributing to the greater wildling density observed in Portugal. While the effect of replanting could not be tested, coppicing was proven to increase wildling density as discussed above. However, even after accounting for coppicing, we still found a large effect of 'country' in the GLM analysis, suggesting other factors may be contributing to higher establishment in Portugal. These may include other non-studied plantation-scale factors, but also other more general biogeographical features that determine the ecological context in each of the two countries.

Genetic factors may partly explain these differences. It has been shown that the Portuguese landrace had its origin predominantly in southern and south-eastern Tasmania (Lopez *et al.*, 2001; Freeman *et al.*, 2007) while Australian plantations are likely to have a larger component of their germplasm from the three Victorian subraces, including the Strezlecki Ranges subrace (Potts *et al.*, 2014). It has been reported that this subrace has lower fecundity and produces smaller seeds than those from southern and south-eastern Tasmania (McGowen *et al.*, 2004b). Bigger heavier seeds produce seedlings with faster initial growth and may improve establishment success (Simberloff, 2009). Thus, differences in germplasm may result in Portuguese plantations producing more seed that is better able to establish than the Australian plantations, possibly contributing to differences in wildling densities between the two countries. This higher reproductive output of Portuguese plantations has potentially been compounded by a longer residence time, when compared to Australia. This is in agreement with Donaldson *et al.* (2014): reproductive output of exotic species increases wildling densities over time, and this effect gradually overwhelms the effects of other factors over the course of residence time.

It is also possible that natural and/or artificial selection has increased the fecundity and/or establishment capacity of the Portuguese germplasm relative to the Australian plantations. *Eucalyptus globulus* was introduced in Portugal in the 1850s where it was extensively planted from the mid-20th century (Radich, 1994) and now corresponds to 26% (around 0.8 million ha) of the forested area (ICNF, 2013). In contrast, almost all Australian plantations of *E. globulus* were established after 1996 and today cover around 0.5 million ha (Gavran and Parsons, 2011; Gavran, 2015), predominantly outside the natural range of the

species (Barbour *et al.*, 2008b; Larcombe *et al.*, 2013). The short history of Australian plantations means that there has been limited opportunity for adaptation. On the contrary, the Portuguese *E. globulus* landrace has been influenced by bottlenecks and genetic drift (Borralho *et al.*, 2007), and by both natural (Almeida, 1993; Freeman *et al.*, 2007) and artificial (Lopez *et al.*, 2001; Potts *et al.*, 2004) selection which may have resulted in better adaptation of *E. globulus* to the plantation environment (Almeida, 1993). There is certainly evidence that after introduction, natural selection has acted to improve *E. globulus* adaption to frost in Portugal (Almeida, 1993) and to drought in Spain (Turnbull and Pryor, 1984; Toro *et al.*, 1998). While artificial selection has been mainly focused on improving growth and wood quality traits (Borralho *et al.*, 1992; Borralho *et al.*, 2007), flowering precocity and fecundity may have also been enhanced (Borralho *et al.*, 1992; L. Leal, pers. com.). There are two sources of evidence suggesting that the selection of mother trees may have increased seed production in Portuguese plantations: (i) 37.6% of trees at plantation borders have capsules in our Portuguese study region (unpublished data; 25 plantations; stem age: 3–10 years), while in the Australian plantation estate, reproductive output of border trees ranges from 7.8% (age 3–10 years) to 29% (age 8–12 years) (Barbour *et al.*, 2008a; Larcombe *et al.*, 2014); and (ii) we observed small wildlings which were reproductive in the juvenile phase in the Portuguese survey but these were never observed in the Australian survey (M. Larcombe, pers. com.). Thus, better adaptation to the plantation environment in Portugal, including frost and drought resistance, may have increased establishment potential, while artificial selection of traits that improve seed production may have increased net fecundity. These signals of adaptation may contribute to increasing reports of naturalization (*e. g.* Silva and Marchante, 2012; Silva and Tomé, 2016) since large areas of plantations were established in the mid-20th century. Concomitantly, adaptation may also help explaining the differences found in wildling densities between Portugal and Australia. In addition, the synergy between longer residence time and larger reproductive output in Portugal than in Australia may strengthen this idea (Donaldson *et al.*, 2014), no matter the origin of fecundity differences between Portuguese and Australian plantations (*i.e.* introduced germplasm or evolution).

It is unlikely that differences in the abiotic environment would favour seedling establishment in Portuguese compared to Australian plantations. Larcombe et al (2013) found that establishment was more likely in conditions that were similar to the native range of *E. globulus*. Therefore, if abiotic factors are important, it would be expected that abiotic conditions in Portugal would be more similar to the species native range than are the Australian planting regions. However, the climate in the studied region in Portugal is relatively distinct from both the native region in Australia and the Australian plantation regions. In central Portugal precipitation tends to be less and seasonality is clearly stronger than in those Australian regions (Kirkpatrick, 1975; Larcombe *et al.*, 2013), and these conditions are compounded by major differences in soil types. In fact, other regions in Portugal, where wildlings are even more abundant than in the region assessed in this study, have climatic conditions that resemble more the native region

(Catry *et al.*, 2015). Hence, abiotic conditions do not seem to be the main factor explaining the high wildling success observed in many sites in the current study.

On the contrary, biotic interactions may have contributed both to higher reproductive success and increased establishment potential in Portuguese plantation environments. These include: release from competition, enemy release, and build-up of mutualistic relationships. Given the industrial setting of both study regions it is unlikely that release from competition would drive large differences in establishment. However, one possible interaction that could influence reproductive success is release from competition for pollinators. Australian plantations may experience competition for pollinator resources with neighbouring native eucalypt species, and such pollinator competition may be absent from Portugal. On the other hand, despite *E. globulus* being exotic in the Iberian Peninsula, there are reports pollination by birds (Calviño-Cancela and Neumann, 2015) and bees (*Apis mellifera*), (H. Águas, pers. com.; Feás *et al.*, 2010) and no obvious pollinator limitation. Indeed, Moncur *et al.* (1995) showed that the introduced *A. mellifera* improves seed production and maintains outcrossing of *E. globulus* in Tasmania. Flowers which are insufficiently fertilised abort (Suitor *et al.*, 2008) and, while higher proportion of trees with capsules in Portuguese plantations most likely reflects selection for fecundity, the possibility of more effective pollination services than in the Australian plantations cannot be dismissed. However, pollinators of *E. globulus* are generalists in its native range (Hingston *et al.*, 2004), and having related plant species nearby may actually increase pollinator services in Australia relative to Portugal where the pollinator community is likely to be less diverse. Conversely, the large area occupied by plantations of this species in Portugal (namely in the studied region), compared to the Australian estate, may significantly facilitate pollination and fruit production.

The enemy release hypothesis may be more relevant to the current study than release from competition. Strauss (2001) reports that Australian eucalypt plantations acquire more pathogens and pests from neighbouring native forests than plantations of species exotic to Australia. In contrast, in Portugal *E. globulus* had no major pests or diseases for more than 100 years, with few local pests or pathogens adapting to eucalypts (Goes, 1977). Only recently, has *E. globulus* been significantly attacked by species of phytophagous insects and fungi, most of which were inadvertently imported from Australia (Branco, 2007). In Portugal local browsing species do not feed on *E. globulus* (Catry *et al.*, 2007), and its juvenile leaves are unpalatable to stock (Jacobs, 1955). This is in marked contrast to the susceptibility of *E. globulus* juveniles to marsupial browsing in Australian plantations (Borzak *et al.*, 2015). In fact key biotic interactions that could contribute to the success of wildlings in Portugal are escape from pre- and post-dispersal seed predators. In Australia, wasps of the genus *Megastigmus* are significant predators of seed held in capsules in the canopy of *E. globulus* (McGowen *et al.*, 2004a). Seed harvesting ants are also known to deplete dispersed eucalypt seeds and limit plant establishment in Australia (Ashton, 1979) contrasting with recent findings in Portugal, showing that a large proportion of post-dispersed *E. globulus* seeds remains unharvested (unpublished data).

The build-up of mutualistic relationships may also be an important factor in the different recruitment profiles between the countries. As discussed above, site-level residence time is far greater in Portugal where multiple plantings and rotations of *E. globulus* have occurred on most sites. However, in Australia, the plantations assessed were typically on sites never previously planted with *E. globulus*. This difference raises the possibility that plantations in Portugal have developed better mutualistic interactions with other organisms that promote wildling establishment, than Australian plantations where they have had less time to develop. Positive soil conditioning, where the presence of a species changes the biological, structural and physio-chemical properties of a soil so that establishment of that species is facilitated, is a prominent theory in the invasion/naturalization literature (Suding *et al.*, 2013). There is evidence that eucalypt plantations do positively condition soils (Orozco-Aceves *et al.*, 2015), and Marchante *et al.* (2001) found frequent ectomycorrhizal associations between several native fungi and *E. globulus* in Portugal. Ectomycorrhizal fungi are potentially a biotic component of soil conditioning, so it is possible that the build-up of mychorrizal communities at the site level over time contributes to establishment of wildlings. It has previously been suggested that these associations may be crucial for exotic establishment of eucalypt seedlings (Rejmánek and Richardson, 2011). It is also conceivable that these relationships could be especially important in our study region where poor soils and water stress are frequent. However, *E. globulus* plantations in Australia are likely to share mycorrhizal species, pest enemies, and pollinators with neighbouring native species (Strauss, 2001), meaning that Australian plantations may come pre-primed with mutualists. Therefore, in the current contrast the effect of mutualistic relationships is unclear but cannot be dismissed.

2.5. Conclusions

This work is the first to assess plantation-scale drivers of wildling establishment in industrial *E. globulus* plantations in Portugal and to compare wildling densities with those in regions within the native range of the *Eucalyptus* genus. Plantation-scale drivers seem to explain a considerable amount of variation in wildling density in the studied Portuguese region. Aspect was the most important variable, leading to the conclusion that water stress is an important factor limiting wildling establishment. The significant effect of stand rotation and stem age also highlight the role of propagule pressure. At present, the observed wildling densities, with plants of different size, some of them showing reproductive capacity, strongly confirms the naturalization status of the species in Portugal. Meanwhile the observed densities at some plantation borders may require control, as they increase fire hazard and may become new propagule sources. Indeed, the location of observed wildlings outside the plantation boundary points to a potentially more relevant issue – their escape to other land uses. Where there is such a concern, it has been suggested that this risk may be minimised with suitable management of plantation boundaries (Calviño-Cancela and Rubido-Bará, 2013; Catry *et al.*, 2015; Fernandes *et al.*, 2016). Comparisons between Portugal and several Australian regions

showed that wildling density in Portuguese plantations was considerably higher. Identifying the precise differences that are causing variation in wildling recruitment between two regions so far apart is virtually an impossible task. Nonetheless it is very likely that factors related with introduction history (namely residence time) should partly explain the observed differences, possibly along with the biogeographic context of the two regions. In fact, biogeographical factors may be at least as important as introduction history, but further studies are needed to shed additional light on the main naturalization drivers of *E. globulus*.

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Chapter 3

Natural establishment of *Eucalyptus globulus* Labill. in burnt stands in Portugal

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3.1. Introduction

Given the expansion of exotic tree plantations in the world (MCPFE, 2007; FAO, 2010), the study of ecological processes associated with these forests is increasingly important. Some of the most relevant issues are related to the naturalization or to the invasive potential of exotic tree species, since significant interactions with the native ecosystems are possible (Richardson, 1998). Disturbances in general and fire in particular are known to facilitate the recruitment of different exotic species (*e. g.* Anderson and Brown, 1980; Mandle *et al.*, 2011; Arianoutsou and Vilà, 2012; Vallejo *et al.*, 2012). Therefore, the fire-mediated naturalization of planted exotic trees is a relevant research topic (Silva and Marchante, 2012).

Eucalyptus globulus Labill. (Tasmanian blue gum) is one of the most widely planted and economically important hardwood species in temperate regions of the world (Potts *et al.*, 2004). This eucalypt is native to SE Australia and it is planted in many regions around the world. Portugal is among the countries that have the largest areas of planted *E. globulus* in the world (Potts *et al.*, 2004). This species was introduced in Portugal in the middle of the 19th century (Radich, 2007) and is now the most widespread tree species in the Portuguese mainland, representing 26% (812 x 10^3 ha) of its forest cover (ICNF, 2013).

Eucalyptus globulus forests in Portugal are planted and mostly managed through a coppice system (10–12 year rotations) (Turnbull and Pryor, 1984; Soares *et al.*, 2007). Their wood is almost exclusively used for pulp production. Water availability and episodic occurrence of temperatures below 0^{o}C are considered the main limiting climatic factors to *E. globulus* development in Portugal (Almeida *et al.*, 1994; Ribeiro and Tomé, 2000; Alves *et al.*, 2012). Wood yields are very variable due to site conditions, but they may exceed 30 m^3 ha^{-1} year^{-1} in the very best sites (Tomé, 2000). However, the good adaptation of *E. globulus* to many Portuguese environmental conditions is having other implications, as the species has become naturalized. The species reproduces by seeds and naturally established plants are commonly found within or close to planted stands nowadays (Marchante *et al.*, 2008; Silva and Marchante, 2012). Although the first reference to naturalization of *E. globulus* in Portugal dates from 1943 (Almeida and Freitas, 2006), we have found no quantitative assessments of this process in the literature. Naturalization processes are known to have resulted in considerable economic and environmental costs for several alien species (Andreu *et al.*, 2009). Naturally established plants may modify ecosystem/plantation dynamics and changes in forest management may be required to control them, since *E. globulus* grows fast (Silva *et al.*, 2007a). Most of the literature concerning the seed regeneration from *E. globulus* plantations reports qualitative assessments in order to infer about the naturalization or the invasive status of the species (Ritter and Yost, 2009; Gassó *et al.*, 2010; Gordon *et al.*, 2012). The few references that provide quantitative data are not comparable due to differences on methods and on considered factors (Virtue and Melland, 2003; Calviño-Cancela and Rubido-Bará, 2013; Larcombe *et al.*, 2013).

Fire is often related with eucalypt recruitment and establishment (Mount, 1964; Cremer, 1965; Mount, 1969; Ashton, 1981; Gill, 1997). Causes for fire facilitated recruitment/establishment of

eucalypts are related with: increased seed shed from canopy (Cremer, 1965; Pryor, 1976; O'Dowd and Gill, 1984; Wellington and Noble, 1985b; Florence, 1996); seed-predator satiation (O'Dowd and Gill, 1984; Wellington and Noble, 1985b; Gill, 1997); increased light availability (Jacobs, 1955; Kirkpatrick, 1975; Gill, 1997); 'ash-bed effect' (Pryor, 1976; Chambers and Attiwill, 1994); reduced competition (Wellington and Noble, 1985a; Whelam, 1995; Gill, 1997); removal of allelopathic substances (Pryor, 1976; Stoneman, 1994); and decreased predator activity (Whelam, 1995). Larcombe *et al.* (2013) demonstrated that fire was associated with higher recruitment levels of *E. globulus*, as it had been suggested earlier by Kirkpatrick (1975).

Portugal has the largest percentage of burnt forest area in Europe and one of the largest in the world (FAO, 2010; JRC, 2012) and the National Forest Strategy (DGRF, 2007) indicates that wildfires are a major threat to sustainable forest management in this country. Moreover, eucalypt stands are highly flammable in comparison to other forest systems in Europe and particularly in Portugal (Nunes *et al.*, 2005; Moreira *et al.*, 2009; Silva *et al.*, 2009; Fernandes *et al.*, 2011; Xanthopoulos *et al.*, 2012).

Effects of post-fire operations on seedling establishment depend on how and when they are performed. Post-fire management of burnt forests often includes salvage logging; tillage; and shrub removal. Post-fire salvage logging usually occurs before establishment of the next generation of trees and its major effects consist of environmental changes derived from removal of burnt trees. If it occurs after seedling establishment, significant seedling mortality may happen (McIver and Starr, 2000). *In situ* germination of seeds from logging eucalypt slash is common under favourable conditions (Fagg, 2001) and *E. globulus* plants may establish and grow normally or to become dominated trees under coppice shoots (Skolmen and Ledig, 1990). Tillage is aimed at improving soil conditions for root development (Madeira *et al.*, 1989), but in burnt areas can largely enhance erosion, if it is not performed with caution (Coelho *et al.*, 1995; Shakesby *et al.*, 1996). Established plants that were born after fire may be destroyed by tillage (Catry *et al.*, 2010). Few years after fire, understorey is well developed in forests. Periodic understorey removal is performed in *E. globulus* stands, because individuals are very sensitive to competition with understorey plants, especially in early years of life, and fuel load build up increases fire hazard (Pereira, 2007; Soares *et al.*, 2007; Moreira *et al.*, 2009; Alves *et al.*, 2012).

To our knowledge, a quantitative assessment of natural establishment of *E. globulus* in burnt areas has never been carried out in Europe. Therefore, we chose to study the seminal regeneration of *E. globulus* in burnt areas because there were recurrent references to fire-induced eucalypt establishment (Jacobs, 1955; Cremer, 1965; Kirkpatrick, 1975; Pryor, 1976; O'Dowd and Gill, 1984; Chambers and Attiwill, 1994; Stoneman, 1994; Florence, 1996; Gill, 1997), as well as frequent observations of *E. globulus* saplings in recently burnt areas in Portugal (Silva *et al.*, 2007a; Silva *et al.*, 2007b; Silva and Marchante, 2012). Stands with *P. pinaster* were included in the study because this species is highly represented in the Portuguese mainland (23% forest cover) (ICNF, 2013), and it has similarities to *E. globulus* on its ecological requirements and geographical range. Additional grounds were the common coexistence of these species in mixed stands (Silva *et al.*, 2011) and the high fire proneness of *P. pinaster* stands (pure or mixed) (Moreira *et al.*, 2009).

The study aimed at answering four questions related to medium-term establishment of *E. globulus* plants originated from seeds under natural conditions in burnt forests (pure *E. globulus* stands, pure *P. pinaster* stands, and mixed stands): a) what is the likelihood of *E. globulus* natural establishment in burnt areas; b) which are the most important factors related with site characteristics, stand type and post-fire management practices influencing this likelihood; c) how do these variables affect the density of post-fire naturally established *E. globulus* cohort and the median height of its individuals; and d) how do these variables influence the size structure (distribution of individuals among size classes) of post-fire naturally established *E. globulus* cohort.

3.2. Material and methods

3.2.1. Study areas

Forty areas that had burnt during 2005 and 2006 were selected in central and northern Portugal (Fig. 3.1), regions where *E. globulus* is common. Burnt areas were identified from existing fire maps created through semi-automated classification of remote sensing satellite data (Marques *et al.*, 2011). Selection of burnt areas was based on time-since-fire (5–7 years), size (largest areas were preferred), accessibility and presence of pure or mixed stands of *E. globulus* and *P. pinaster* (pure stands corresponding to cover of target species ≥ 75%, and mixed stands to cover of either species < 75%) (AFN, 2009). The selected areas ranged in size from 6 to 10,924 ha, with an average of 2078 ha. The sampling grid (500 m x 500 m) created for the National Forest Inventory (NFI) (AFN, 2010) was used to define potential study sites within the selected areas. From these potential sites, those corresponding to pure or mixed stands of *E. globulus* and *P. pinaster* were selected, based on pre-2005 NFI data. Depending on the size of the selected areas, up to 30 sites were chosen per area. The selected sites were checked in the field for eventual land cover changes after fire, and the ones that had been converted to other land uses after fire were excluded. A total of 321 sites was obtained (range = 1-30 sites per burnt area): 37 for refining sampling method (data not presented), and 284 for definite sampling.

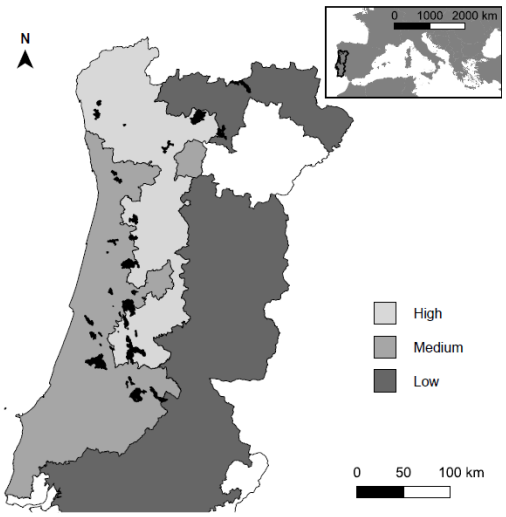

Fig. 3.1 - Location of the 40 studied burnt areas in Portugal (in black). Limits for the three *Eucalyptus globulus* productivity regions are also shown. White areas refer to regions where sampling did not occur.

3.2.2. Field sampling

Sites were sampled between June 2010 and June 2012. The sampling design was an adaptation of the method used in Portuguese National Forest Inventory (AFN, 2009) for minor trees assessment, and consisted of a combination of transect sampling (to allow a more effective detection of eucalypt presence) with area surveys (subplots) to measure plant densities. Each sampling site consisted of a 6.78-m radius circular plot centred on the site coordinates (located using a hand-held GPS – Garmin, e-Trex, Taiwan). Four 5 m x 2 m transects were established at every site, diverging from the plot centre and oriented towards the North, the South, the East and the West. At the end of each transect, a 1.78 m radius subplot was established, with its centre 5-m distant from the plot centre. Presence of post-fire naturally established *E. globulus* plants was checked inside the four subplots and along the four transects. In order to distinguish these individuals from pre-fire, artificially sown or planted individuals, we observed cumulatively the following features: absence of charred parts; presence of a conspicuous lignotuber; and location within the plantation inconsistent with spacing. The number of target plants and the height of the median individual were measured *in loco* inside each of the four subplots. Each individual was assigned to one of the four size classes using a combination of height (h) and diameter at breast height (DBH) – size class 1: h ≤ 1.3 m; size class 2: h > 1.3 m and DBH ≤ 5 cm; size class 3: h > 1.3 m and 5 < DBH ≤ 7.5 cm; size 4: h > 1.3 m and DBH > 7.5 cm. At all sampling sites, evidences of several post-fire stand management operations (Table 3.1) were collected by field observation and, whenever possible, by inquiring land owners. Information about site physiographic position, slope, and aspect was also collected (Table 3.1). A hypsometer (Haglöf Vertex III, Sweden) was used to measure slope. Aspect was measured using a compass. Additionally, the presence of potential mother trees, either in the plot or in its surroundings (up to 100 m), was registered.

Table 3.1 – Description of assessed explanatory variables.

Variable	Data description	Data type	Data source	Frequency (%)
Pre-fire forest type	Dominant species in the plot	Categorical	Forest inventory data and field evidence	*P. pinaster* (29.6) *E. globulus* (45.8) Mixed (24.6)
Productivity region	*E. globulus* productivity classes adapted from Ribeiro and Tomé (2000), based on annual precipitation and number of frost days per year	Categorical	Ribeiro and Tomé (2000)	High (35.6) Medium (53.9) Low (10.6)
Post-fire salvage logging	Cut of *Eucalyptus globulus* poles and/or *Pinus pinaster* trees (all or only dead)	Binary (0/1)	Inquiries and field evidence	69.4
Post-fire tillage	Mechanical disturbance of the forest floor, by harrowing, ploughing or ripping	Binary (0/1)	Inquiries and field evidence	15.8
Post-fire understorey removal	Mechanical removal of understorey shrubs and small trees	Binary (0/1)	Inquiries and field evidence	16.2

(to be continued)

Table 3.1 – (Continuation)

Variable	Data description	Data type	Data source	Frequency (%)
Terrain physiography	Plot physiographic position	Categorical	Field evidence	Flat or valley bottom (10.9) Slope (78.5) Ridge (10.6)
Slope	In degrees	Continuous	Field measurement	[0º;15º] (50.0)]15º;30º] (44.0)]30º;45º] (6.0)
Aspect	Aspect classes based on Kutiel and Lavee (1999): unfavourable – SE, S, and SW aspects; favourable – remaining aspects.	Categorical	Field evidence	Unfavourable (31.3) Favourable (68.7)

3.2.3. Data analysis

Considering that there is no accurate and quick method of determining age of *E. globulus* plants, we decided to use a broad concept of cohort in this study – cohort as a group of individuals of the same species that experienced the same event within the same time interval (Ryder, 1965), using as cohort definer (*sensu* Schaie (1984)) the natural establishment in a burnt stand. Accordingly, we included all *E. globulus* plants that had been naturally established within 5-7 years after fire in a single cohort, which was our study subject.

For simplicity purposes, we will refer to post-fire natural establishment of *E. globulus* as establishment. Similarly, post-fire naturally established *E. globulus* plants/individuals and post-fire naturally established *E. globulus* cohort, will be respectively named as plants/individuals and cohort. Nevertheless, the extensive names will be used whenever the use of short names results in ambiguity.

Three response variables were modelled using generalized linear models (GLM): occurrence of establishment (presence/absence), cohort density (plants m^{-2}), and plant median height (m). A plot was considered as having established plants if they occurred in at least one of its transects or subplots. Plant density was estimated by averaging the densities from the different subplots. Median height was also calculated across subplots. Nine explanatory variables were used (Table 3.1): stand type; tillage; understorey removal; salvage logging; terrain physiography; slope; aspect (based on Kutiel and Lavee (1999)); and *E. globulus* productivity regions (Fig. 3.1; adapted from Ribeiro and Tomé (2000) and Tomé *et al.* (2001)). Productivity region factor was chosen as it could be a surrogate of habitat quality for *E. globulus* and, consequently, for seedling establishment and growth of this species.

Occurrence of establishment (presence/absence) was modelled by setting a binomial distribution for the response variable and a logit link (occurrence model) (Quinn and Keough, 2002). The cohort density (Fig. 3.2) was modelled using a gamma distribution and a log link (density model). In this case, only sites with plant establishment were considered. The median heights of established plants had a log normal distribution (Fig. 3.3), so they were log-transformed and modelled through a Gaussian distribution and an identity link (height model) (Quinn and Keough, 2002). The only three sites with regeneration at subplots located in low productivity regions were discarded in density and height models as these regions were not sufficiently represented. Model selection followed Zuur *et al.*

(2009), starting with a model that included all nine explanatory variables and sequentially removing the variables that did not contribute significantly (α = 0.05) to the explained deviance (according to analysis of deviance tests). Modelling was performed using R statistical software (R Core Team, 2012).

Assessment of each model performance was based on the fraction of total deviance explained by the model. Performance of occurrence model was additionally assessed through the area under the receiver operating characteristics curve, commonly known as area under the ROC curve (AUC) (Pearce and Ferrier, 2000). AUC was estimated using package ROCR for R (Sing *et al.*, 2009). The eventual presence of spatial autocorrelation in the model residuals was tested through a spatial correlogram using the Moran's I autocorrelation coefficient (Fortin and Dale, 2005), employing the function correlog of the ncf package for R statistical software (Bjornstad, 2009). Significance was tested using 100 permutations and the progressive Bonferroni correction (Legendre and Legendre, 1998).

Relationship between regeneration occurrence and presence of potential mother trees was analysed through a contingency table, X^2 test, and Φ_2 coefficient (Zar, 1996).

Differences on size structure of the naturally established cohort (given by the distribution of individuals into size classes) among sites were assessed, using analysis of similarity (ANOSIM) and non-metric multidimensional scaling (MDS) (Clarke, 1993). Data on distribution of individuals among sizes classes in different sites were standardized. Then, a similarity matrix of those frequencies was computed using the Bray-Curtis coefficient of similarity (Bray and Curtis, 1957; Clarke, 1993). MDS was performed with 20 restarts and a two-dimension MDS diagram was built. A one-way ANOSIM test was performed for each factor separately. Then, obtained global R value was compared with the R probability distribution, previously produced with a maximum of 9999 random permutations, considering α = 0.05. ANOSIM and MDS were performed using the software Primer 5 for Windows (version 5.2.9) (Primer-e, 2002).

3.3. Results

The 284 sites were unevenly distributed among different stand types, productivity regions, and topographic conditions (Table 3.1).

Post-fire management occurred in 78.5% of sites. Salvage logging was the most common operation (69.4%), while understorey removal (16.2%) and tillage (15.8%) were less common (Table 3.1).

A total of 3062 naturally established *E. globulus* plants were observed across 72.5% of the 284 sampling sites. Almost every site (99.5%) with natural regeneration had or had had potential mother trees inside the plot or nearby. At sites with no regeneration, these seed trees were, or had been present in 29.5% of cases. This difference was significant ($\varphi^2 = 0.79$, $P < 0.001$). At the sites where studied plants were present at the subplots, cohort density had its median at 0.20 plants m^{-2} and its maximum at 4.55 plants m^{-2} (mean ± SD = 0.48 ± 0.73 plants m^{-2}). Plant median height was 2.0 m, and the values ranged from 0.2 to 12.1 m (mean ± SD was 2.7 ± 2.4 m). These two variables had positively skewed distributions (Fig. 3.2; Fig. 3.3).

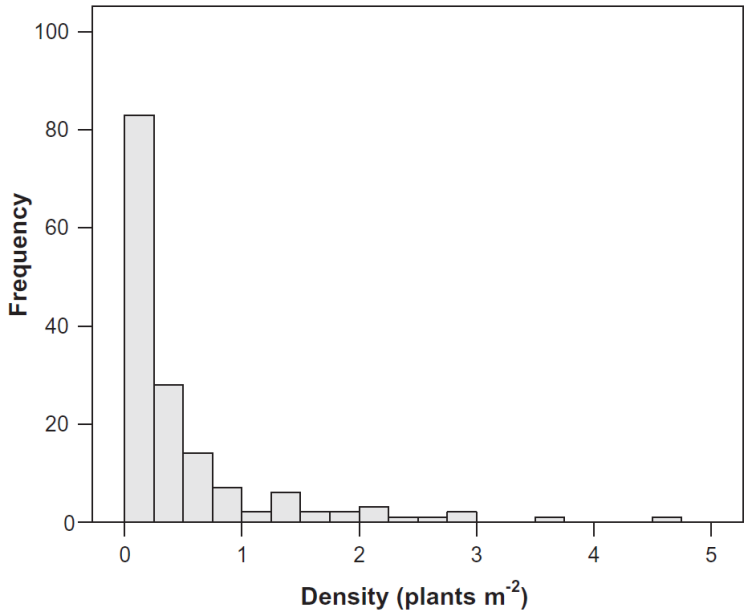

Fig. 3.2 – Observed densities of post-fire naturally established *E. globulus* cohort, in sampled sites where it was present. *n* = 153

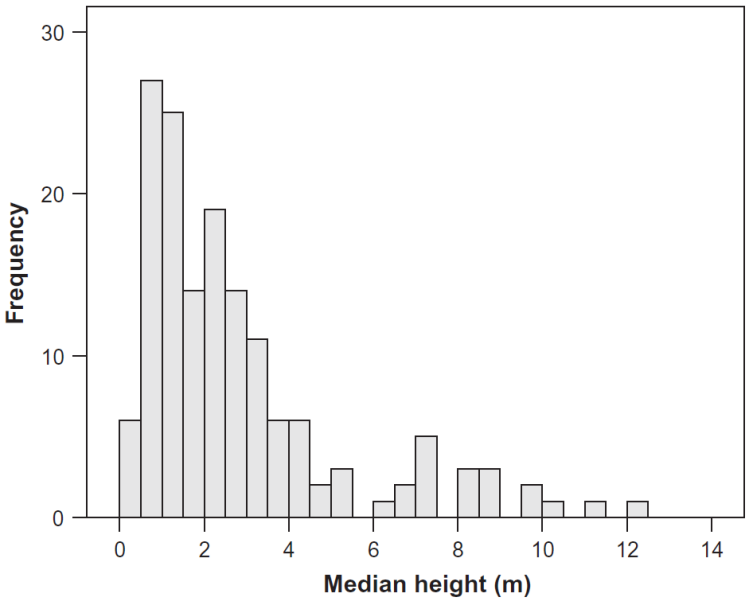

Fig. 3.3 – Median heights of post-fire naturally established *E. globulus* plants in sampled sites. *n* = 152.

The occurrence model showed that establishment was significantly influenced by stand type, productivity region, and understorey removal, together explaining 56.8% of the deviance (Table 3.2). AUC value for occurrence model was 0.941, revealing its high accuracy (Swets, 1988). Establishment probability was higher in mixed and pure *E. globulus* stands, in medium and high productivity regions, and when understorey vegetation was not removed, although this latter effect was less important and appeared to be overshadowed by other factors (Fig. 3.4a). The main explanatory variables in the density model were productivity region and two post-fire management operations, although altogether they only explained 11.6% of the deviance (Table 3.2). Density was higher in the high productivity region, and when salvage logging had occurred, while it was lower following tillage (Fig. 3.4b).

According to height model, plants were taller in the high productivity region and smaller where the soils were tilled (Fig. 3.4c). However, this model only explained 8.0% of the deviance (Table 3.2). There was no significant spatial autocorrelation pattern in any of the models' residuals.

Table 3.2 – Generalized linear models for natural establishment of *E. globulus* – occurrence likelihood, cohort density, and plant median height. For each response variable, explanatory variables kept in the respective final model are indicated through their coefficient (± SE), as well as their significance (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$).

	Occurrence	**Density**	**Height**
Intercept	1.002 ± 0.925	- 0.812 ± 0.260 *	0.889 ± 0.113 ***
Stand type	***		
E. globulus	0		
P. pinaster	- 4.000 ± 0.521		
Mixed	1.717 ± 1.073		
Productivity region	**	*	*
High	1.732 ± 0.900	0	0
Medium	2.299 ± 0.877	- 0.501 ± 0.259	- 0.293 ± 0.146
Low	0	–	–
Salvage logging		0.597 ± 0.278 *	
Tillage		- 0.864 ± 0.333 *	- 0.436 ± 0.193 *
Understorey removal	- 1.462 ± 0.624 *		
n	284	150	149
Explained deviance	56.8%	11.6%	8.0%
AUC	0.941		

The MDS analysis performed to compare the size structure of the spontaneous cohort among different sites had a stress of 0.03, revealing an excellent representation with no prospect of misinterpretation (Clarke, 1993). The 2-dimension MDS diagram (Fig. 3.5) showed two main groups of sites: one group of 52 sites lacking plants of size 1, located along the vertical axis (group A); and another group of 119 sites lacking of sizes 3 and 4, located along the horizontal axis (group B). The two groups partially overlapped. This overlap involved 38 sites that only had plants of size 2. The far end of group B comprised sites only with plants of size 1 (C; $n = 23$). In between groups A and B, there were sites with different proportions of plants belonging to several size classes (1, 2, and 3 or/and 4). Size classes 1 and 2 were prevalent in the study, 95.4% of plants belonged to one of these classes, and they were present in all sites that had natural regeneration at the subplots. Size classes 3 and 4 were the rarest, together they occurred in 20.6% of sites which had natural regeneration at subplots (all but group B) and they represented only 4.5% of plants. ANOSIM showed that tillage was the only explanatory variable that significantly affected cohort size structure ($P = 0.048$) but its

influence on relative abundances of different size classes was not very strong (global R = 0.064). Plants of sizes 3 and 4 were absent in tilled sites (Fig. 3.5).

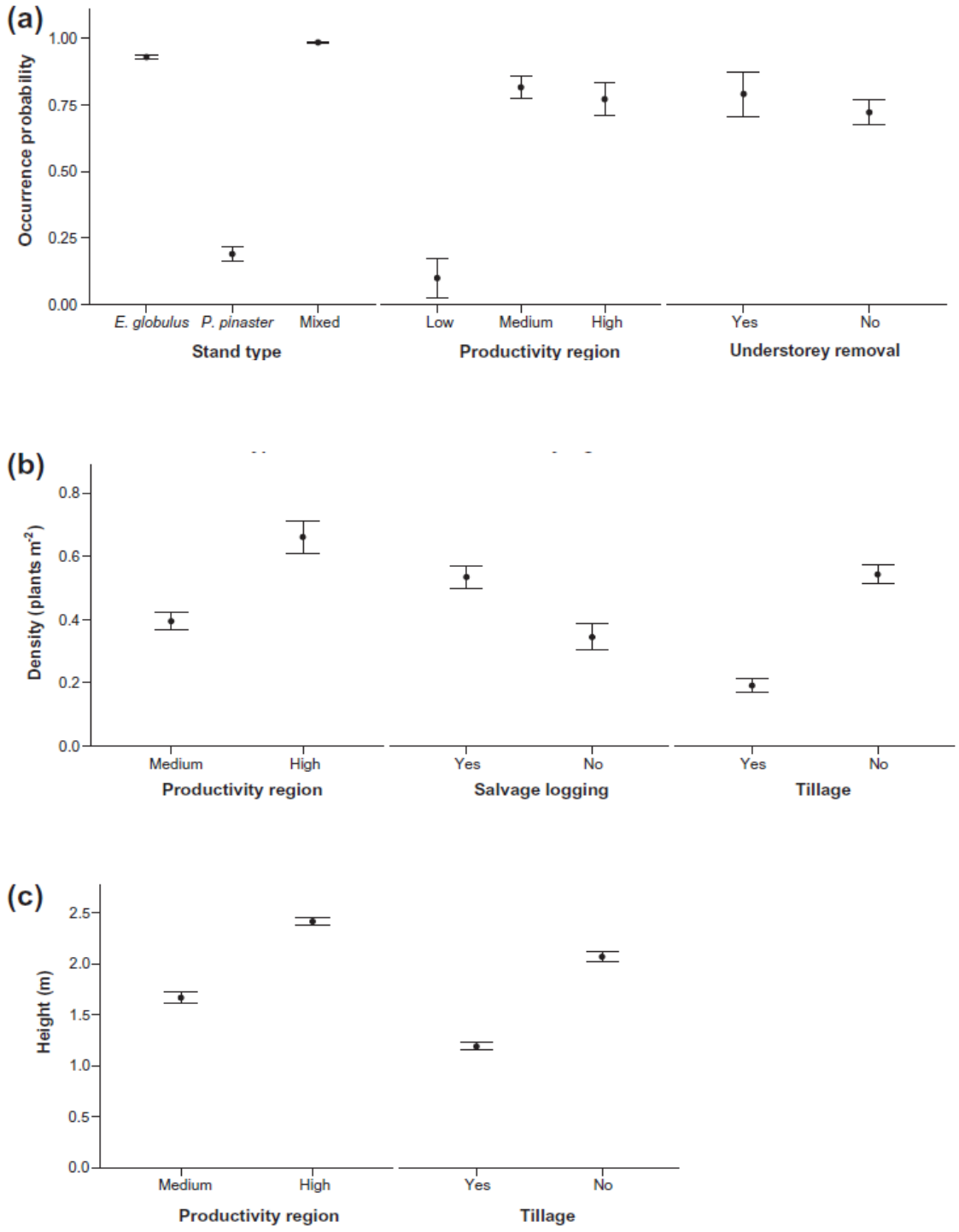

Fig. 3.4 – Predictions of produced models (mean ± 95 CI) for *E. globulus* post-fire natural establishment: (a) occurrence model; (b) density model; (c) median height model.

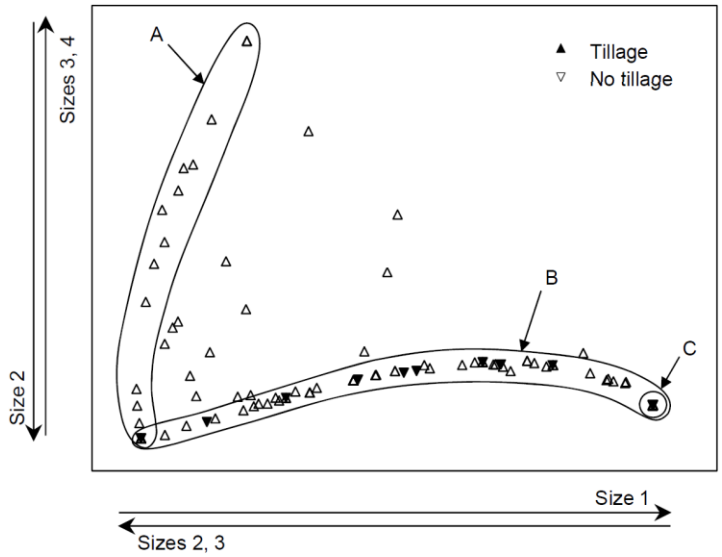

Fig. 3.5 – Ordination diagram of size structure of post-fire naturally established *E. globulus* cohort in sampled sites, obtained from a non-metric multidimensional scaling analysis. Size classes of plants, by height (h) and diameter at breast height (DBH): size 1 – h ≤ 1.3 m; size 2 – h > 1.3 m and DBH ≤ 5 cm; size 3 – h > 1.3 m and 5 < DBH ≤ 7.5 cm. *n* = 153. See text for description of groups A, B, and C

3.4. Discussion

Considering the wide geographical range of the survey, the diversity of sampled stands and the relatively small size of the sampling plots, it is relevant that 72.0% of plots from all stands had naturally established *E. globulus* plants. The positive association between the occurrence of these plants and the presence of potential mother trees suggested the existence of a widespread establishment in burnt stands where close seed sources were available. The occurrence of progeny restricted to vicinity of these trees is in accordance with the limited dispersal capacity of the species (Cremer, 1977) and occasional occurrence of regeneration some tens of meters away from these trees are compatible with results obtained by Larcombe *et al.* (2013) and Calviño-Cancela and Rubido-Bará (2013).

The occurrence model revealed high accuracy in predicting the likelihood of establishment, pointing at stand type, productivity region, and understorey removal as main explanatory variables.

Natural establishment was very common in pure *E. globulus* stands (90.9%) and mixed stands (94.9%), while it only existed in 19.1% of pure *P. pinaster* stands. This difference was very significantly reflected in the occurrence model. While this result is not surprising, since the presence of adult *E. globulus* trees (seed sources) naturally increases the likelihood of recruitment, it is noticeable that this type of establishment also occurred often in pure *P. pinaster* stands. However, all but one of concerned sites had adult *E. globulus* trees or its burnt remnants within the plot or had conspicuous potential mother trees in the surrounding area. The presence of sexually mature *E. globulus* trees in pure *P. pinaster* stands is common in Portugal (Godinho-Ferreira *et al.*, 2005).

Likelihood of establishment was higher in better productivity regions, based on classification proposed by Ribeiro and Tomé (2000). The criteria used in this classification to define homogeneous climatic regions for *E. globulus* productivity (~growth) were essentially based on indicators of water availability and frost occurrence. These factors are known to affect not only *E. globulus* growth, but

also this species's recruitment, establishment, and survival. In fact, water deficit negatively affects: *E. globulus* seed germination (López *et al.*, 2000; Humara *et al.*, 2002); *E. globulus* seedling establishment success (González-Muñoz *et al.*, 2011); and summer survival of young eucalypts (Jacobs, 1955; Whelam and Main, 1979; Wellington and Noble, 1985a; Stoneman *et al.*, 1994; Richards and Lamont, 1996). Additionally, frost may directly kill foliage and buds and sometimes the whole plant (Cremer *et al.*, 1984). The median lethal temperature (T50) for *E. globulus* seedlings leaves is -5.5ºC (Almeida *et al.*, 1994). These facts may explain why higher productivity regions are more likely to have *E. globulus* establishment. Results obtained by Larcombe *et al.* (2013) and ours point at the same direction: Australian sites where precipitation seasonality was lower had higher probability to have natural establishment of this species; similarly to what happens in Portuguese areas in the higher productivity regions in Portugal, probably due to reduced water stress in late summer.

Most of the surveyed sites (68.3%) did not show any evidences of management activities, except for salvage logging. Establishment occurred in all but one of those "unmanaged" sites in pure *E. globulus* stands, and the same happened in mixed stands, while only 15.8% of "unmanaged" pure *P. pinaster* sites had spontaneous *E. globulus* plants. This suggests that poor management or total lack of it favoured establishment both in pure *E. globulus* stands and in mixed stands; while other factors may have major influence on the phenomenon in pure *P. pinaster* stands. However, a more detailed analysis shows that different management operations had different effects.

Sites where understorey removal had occurred were less likely to have naturally established *E. globulus* plants, although this effect was minor. This operation is aimed at destroying understorey vegetation, which may include small *E. globulus* individuals. In some sites where understorey was removed, probably all previously recruited *E. globulus* seedlings/saplings were killed and no subsequent establishment succeeded, resulting in absence of plants at sampling time. This would be compatible with the idea that most post-fire establishment happens shortly after fire (Pryor, 1976; Florence, 1996).

Densities of naturally established *E. globulus* cohort observed in our study were not easy to compare with other quantitative references on this subject because of differences in methodology and considered factors (Virtue and Melland, 2003; Calviño-Cancela and Rubido-Bará, 2013; Larcombe *et al.*, 2013). In order to compare our data to those from other studies, we needed to consider only pure *E. globulus* stands. Densities observed in our study (maximum = 4.55 plants m^{-2}; mean = 0.48 plants m^{-2}) were largely higher than those registered by Larcombe *et al.* (2013) (maximum = 1.98 x 10^{-3} plants m^{-2}; mean = 8.52 x 10^{-4} plants m^{-2}) within 10 m from plantation borders, including both burnt and unburnt areas. Meanwhile, the absolute values of maximum density mentioned in the other two references had the same order of magnitude that we observed: 1-2 plants m^{-2} (Virtue and Melland, 2003) and about 2 plants m^{-2} (Calviño-Cancela and Rubido-Bará, 2013), both located next to unburnt plantations edges. However, we cannot evaluate if these values are lower or higher than ours because they might result from potential periods of recruitment with very different time lengths.

The density model pointed at productivity region and two management operations as the most important studied variables for explaining young cohort density.

Cohort density was higher in the most productive region. Since water deficit can negatively affect eucalypt recruitment, establishment and survival, as discussed before, it may explain this density difference. Our results on density also agreed with those reported by Larcombe *et al.* (2013) for Australia, with respect to annual precipitation and precipitation seasonality.

Salvage logging had a positive effect on cohort density. Although logging can destroy post-fire established plants (McIver and Starr, 2000), it is not likely because it is recommended to harvest burnt *E. globulus* trees shortly after fire (Shakesby *et al.*, 1996). In fact, logging has been associated with plant recruitment in *E. globulus* stands and in other eucalypt stands (Skolmen and Ledig, 1990; Fagg, 2001), since seedlings may originate from the so-called 'slash seed' (Fagg, 2001), which in the specific case of salvage logging may be enhanced by the seed shed caused by fire (Cremer, 1965). Additionally, reduction of competition caused by salvage logging might have improved establishment success of youngsters, resulting in higher densities of their cohort in fallen stands. Eucalypts are not able to become established and develop normally under a complete overstorey canopy (Florence, 1996). *Eucalyptus globulus* usually regenerates only when the overstorey is removed (Stoneman, 1994). Asymmetrical competition was observed in *E. globulus* by Tomé *et al.* (1994). Several Australian studies under temperate and Mediterranean climates have shown that water deficits are stronger in eucalypt seedlings in sites with overstorey than in sites where it did not exist, resulting in higher mortality rates in the former (Bowman and Kirkpatrick, 1986; Battaglia and Wilson, 1990; Stoneman *et al.*, 1994). Negative effects of water stress on *E. globulus* recruitment and establishment were already discussed.

Tillage had a negative effect on cohort density. This operation can destroy post-fire regeneration from seeds (Catry *et al.*, 2010). Direct killing of most of the naturally established *E. globulus* plants was probably the major cause of the low density of this cohort in tilled sites. Harrowing reduces the development of understorey biomass in *E. globulus* plantations (Carneiro *et al.*, 2008) and this may be related to a reduction of nutrients in soil caused by tillage (Madeira *et al.*, 1989; Carneiro *et al.*, 2008). Moreover, increased soil erosion due to tillage probably resulted in harsher soil conditions that might have hindered plant establishment (Shakesby *et al.*, 1996) and increased mortality since then, contributing to an even lower density of the cohort in those sites.

Median height of spontaneous *E. globulus* plants in surveyed plots had a broad variation across sites (0.2–12.1 m). This fact could be related either with different times of recruitment or with environmental differences among the sites where these plants were growing. As *E. globulus* age is difficult to determine (Williams and Brooker, 1997; Leal *et al.*, 2004), the relationship between size and age of observed plants is partially speculative. Even so, plant size can be used for demographic interpretations in eucalypt communities (Florence, 1996). Therefore, the existence of continuous recruitment or occurrence of several recruitment episodes might explain the diversity of plant sizes observed in many sites and especially the abundance of the smallest plants. Nevertheless, according to literature, it is probable that most of these plants have been recruited in the first year after fire, since: fire improves conditions for eucalypt recruitment (Cremer, 1965; Kirkpatrick, 1975; O'Dowd and Gill, 1984; Chambers and Attiwill, 1994; Stoneman, 1994; Gill, 1997); eucalypt seeds are short lived in soil (Jacobs, 1955; Cremer *et al.*, 1984; Wellington and Noble, 1985b) and germinate as soon as they

have favourable conditions to do so in nature (Penfold and Willis, 1961); and *E. globulus* plants take 4–7 years to produce their first seeds (Kirkpatrick, 1975; Turnbull and Pryor, 1984; Jordan *et al.*, 1999) and seven years to produce seeds after canopy burn (Kirkpatrick, 1975).

More than 95% of sampled individuals were of sizes 1 or 2, and were at most 6.77 m tall (height estimate based in Marques *et al.* (2011)). If we presume a major event of recruitment in the first year after fire, these plants were shorter than it would be expected (data from WebGlobulus 2.1 simulator (Palma, 2009)). Additionally, they were also much shorter than the resprouts of most of the coexisting burnt trees. Therefore, we may think about the former as dominated, suppressed or growth restricted trees (Skolmen and Ledig, 1990; Florence, 1996). Eucalypt lignotuberous seedlings/saplings are very resistant, therefore they can survive for long years in the understorey, and may be considered as a 'regeneration pool' in eucalypt forests (Florence, 1996; Ashton, 2000), since they can speed growth up after release from overstorey competition (Florence, 1996). If we consider the observed persistence of growth restricted *E. globulus* trees, their latent growth capacity, and their ability to attain reproductive state (pers. observ.); we may say that conditions might exist for natural perpetuation of this species in many surveyed sites. This means that the naturalization process (*sensu* Richardson *et al.* (2000)) is undergoing.

Plants with the above features are often used in forestry as advance growth (Jacobs, 1955; Florence, 1996; Alves *et al.*, 2012), being a most important aspect of eucalypt forest regeneration cycle in Australia (Ashton, 2000). Although this practice is possible with *E. globulus* in Portugal, the species invasive potential in this territory (Marchante *et al.*, 2008; Silva and Marchante, 2012) must be taken into account.

The height model indicated that productivity region and tillage were significant explanatory variables. Tillage also affected the size structure of the regenerating population. However, other non-studied variables might have significantly influenced the observed plant height.

Plants were taller in the highest productivity region. Climate factors (frost and water availability) that influence *E. globulus* growth, and consequently height, are the basis of this classification. Negative effect of water stress on *E. globulus* growth is broadly known (Wang *et al.*, 1988; Tomé *et al.*, 1994; Osório *et al.*, 1998; Pita and Pardos, 2001; Humara *et al.*, 2002). Actually, lack of water is the main limiting factor to *E. globulus* growth in Mediterranean type ecosystems (Alves *et al.*, 2012), even though this species can deal with more severe water stress in Portugal and Spain than in its native range (Turnbull and Pryor, 1984; Alves *et al.*, 2012). Cold is also an important limiting factor to *E. globulus* growth. Temperatures around 0 ± 2ºC inhibit water uptake and growth of *E. globulus* seedlings (Almeida *et al.*, 1994; Costa-e-Silva *et al.*, 2008). Our results on relative height of plants among productivity regions were obviously consistent with the rationale underlying classification produced by Ribeiro and Tomé (2000). They also coincide with the trends predicted by model Globulus 2.1 (Tomé *et al.*, 2001).

Tillage had a negative effect on median height of plants. Wherever performed, this management operation also slightly affected the size structure of the cohort; it completely eliminated plants of sizes 3 and 4 and tended to favour the presence of size 1 plants. If we admit the existence of recruitment along time, since fire until sampling, many of the observed smaller plants might have

established after tillage, while all or part of pre-existing plants was destroyed by tillage, both contributing to reduced median height of plants at sampling time at tilled sites. Tillage reduces nutrient availability in soil (Madeira *et al.*, 1989). The synergetic effects of fire and tillage risk aggravating erosion even more (Coelho *et al.*, 1995; Shakesby *et al.*, 1996). Carneiro *et al.* (2008), found that harrowing reduced understorey development in *E. globulus* plantations and related it with reduction of nutrients in soil. The indirect effect of tillage hampering plant growth can be an additional explanation for lower plant median height at tilled sites.

Aspect, slope and topographic position influence incident solar radiation, water flow and soil erosion; whose effects on temperature, radiation, water and nutrients availability to plants may be determinants of their successful establishment and development (Jacobs, 1955; Moore *et al.*, 1988; Shakesby *et al.*, 1996; Kutiel and Lavee, 1999; Taiz and Zeiger, 2002; Pereira, 2007). Notably, none of topographical explaining variables was considered significant in any of the analyses. Similarly, Larcombe *et al.* (2013) found no significant influence of slope and aspect either on occurrence of natural establishment of *E. globulus* or on its density. This probably means that potential effects of topography on our response variables were overwhelmed by other factors effects, or even hidden by background noise of data.

The built models didn't explain all the observed variability. Some other factors, like fire severity and intensity, might have affected observed establishment (Mount, 1969; Pryor, 1976; Florence, 1996; Gill, 1997; Martínez *et al.*, 2002; Bailey *et al.*, 2012), but they were not considered in this study because accurate evaluation of these features is not feasible 5-7 years after fire, when the study was done.

3.5. Conclusions

This study showed that the natural establishment of *E. globulus* was widespread in two types of burnt stands (pure *E. globulus* and mixed) and that this establishment was favoured by climatic conditions that enhance the productivity of this species. Considering the frequency of occurrence and the characteristics (presumable age, size, lignotuber) of observed plants, we can say that conditions for natural persistence of *E. globulus* were probably met in those types of stands in the studied regions. However, further studies are needed to assess the capacity that this type of plants has to complete the life cycle under the conditions they are growing at, in order to better understand the naturalization process of this species in Portuguese territory.

The use of a naturally regenerating cohort as advance growth for production purposes seems possible. Nevertheless, whenever this possibility is considered, the fact that *E. globulus* is an exotic species with some invasive potential in Portugal must be kept in mind.

On the other hand, the increased stand density that results from the existence of spontaneously established plants may have detrimental consequences in terms of forest management and may lead to a higher fire hazard. Attention should be paid to the very high prevalence of these plants in two of the studied stand types, regarding the wide distribution of *E. globulus and the* high incidence of forest fires in Portugal. Our results suggested that both the lack and the type of post-fire management operations strongly influenced seminal regeneration of *E. globulus* in burnt stands.

Tillage has detrimental effects on this regeneration and may be considered in management programs for its control, in the geographical range of this study.

Mechanisms responsible for this species establishment are still poorly understood. It is not clear yet if post-fire recruitment of plants occurs mainly in a single initial recruitment event or in successive minor events or even if it is continuous along time. Comparative studies on this subject, in burnt and unburnt areas, are needed to clarify the role of fire on this species recruitment and establishment. For instance: effects of fire severity and intensity on natural regeneration of the species are still unknown; and there is no quantitative evidence on the importance of fire-stimulated seed shed and how it interacts with post-fire management. Considering the wide expansion of *E. globulus* in the world, the study of such mechanisms is undoubtedly a fertile field for future research initiatives.

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Chapter 4

Microsite drivers of natural seed regeneration of *Eucalyptus globulus* Labill. in burnt plantations

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4.1. Introduction

Many species are cultivated outside their native ranges worldwide including in industrial plantations. Fire is a factor that may influence the success of these species in their new environments (Richardson *et al.*, 1990; Contreras *et al.*, 2011), and this influence is heterogeneous both over time and space (Raison, 1979; DeBano *et al.*, 1998; Schoennagel *et al.*, 2008). Moreover, fire regimes are changing in many regions of the world, mostly as a result of changes in climate and land use (Keeley *et al.*, 1999; Pausas, 2004). In such contexts, some exotic species are better adapted to new fire regimes than the native ones (Richardson *et al.*, 1990; Contreras *et al.*, 2011). In the case of cultivated exotics, active management may be required, in order to control growing populations, both inside or around cultivated areas (Le Maitre *et al.*, 2011; van Wilgen and Richardson, 2014). Several studies have been made with widely cultivated trees outside their native ranges, on several scales down to tens of square metres (*e. g. Eucalyptus globulus*: Águas *et al.*, 2017; Fernandes *et al.*, 2018). However, propagules and young plants are very small, thus microsite characteristics are particularly relevant for recruitment and early survival of plants (Lamont *et al.*, 1991; Nathan and Muller-Landau, 2000). Although microsite scale has not deserved so much attention as other scales, the knowledge about microsites of young plants in burnt plantations is important for plantation management.

The ecological niche is a keystone for understanding species distribution (Hutchinson, 1957; Pulliam, 2000; Holt, 2009), as it encompasses the whole environment that allows species to exist indefinitely (Hutchinson, 1957). However, species have different ontogenic phases which do not have necessarily the same requirements (Grubb, 1977; Poorter, 2007). Indeed, it is proven that some species undergo ontogenic niche shifts (*e. g.* Battaglia and Reid, 1993; Young *et al.*; 2005; Quero *et al.*, 2008; Bailey *et al.*, 2012). Thus, ecological niche is sometimes divided for study purposes.

The regeneration niche includes only the environmental factors that enable species to replace mature individuals by mature descendants (Grubb, 1977). Both reproduction and early ontogenic development are very sensitive stages for species perpetuation everywhere (Grubb, 1977; Schupp, 1995). Availability of both seeds and safe sites for germination (*sensu* Fowler, 1988) is important for plant recruitment (Eriksson and Ehrlén, 1992; Setterfield, 2002). However, recruitment does not guarantee the species perpetuation in a site because seedling mortality can be very high (Jacobs, 1955; Wellington and Noble, 1985a; Stoneman, 1994). Successful establishment underpins that perpetuation. The persistence niche encompasses the factors that affect permanence of established plants *in situ* (Bond and Midgley, 2001). Survival and growth of juvenile plants are influenced by: resource availability; temperature, early protection from stresses; litter accumulation; disturbance; competition; predation; symbioses; and pathogens (Lamont *et al.*, 1993; Gill, 1997; Ashton, 2000; Setterfield, 2002). Therefore, knowledge about ecological niche, especially from early life stages until establishment, is crucial when it is intended to keep plant populations under control.

Fire is an ecological factor that can influence plant distribution. The genus *Eucalyptus* is planted worldwide (Jacobs, 1979) and includes many species adapted to fire (O'Dowd and Gill, 1984). Fire has influenced reproductive biology of *Eucalyptus* species during the evolution of the genus (House, 1997). Nowadays, fire is still frequent in *Eucalyptus* forests, promoting regeneration from seeds, especially when it is intense (Wilkinson *et al.*, 1993; Florence, 1996). It can affect *Eucalyptus* seed supply, as well as the availability and quality of microsites (Cremer, 1965; Setterfield, 2002). Short-time after fire, *Eucalyptus* trees may release massive amounts of canopy-stored seeds (Cremer, 1965; Wellington and Noble, 1985b; Silva *et al.*, 2016). Burnt sites are usually much more suitable for *Eucalyptus* seedlings, when compared to unburnt sites (Jacobs, 1955; Wellington and Noble, 1985a; Florence, 1996; Pennington *et al.*, 2001). Hence, fire may open a window of opportunity for regeneration from seeds, which lasts as long the niche is still vacant (Grubb, 1977; Bailey *et al.*, 2012). Some authors go further with this idea. For instance, Mount (1964) states that fire is required for *Eucalyptus* regeneration, in order to ensure stands persistence; and Kirkpatrick (1975) deems that site fire-regime deeply influences perpetuation of *E. globulus*. Considering that many *Eucalyptus* forests are very flammable (Florence, 1996; Ganteaume *et al.*, 2010; Fernandes *et al.*, 2011), the paradoxical idea of flammability as an adaptation to fire (Bond and Midgley, 1995; Keeley *et al.*, 2011) is probably true for many *Eucalyptus* species. Calviño-Cancela *et al.* (2018) also postulate that increased seedling recruitment in burnt areas, together with high fire-proneness of *Eucalyptus*, may generate positive feedbacks, favouring colonization of new areas. Therefore, fire is a strong determinant of *Eucalyptus* distribution.

Nevertheless, fire influence on plant distribution is not spatially uniform. Fire behaviour depends on weather, topography, and fuel (type and distribution) (Raison, 1979; Fernandes, 2006). Consequently, different microsite types are generated by fire within each burnt area (Raison, 1979; Schoennagel *et al.*, 2008), giving plants different chances to succeed. As a consequence, regeneration success of *Eucalyptus* is also heterogeneous within burnt areas (Bailey *et al.*, 2012). However, a few studies have addressed fire consequences on *Eucalyptus* regeneration at microsite level (Potts, 1986; Setterfield, 2002; Bailey *et al.*, 2012). In reverse, several studies on *Eucalyptus* regeneration were focused at microsite level but did not assess fire effects (*e. g.* Battaglia and Reid, 1993; Sanger *et al.*, 2011; Wilson and Gibbons, 2014).

Although *Eucalyptus* native range is restricted to Australia and a few islands in the Indian Ocean (Ladiges, 1997), this genus is the most cultivated hardwood genus worldwide (2.0×10^7 ha, in 2008; Richardson and Rejmánek, 2011). Cultivation of exotic species protects juvenile individuals against environmental stochasticity and preserves founder populations, facilitating their naturalization and invasion in the arrival territories (Mack, 2000; Lockwood *et al.*, 2005; Wilson *et al.*, 2009). Moreover, synergies may happen between the wide recurrent cultivation of *Eucalyptus* outside its native range and the high flammability of its forests. The success of eucalypts may be increased even further, if fire occurrence and high propagule pressure coincide with good environmental conditions for regeneration from seeds. Indeed, such a lucky coincidence often happens when fire burns seed-bearing plantations and is

followed by favourable weather for plant growth (Wellington and Noble, 1985a; Calviño-Cancela *et al.*, 2018). For instance, late summer fires which are followed by a mild rainy autumn. Unfortunately, excepting studies on *Pinus* spp., only a few others have analysed fire effects on the reproductive success of trees cultivated in fire-prone introduction ranges (Richardson *et al.*, 1990; Calviño-Cancela *et al.*, 2018).

Eucalyptus globulus Labill. is the most planted *Eucalyptus* species worldwide (2.3×10^6 ha, in 2008; Richardson and Rejmánek, 2011) and its stands are often in fire-prone regions (Manuel-Valdés and Gil-Sánchez., 2002; Moreira *et al.*, 2009; Nunes, 2012). The species is naturalized in several regions of the world and it is invasive in some of them (Rejmánek and Richardson, 2013). Western Iberian Peninsula has the *Eucalyptus globulus* largest area outside the native range (8.12×10^5 ha in Portugal; 1.74×10^5 ha in Galicia, Spain) (CMR, 2010; ICNF, 2013). There, stands dominated by this species are among the most fire susceptible (Moreira *et al.*, 2009). After fire, its regeneration from seeds is frequent, spatially heterogeneous, and sometimes attains high densities there (Águas *et al.*, 2014). Actually, this species is often involved in post-fire land-use transitions related to land abandonment, tending to increase its representativeness in that territory (Silva *et al.*, 2011). Nevertheless, interest about fire effects on regeneration from seeds of this species is fairly recent (Reyes and Casal, 1998, 2001; Águas *et al.*, 2014; Águas *et al.*, 2018; Calviño-Cancela *et al.*, 2018), only a couple of studies were field based, and none was focused at microsite level. Studies at this level would be useful to better understand the formerly reported spatial heterogeneity of this regeneration at plantation level (Larcombe *et al.*, 2013; Águas *et al.*, 2017; Fernandes *et al.*, 2018), and would provide useful information for forest management. Furthermore, studies inside the introduction range of *E. globulus* would shed light onto the naturalization status and the invasion risk of this species there.

Therefore, this study is aimed at identifying microsite factors that are associated to the occurrence of *E. globulus* regeneration from seeds in burnt plantations. More specifically, it is aimed at identifying factors related to: recruitment, establishment, and growth of this regeneration.

Henceforth, we will refer to post-fire natural regeneration of *E. globulus* from seeds as post-fire regeneration. Similarly, post-fire naturally established *E. globulus* seedlings, saplings, or wildlings will be named as seedlings, saplings, or wildlings, respectively. However, the long names will be preferred whenever the use of short names would cause ambiguity.

4.2. Material and methods

4.2.1. Study sites

Study sites were four industrial plantations of *E. globulus*: two in central Portugal (Casal do Malta I, CM1; Casal do Malta II, CM2) and two in northern Portugal (Santo António, SA; Currelos Valdeias, CV). The main characteristics of these study sites are presented in Table 4.1. Sampling occurred in August–September 2014. The sampled plantations shared

several attributes. They were burnt in September 2012, approximately two years before sampling. They were at second rotation and their poles were reproductively adult, at the time of fire. Finally, they were coppiced between fire and sampling time. In addition, all the sites have temperate climate with dry and mild summer (Csb), according to the Köppen-Geiger classification (IPMA, n. d.). However, important differences exist between the climatological standard normals of the closest weather stations (south: Dois Portos; north: Nelas; 1961–90 period) (downloaded from http://home.isa.utl.pt/~joaopalma/tools/nc6090/, on September 12, 2015. Annual rainfall is much lower in the central region (650.1 mm) than the northern (1007.8 mm). Mean annual temperature is higher (15.08 *vs.* 13.68ºC), frost days are less common (23.2 *vs.* 39.9 days year^{-1}), and temperature seasonality is weaker in the central region than in the northern.

Table 4.1 - Characteristics of plantations studied in central (Casal do Malta I and II) and northern (Currelos Valdeias and Santo António) Portugal.

	Casal do Malta I (CM1)	**Casal do Malta II (CM2)**	**Currelos Valdeias (CV)**	**Santo António (SA)**
Coordinates (centroid)	39º07'36"N 9º13'06"W	39º07'53"N 9º12'55"W	40º23'59"N 8º00'25"W	40º18'05"N 7º57'28"W
Altitude	75–100 m	50–74 m	176–226 m	249–338 m
Litology	Paleogene sandstone	Paleogene sandstone	Granite	(Pre-)cambrian schist-greywacke
Soil type	Eutric cambisol	Eutric cambisol	Humic cambisol and dystric cambisol	Humic cambisol
Tree density (trees ha^{-1})	1263	1041	788	840
Pole age at fire date	10 years	8 years	6 years	13 years
Burnt area	14.90 ha	10.59 ha	7.35 ha	14.00 ha
Wildfire date	3rd.Sep.2012	3rd.Sep.2012	5th.Sep.2012	15th.Sep.2012
Tree harvesting date	May.2014	May.2014	Jan-Feb.2013	Nov.2013
Sampling date	Aug-Sep.2014	Sep.2014	Aug.2014	Aug.2014

Note: Lithological data from Silva (1983). Edaphic data from Cardoso *et al.* (1971).

4.2.2. Sampling design

The four plantations were surveyed for *E. globulus* regeneration from seeds. Forty circular plots, grouped into twenty blocks, were established within each burnt plantation in the field, to study the microsites of post-fire regeneration. Each block included two plots representative of different microsite types: one microsite with no *E. globulus* regeneration from seeds (control, C) and another with at least one short sapling. As CM1 was the plantation which had the widest variety of wildling sizes, this plantation was chosen to study microsites of wildlings in different developmental stages. Therefore, in CM1, the twenty blocks included four microsite types: the two previously described; one with at least one seedling and another with at least one tall sapling.

Target plants of different sizes were representative of the size range of post-fire regeneration. As an attempt to study plants that were at different developmental stages, only plants from clearly different size classes were chosen, as follows. Firstly, seedlings (Se) were plants shorter than 18 cm which had: less than 12 pairs of leaves (including scars); non-branched stem; no lignotuber; and no evidence of growth for more than one season. They were considered non-established plants, as *Eucalyptus* establishment takes at least 12 months (Forestry Tasmania, 2009). Secondly, saplings were plants with: branched stem; lignotuber; evidences of aging for more than one growing season; and evidences of some physical damage. Short saplings (SSa) were designated as being between 30 and 100 cm in height, with a lignotuber narrower than 25 mm wide. They were the smallest *E. globulus* wildlings that were considered to be established in the plantations. Tall saplings (TSa) were taller than 150 cm and their lignotuber was generally wider than 30 mm.

The distance between sampled microsites within any block was always shorter than between different blocks. Microsite plots within each block were usually 10–20 m away from each other. The blocks were at least 30 m away from each other and from the border of the burnt areas. Apart from the aforementioned restrictions, the blocks were randomly placed, using a list of random numbers to choose the coordinates where to start the search for each microsite type.

In every block, one plot was established for sampling each microsite type. The plots were circular and had a 25-cm radius. The control plots had their centre at a place with no *E. globulus* wildlings closer than 5.5 m. All the other plots were centred on a target plant (TSa, SSa, or Se), which was randomly selected amongst the available wildlings of the respective size class.

4.2.3. Field sampling

In order to characterise each microsite, several attributes were assessed in each plot. Firstly, microtopography was characterized: aspect; slope; and microtopographic position of the central point (flat, slope, top, and depression). Then, cover by different vegetation guilds and ground cover were visually estimated. The considered vegetation guilds were: fungi; mosses; grasses; herbs; ferns; small shrubs ($h < 1$ m); tall shrubs ($1 \leq h < 5$ m); and trees. Ground cover was classified as: bare soil; rocks (diameter ≥ 2 cm); ash; charcoal; litter (leaves and twigs); and coarse woody debris (diameter ≥ 5 cm). The cover of each layer was independently estimated, using cover classes: **0**—absent; **1**— < 1%; **2**—1–4%; **3**—5–10%; **4**—11–25%; **5**—26–33%; **6**—34–50%; **7**—51–66%; **8**—67–75%; **9**—76–90%; and **10**—91–100%. Distance between the plot centre and the nearest vascular plant was measured. Afterwards, conspicuous objects surrounding the plot centre (distance ≤ 2 m) were characterized according to: type (rock, balk/talus, deadwood, living stump, shrub, or cluster of small plants); width (in degrees, measured from the plot centre); height; and distance from the plot centre. Presence of *E. globulus* capsules was also checked inside every plot. Then, depth of mulch produced after fire was measured within 10 cm from the plot centre (5 readings), and so were ash depth

(5 readings), and soil hardness (10 readings with penetrometer, Geotester, Italy). In addition, a composite sample of surface soil was collected, using a soil borer (diameter = 2 cm; depth ≤ 5 cm; 5 cores). Finally, maximum and median heights of all *E. globulus* wildlings inside plots were measured, and wildlings were counted within 1.78 m from plot centre (area = 10 m^2).

4.2.4. Laboratory analyses

Soil hydrophobicity

Soil samples were sieved (mesh = 2 mm), and a 15-g subsample of each one was put into an individual Petri dish and left to air-dry until reaching constant weigh. Then, the subsamples were used to assess persistence of hydrophobicity, using the 'water drop penetration time test' (WDPT), and severity of hydrophobicity, using the 'molarity of an ethanol drop test' (MED) (Doerr, 1998; Letey *et al.*, 2000). Air temperature and moisture were controlled during soil drying and hydrophobicity tests (Dekker and Ritsema, 1994). Temperature was kept at 19±1ºC and relative air moisture at 50±2%.

The WDPT test consisted in carefully dripping 5 drops of distilled water on the smoothed surface of each sample and measuring the median time to complete absorption (t) (Doerr, 1998). The Petri dishes were covered with a lid to reduce water evaporation, whenever t > 1'. When t > 3 h, it was just registered as so. The hydrophobicity persistence class of each sample was identified using its t (Table 4.2).

The MED test consisted in dripping 5 drops of an aqueous solution of ethanol on the smoothed soil surface and waiting for their absorption. Different solutions of decreasing concentrations were successively applied to each soil sample, until a single solution had t > 3'' (Doerr, 1998; Leighton-Boyce *et al.*, 2005). The concentration of the last solution to be applied on each sample was used to identify the sample's class of hydrophobicity severity (Table 4.2) (Leighton-Boyce *et al.*, 2005).

Soil samples from the same block were analysed in a single batch in tests.

Table 4.2 - Specifications of soil hydrophobicity. **a.** persistence of soil hydrophobicity measured by water drop penetration time test (WDPT, adapted from Doerr (1998) and Dekker and Ritsema (1994)). **b.** severity of soil hydrophobicity measured by molarity of ethanol droplet test (MED, from Leighton-Boyce *et al.* (2005)).

a) Persistence of soil hydrophobicity

Water drop penetration time (s)	< 5	5 - 9	10 - 29	30 - 59	60 - 179	180 - 299	300 - 599	600 - 899	900 - 3,599	3,600 - 7,199	7,200 - 10,799	≥10,800
Persistence class	1	2	3	4	5	6	7	8	9	10	11	12
Descriptive category	Wettable		Slight		Strong			Severe		Extreme		

b) Severity of soil hydrophobicity

Ethanol concentr. (%, v/v)	0	1	2	3	5	8.5	13	18	24	36	50	>50
Severity class	1	2	3	4	5	6	7	8	9	10	11	12
Descriptive category	Wettable	Low			Moderate			Severe		Extreme		

Soil nutrients

Soil samples from SSa plots and control plots were further analysed to determine: texture; pH (in water); organic matter (%); and contents of extractable nutrients (P_2O_5, K_2O, and Mg^{++}; in mg kg^{-1}).

Firstly, all the samples were sieved (mesh = 2 mm) and oven dried (35-37ºC). Then, they were separated into subsamples for the different analyses. The textural class (coarse, medium, or fine) was expeditiously ascertained rubbing moist soil samples by hand. Soil pH was determined by potentiometry (Radiometer Analytical, France), in a water suspension (1:2.5, v/v; 1h). Colourimetry was used for quantifying organic matter (modified Tinsley method) (Gonçalves, 1985; Dias *et al.*, 2005). Phosphorous and potassium were extracted from the soils with Egner-Riehm solution (soil/solution 1:20, m/v; 2h), whereas magnesium was extracted with ammonium acetate at 1 M (soil/solution 1:10, m/v; pH 7; 30'). The concentrations of these nutrients were determined by: inductively coupled plasma optical emission spectrometry for P_2O_5; flame emission spectrometry (Corning, UK) for K_2O; and flame atomic absorption spectrophotometry (GBC Scientific Equipment, Australia) for Mg^{++}. As far as it was possible, soil subsamples from the same block were analysed in a single batch in every analysis.

4.2.5. Data analyses

Exploratory analyses were made using the collected data from all plantations. These data included attributes observed in the field and in the laboratory, corresponding to control, Se, and SSa microsites. Covers of fungi, lichen, and rotten wood were discarded from the analyses because these guilds occurred in four or less plots. Exploratory analyses consisted in two steps. Firstly, a correlation matrix was built (Pearson r^2) using all measured attributes. Secondly, a forward stepwise discriminant analysis was performed. For this analysis, the grouping variable was 'plantation' and candidate dependent variables were the 14 data attributes which had more than 10 significant correlations ($P < 0.05$) on the correlation matrix. Some variables were ln transformed, in order to accomplish discriminant analysis assumptions. Model quality was assessed through a classification matrix.

Preliminary analyses showed that plantations had substantially differed in their characteristics (eqs. 1–7, Fig. 4.1). Hence, data from different plantations were separated for further analyses.

To compare SSa microsites with control microsites, the paired-sample Student's t test was used when data of continuous variables were normally distributed. The Wilcoxon test was used when those data distributions were not normal and for comparisons concerning ordinal variables. The latter test was only used when the data were symmetrically distributed around the median. When this assumption was not met, the sign test was used instead (Zar, 1996). The McNemar's test was employed for comparisons concerning binary variables.

To compare the four types of microsites from CM1, a 2-way mixed-effects ANOVA (fixed factor—microsite type; random factor—blocks; no interaction between factors) and the *post-hoc* Tukey test were used, when data had normal distributions. The Friedman test and the

post-hoc Dunn test were used instead, when data distributions were not normal and for comparisons concerning ordinal variables. The Cochran's Q test and the Dunn test were chosen for comparisons concerning binary variables. Relationships between the microsite types and other nominal variables were analysed through contingency tables and χ^2 test (Zar, 1996).

Statistica 6.0 release (StatSoft, 2000) was used for preliminary analyses, while SPSS 22.0.0.0 version (IBM, 2013) was used for all the other analyses. Analysis results were considered significant at $P < 0.05$.

4.3. Results

4.3.1. Preliminary note

No pre-fire *E. globulus* regeneration (alive or remains) was found in the plantations during the wildling survey for plot establishment, with the only pre-fire individuals being those that were planted. The post-fire regeneration consisted of wildlings of different sizes. Saplings were absolutely dominant in all plantations, whereas Se were a minority in CM1 and nearly absent in the other plantations.

4.3.2. Exploratory analyses

Fourteen observed variables were significantly correlated to more than ten other observed variables ($P < 0.05$). They were: southern aspect; cover of bare soil; cover of ash; cover of small shrubs; soil hardness; soil texture; soil pH; content of organic matter in soil; soil [P_2O_5]; soil [K_2O]; soil [Mg^{++}]; persistence of soil hydrophobicity; number of wildlings; and median height of wildlings.

Discriminant analysis for plantations produced a model which had an average Wilk's lambda of 0.36, and three discriminant functions with standardized coefficients (Eqs. 1-3):

D1 = - 0.60 ln (median height of wildlings) - 0.86 ln (soil K2O) + 0.34 (persistence of soil hydrophobicity) + 0.39 ln (ash cover +1) **(Eq. 1)**

D2 = 0.73 ln (median height of wildlings) - 0.13 ln (soil K2O) - 0.05 (persistence of soil hydrophobicity) + 0.60 ln (ash cover +1) **(Eq. 2)**

D3 = - 0.22 ln (median height of wildlings) + 0.25 ln (soil K2O) - 0.81 (persistence of soil hydrophobicity) + 0.54 ln (ash cover +1) **(Eq. 3)**

This model provided a classification matrix with 55% to 63.5% of correct classifications, obtained through the following classification functions (Eqs. 4-7):

CM1 = 13.63 ln (median height of wildlings) + 32.33 ln (soil K2O) - 0.78 (persistence of soil hydrophobicity) - 0.07 ln (ash cover +1) - 97.02 **(Eq. 4)**

CM2 = 15.31 ln (median height of wildlings) + 32.32 ln (soil K2O) - 0.78 (persistence of soil hydrophobicity) - 0.07 ln (ash cover +1) - 103.95 **(Eq. 5)**

CV = 16.24 ln (median height of wildlings) + 37.06 ln (soil K2O) - 1.16 (persistence of soil hydrophobicity) - 1.15 ln (ash cover +1) - 128.56 **(Eq. 6)**

SA = 16.50 ln (median height of wildlings) + 36.50 ln (soil K2O) - 0.86 (persistence of soil hydrophobicity) - 2.26 ln (ash cover +1) - 127.64 **(Eq. 7)**

According to this model, the four plantations can be distinguished using four variables (Fig. 4.1; Eqs. 4-7). Firstly, soils in CM1 and CM2 were poorer in K_2O than those in SA and CV (Fig. 4.1). Secondly, CM1 had the shortest wildlings comparatively with all the other plantations (Fig. 4.1a), a fact that was very evident in the field as CM1 was the only plantation that had Se. Thirdly, SA microsites were the least covered by ash, followed by those of CV (Fig. 4.1). Finally, CV soil had the least persistent hydrophobicity (Fig. 4.1b).

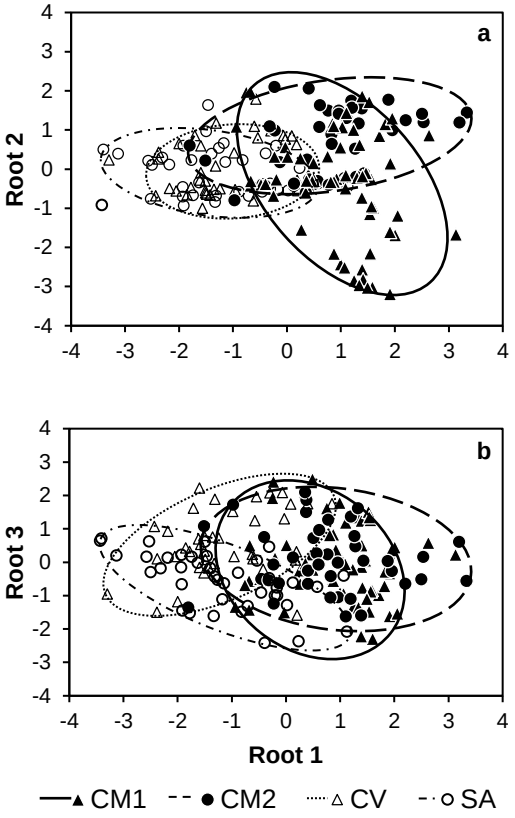

Fig. 4.1 – Scatter plots of canonical scores obtained in discriminant analysis of the plantations, using microsite attributes as dependent variables (see eqs. 1–3 and text for detailed explanation). **a.** root 1 *vs.* root 2; **b.** root 1 *vs.* root 3. Plantations: CM1 – Casal do Malta 1; CM2 – Casal do Malta 2; CV – Currelos Valdeias; SA – Santo António. (*n* = 183)

4.3.3. Microsites of established wildlings

Microsites with SSa (regarded as the smallest established wildlings) differed from those with no wildlings, in several features (Fig. 4.2), predominantly at the plantations of the central region (CM1 and CM2). While similar trends were found at the more northerly plantations (CV and SA), few of the differences (namely, slope and ash depth at SA) were statistically significant (Fig. 4.2). Microsites containing saplings, in comparison to those without, tended to have: steeper slope; higher degree of shelter; taller sheltering objects; closer proximity to other plants; greater amount of small shrub cover; more charcoal cover; greater ash cover and depth; and

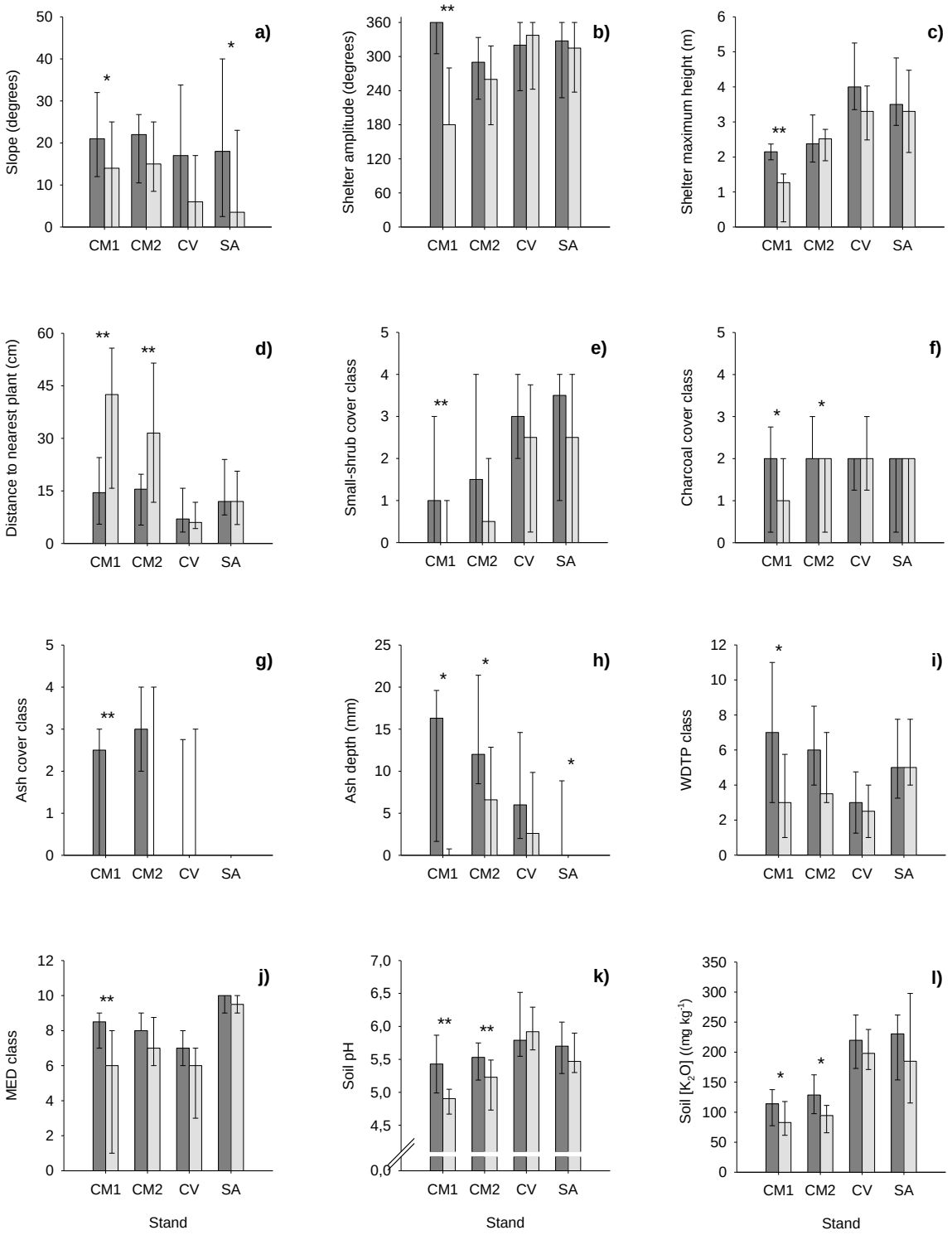

Fig. 4.2 – Microsite attributes (**a** to **l**) in studied plantations. Microsite types: short sapling microsites (■) and control microsites (□); (n = 20). Plantations: CM1 – Casal do Malta 1; CM2 – Casal do Malta 2; CV – Currelos Valdeias; SA – Santo António. Columns show medians and bars show first and third quartiles. Significant differences are indicated by: * $0.01 \leq P < 0.05$; ** $0.001 \leq P < 0.01$. See text for detailed explanation about classes of: cover; persistence of soil hydrophobicity (water drop penetration time test, WDPT); and severity of soil hydrophobicity (molarity of an ethanol droplet test, MED).

soils with more persistent and severe hydrophobicity, higher pH, and greater concentrations of K (Fig. 4.2). None of the other ground cover, plant guild cover, or soil properties differed significantly between SSa and control microsites.

The height of objects surrounding the plot centres varied largely, even in a single plot, and ranged from a few centimetres to several metres. Each plot usually had several types of objects around its centre. No significant differences were found on the occurrence of each type of surrounding objects between SSa microsites and control microsites. Abiotic objects were almost as much represented (in 87.0% of the microsites) as biotic objects were (in 93.8% of the microsites). Most common biotic objects around the plot centres were *E. globulus* stump resprouts (in 77.8% of the microsites) and plants of other species (in 50.6% of the microsites). Abiotic objects were mainly features derived from soil preparation for planting, namely 77.8% of the microsites had balks or taluses nearby. Distance from the plot centre to closest object was not significantly different between SSa microsites and control microsites, in any plantation (mean = 28.1 cm).

4.3.4. Microsites of wildlings of different sizes

Wildlings were observed in a wide range of sizes at all studied plantations. However, only CM1 had plentiful seedlings which presumably represented a more recent cohort than the taller and more developed saplings. Comparisons of the respective microsites at CM1 detected several significant differences (Fig. 4.3 and Table 4.3). Seedling microsites were characterised by significantly shallower slope, less shelter by objects, more bare ground, less ash cover and depth, and lower levels of soil hydrophobicity severity/persistence than the microsites containing TSa (Fig. 4.3a–j). These variables tended to have intermediate values in SSa microsites. Although small shrubs seemed to cover substantially smaller areas in Se microsites (cover class: quartiles, Q1 = Q2 = Q3 = 0) and controls (cover class: Q1 = Q2 = 0; Q3 = 1) than in sapling microsites (cover class: SSa – Q1 = 0; Q2 = 1; Q3 = 3; TSa – Q1 = 0; Q2 = 2; Q3 = 3), *post-hoc* tests were not able to determine where the differences were. Moreover, the three types of wildlings microsites significantly differed in terms of the abundance of other *E. globulus* wildlings in the surroundings, since these plants occurred much more frequently around TSa (Q1 = 1.70; Q2 = 2.80; Q3 = 4.30 wildlings m^{-2}) than around SSa (Q1 = 0.55; Q2 = 1.00; Q3 = 1.40 wildlings m^{-2}) and Se (Q1 = 0.50; Q2 = 0.40; Q3 = 1.15 wildlings m^{-2}). Maximum density observed was 9.90 wildlings m^{-2}, around TSa. While the most represented height class around TSa was '50 < h ≤ 130 cm', the one around SSa and Se was 'h ≤ 50 cm'. The sharpest difference between microsites with SSa and TSa was the more than four-fold greater occurrence of live stumps close to SSa than to TSa (Fig. 4.3k). Finally, the only difference between microsites containing Se and microsites without wildlings was the much greater presence of *E. globulus* capsules in the Se microsites (Fig. 4.3l).

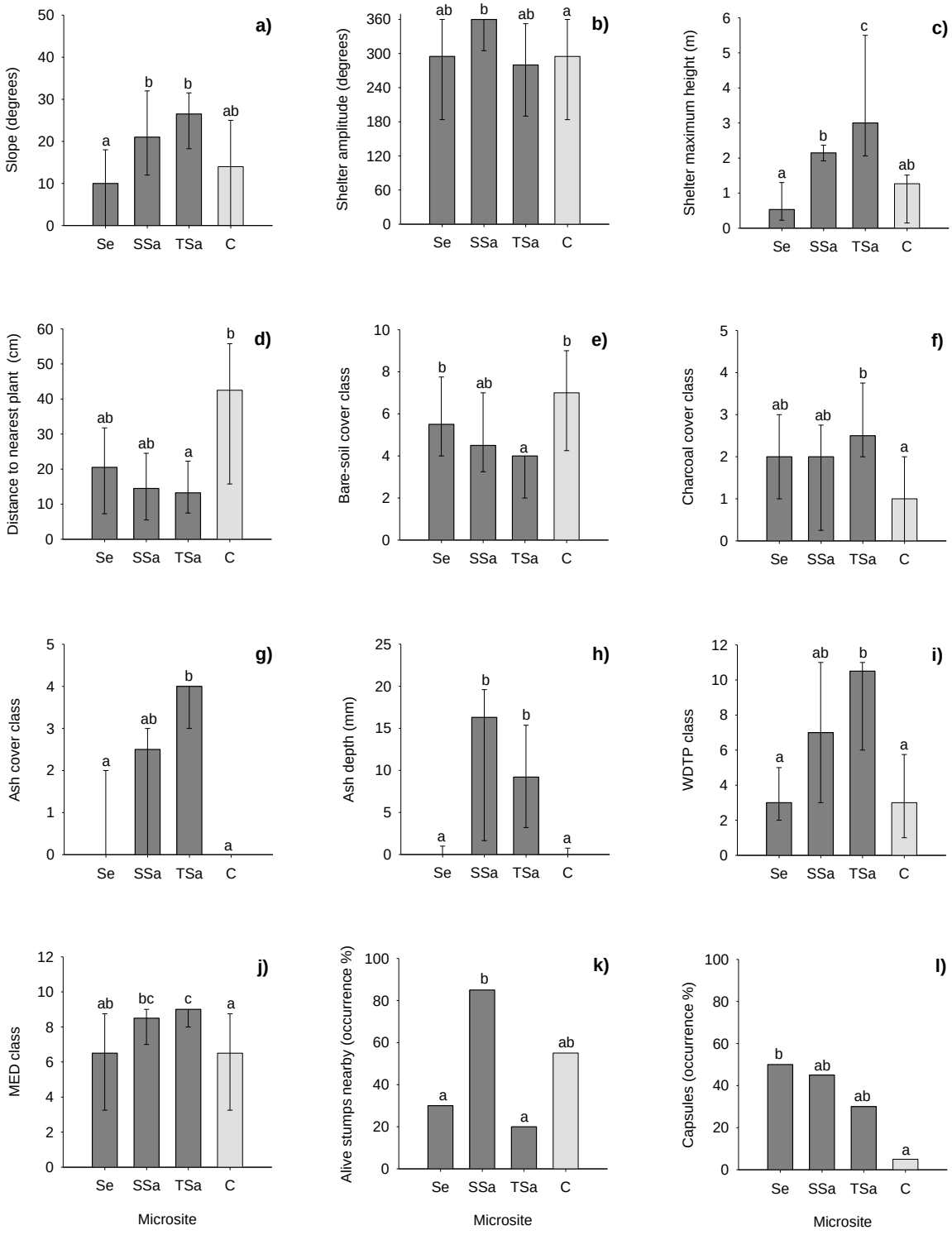

Fig. 4.3 – Microsite attributes (**a** to **l**) in the plantation Casal do Malta 1 (*n* = 20). Microsite types: Se – seedling; SSa – short sapling; TSa – tall sapling; C – control. Columns show medians (■ – wildlings; □ – controls) and error bars show first and third quartiles. Microsite types that are significantly different ($P < 0.05$) are indicated by different letters. See text for detailed explanation about classes of: cover; persistence of soil hydrophobicity (water drop penetration time test, WDPT); and severity of soil hydrophobicity (molarity of an ethanol droplet test, MED).

Table 4.3 - Results of statistical analyses comparing different microsite types in Casal do Malta 1 plantation (n = 20). Microsite types: seedling; short sapling; tall sapling; control.

Microsite attribute	Test statistic	*P* value
Slope	$F_{3,56} = 5.313$	0.003
Shelter amplitude	$\chi^2_r = 5.913$	0.001
Shelter maximum height	$F_{3,56} = 16.207$	< 0.001
Distance to nearest plant	$\chi^2_r = 10.600$	0.014
Small-shrub cover	$\chi^2_r = 12.210$	0.007
Bare soil	$\chi^2_r = 4.968$	0.002
Charcoal cover	$\chi^2_r = 10.399$	0.015
Ash cover	$\chi^2_r = 22.288$	< 0.001
Ash depth	$\chi^2_r = 22.288$	< 0.001
Persistence of soil hydrophobicity	$\chi^2_r = 25.532$	< 0.001
Severity of soil hydrophobicity	$\chi^2_r = 21.475$	< 0.001
Occurrence of alive stumps nearby	Q = 16.833	0.001
Capsule occurrence	Q = 12.488	0.006
Abundance of other wildlings	$\chi^2_r = 21.221$	< 0.001

4.4. Discussion

This study shows that changes at microsite level, caused by fire and the subsequent salvage logging, can create opportunities for wildling recruitment and establishment within burnt *E. globulus* plantations outside the species' native range. No pre-fire wildlings were found within four surveyed plantations in Portugal. However, many post-fire wildlings of different sizes existed in all sites, with most of them being saplings two years after the fires. Most differences between microsites with and without saplings concerned fire-related features and protective features in the surroundings. Microsites of wildlings from different size classes had significantly different characteristic; possibly indicating different recruitment events, as well as effects of competition from recently coppiced adults.

4.4.1. Wildling origin

The studied plantations had thousands of wildlings inside burnt areas (range of measured density: 0–9.90 wildlings m^{-2}). As no artificial sowing was made there, these plants must have emerged from seeds of natural origin. Stems of planted trees (2nd rotation) were old enough to produce seed crops at the time of fire (Kirkpatrick, 1975; Jordan *et al.*, 1999). Considering the seed dispersal distance for this species (Cremer, 1977), the height of planted trees when fire occurred, and the plantation density (Table 4.1), seeds from planted trees could easily reach any place within the plantations. This was confirmed by the detection of *E. globulus* capsules at similar frequencies at sapling microsites and the other microsites. The relevance of a soil seed bank should have been negligible because free eucalypt-seeds usually do not last more than 6–12 months in the soil (Florence, 1996) and they are susceptible to heat (Santos *et al.*, 2015). However, many seeds could be stored in the canopies at fire moment, protected by capsules (Silva *et al.*, 2016), and others could be produced by surviving trees later on. Therefore, the observed wildlings were the offspring of burnt planted trees.

The observed wildlings ranged widely in size and had no fire injuries. Although *E. globulus* age is difficult to determine (Williams and Brooker, 1997; Leal *et al.*, 2004), the height of these wildlings is compatible with ages under two years (Tomé *et al.*, 2001). Thus, wildling recruitment should have occurred after fire. Requisites for *Eucalyptus* regeneration are: seed availability; mineral seedbed; suitable weather; and release from both predation and competition (Jacobs, 1955; O'Dowd and Gill, 1984; Wilkinson *et al.*, 1993; Florence, 1996). In fact, *E. globulus* only regenerates where tree cover is absent (Stoneman, 1994). The conjunction of these conditions existed twice recently in the sampled plantations. The first opportunity was just after fire and the second one was after salvage logging. Fire destroys or damages competitors, destroys litter layer, induces seed release from eucalypt trees, and reduces populations of seed predators and browsers (Gilbert, 1959). In fact, regeneration of *Eucalyptus* natural forests is predominantly triggered by fire (Jacobs, 1955; Wilkinson *et al.*, 1993; Gill, 1997). In addition, logging activities can also elicit events of *Eucalyptus* regeneration, because they can provide an additional input of seeds and expose mineral soil in some areas (Wilkinson *et al.*, 1993; Florence, 1996; Fagg, 2001; Neyland *et al.*, 2009). Hence, fire and salvage logging enabled the emergence of two wildling cohorts in studied plantations. These two cohorts were more obvious in the CM1 plantation which was salvage logged only 3 months prior to sampling (compared to 9 and 18 months prior to sampling at the two northern plantations). At the time of sampling CM1, the observed Se had a size which was compatible with germination short time after logging, and their aging traits were incompatible with a second growth season. Conversely, the observed saplings had traits compatible with older age (height, aging signs, and lignotuber) and had physical damages that could have been caused by salvage logging. Thus, saplings would have been recruited before logging.

When fire occurred, tree canopies probably bore ripe capsules from the 2012/13 crop (Goes, 1977) and serotinous capsules from previous crops (Tng *et al.*, 2012). Canopy seed bank might have been nearly exhausted after a massive post-fire release of seeds (Cremer, 1965; Silva *et al.*, 2016). Seeds arriving soil at early autumn found favourable weather conditions for germination (López *et al.*, 2000; Rix *et al.*, 2011) and growth (Pita and Pardos, 2001). The resultant wildlings had the chance to grow for some months before winter frost and summer drought, thus improving their ability to endure those stresses (Ashton, 2000; Calviño-Cancela and Rubido-Bará, 2013). Hence, this sequence of events and their timing must have enabled a numerous first cohort (observed saplings) to thrive in all plantations just after fire.

Thereafter, first cohorts of *E. globulus* and other species, as well as resprouts, occupied the suitable space that was available. This way, both seed source and safe sites were absent for a while, preventing new episodes of *E. globulus* recruitment. This recruitment could be resumed, only after 2013/14 seed crop was ripe and ready for release (since august 2013, Goes, 1977), and new safe sites for Se were created by tree harvesting. Then, germinants could emerge from both the viable seeds that already were on the ground at logging time and those released from fallen trees (Fagg, 2001; Pennington *et al.*, 2001). Hence, this recruitment resumption was the most likely origin of the second cohort (observed Se).

This second cohort was frequent only in one out of four plantations. Whereas fire was almost simultaneous in the four plantations, salvage logging was not. Differences in harvesting timing determined the amounts of viable seeds that could land on the new safe microsites created by this operation, as well as the weather conditions that seeds released from fallen trees and their germinants would have to cope with. Moreover, biotic interactions between *E. globulus* (seeds and Se) and other species depended on both phenology and spatial distribution of the coexisting species, which were different amongst plantations. Therefore, differences in second cohort density across plantations could have resulted not only from the tree harvesting timing, but also from other factors which are out of the scope of this study.

Two years after fire, Se were much scarcer than saplings, in sampled populations. Both seeds and safe microsites would have been very abundant just after fire and steadily decreased thereafter. Subsequent salvage logging interfered with this trend. The microsites with Se were very similar to the controls, suggesting that the availability of safe microsites should not have been limiting for the recruitment of the second cohort (observed Se). Conversely, capsules were more often observed in Se microsites than in controls, suggesting that wildling absence in controls could be related to seed limitation (Turnbull *et al.*, 2000). Therefore, the occurrence of a major recruitment event just after fire, followed by a period of much lower recruitment due to seed limitation, is the most likely explanation for the observed population structure.

4.4.2. Establishment niche

Established wildlings occupied microsites that differed from vacant microsites in several features, including; slope; shelter dimensions; proximity to other plants; small-shrub cover; ash and charcoal abundance; and soil traits. Consistent patterns were detected across plantations (SSa *vs.* control; Fig. 4. 2), despite the geographical and management differences (Table 4.1).

The only microtopographical feature that distinguished SSa from control microsites was slope, which was significantly steeper in microsites with SSa. These results are opposite to those found by Sanger *et al.* (2011) with *Eucalyptus gunii* of a broader age range in a native forest setting. In that study, wildling occurrence was related with increased water availability in shallower slopes. However, it is likely that wildlings occurrence was not strongly water limited in our study. In *E. globulus* native range, mean annual precipitation is between 600 mm and 1400 mm (Boland *et al.*, 1980). Two plantations (CV and SA) had quite high precipitation for the species requirements; while the others (CM1 and CM2) were close to the species lower precipitation limit (Boland *et al.*, 1980), but had bioindicators of soil moisture (*e. g.* mosses, *Juncus* sp., and *Erica ciliaris*). Our results are, however, in accordance with those obtained by Wilson and Gibbons (2014), in a study of three species of *Eucalyptus* of a similar age. There, steeper slopes were associated with higher herbaceous cover, which may have allowed the trapping of seeds and resources, transported by superficial water flow. Ashton and Spalding (2001) found that vigour and density of eucalypt seedlings was patchy and related to the pattern of redistributed nutrient-rich hillwash in burnt sites. This could be also the case in our study, since SSa plots tended to have plants closer to their centres and a larger cover of small shrubs,

comparatively to the controls. Actually, under stressing or low productive conditions, competition can be surpassed by facilitation as a determinant factor for seedling establishment (Ryser, 1993; Callaway, 1995; Gómez-Aparicio *et al.*, 2004). Thus, the clustered plants observed in our study might have created a synergy, benefiting altogether from the resource availability and other favourable conditions, rather than just competing among themselves. Results from Fowler (1988) corroborate this idea, insofar as the effects of aggregation outweighed the effects of competition in seedlings of two grass species living among other juvenile plants. Moreover, the nurse effect of shrubs is a common pattern in Mediterranean environments (Callaway, 1995; Quero *et al.*, 2008), and *E. globulus* wildlings are much more common in shrublands than in other habitats nearby plantations in Portugal (Fernandes *et al.*, 2018). Finally, steep slopes may also contribute to the occurrence of wildlings, through enabling small landslides which may bury seeds under a thin soil layer. The presence of the seeds in the topsoil, rather than on it, could protect them against predation, provide more favourable conditions for germination, and enable the ready penetration of radicles in soil (Florence, 1996; Hulme, 1998; Clarke and Davison, 2001). Accidental burying of seeds frequently decides which individuals have the chance to become established (Jacobs, 1955). *Eucalyptus globulus* individual seeds and heaps of them germinate from depths down to 1.3 cm and 3.9 cm, respectively (Jacobs, 1955). Such depths are compatible with the occurrence of microtopographical landslides. Therefore, steep slopes may have improved the chances of plant recruitment and establishment, directly, by promoting seed burying and, indirectly, by facilitation phenomena associated to plant clusters.

The type of objects surrounding the plot centres did not differ between the SSa microsites and the control microsites, but the set of those objects tended to be taller and wider in the wildling microsites. These results can be related to the protective properties that those objects have in common, which can assist young plants to overcome climatic adversity (Battaglia and Reid, 1993).

Fire related traits had stronger presence in SSa microsites than in control microsites. Charcoal (cover) and ash (cover and layer thickness) were the only ground cover features that differed significantly between these two microsite types. The presence of ash beds (*sensu latu*: soils covered by either ash or charcoal) has been long referred as a facilitator of *Eucalyptus* regeneration (*e. g.* Jacobs, 1955). Pryor, in 1960, created the concept of 'ash-bed effect' (Pryor, 1963; Wilkinson *et al.*, 1993) to name the enhanced plant growth achieved on soils that have been heated to temperatures usually in excess of 150ºC (Willis, 1999). This stimulation of growth at early ontogenic stages increases the establishment chances of *Eucalyptus* individuals (Cremer and Mount, 1965; Wilkinson *et al.*, 1993; Ashton and Spalding, 2001), which can in turn help to explain the presence of SSa in places where fire effects on soil were more evident.

Charcoal cover was wider at SSa microsites than at controls in CM1 and CM2. Combustion can destroy phytotoxic substances (Águas *et al.*, 2018), such as the phenolics contained in *E. globulus* leaves (del Moral and Muller, 1969). Charcoal itself can adsorb and deactivate phenolics in soil (Hille and den Ouden, 2005). Their destruction or adsorption can have a positive effect on germination and plant growth (Hille and den Ouden, 2005; Águas *et*

al., 2018). Nitrifying microbial community is also affected by charcoal, increasing N availability to plants (Wardle *et al.*, 1998; DeLuca *et al.*, 2006). Charcoal can also adsorb nutrients, improving the cation exchange capacity of soil (Glaser *et al.*, 2002). Furthermore, it can improve water retention and availability in coarse textured soils and, consequently, reduce nutrient leaching (Glaser *et al.*, 2002). Charcoal role as a facilitator of germination, plant survival, and growth might have contributed for SSa presence at microsites with broader charcoal cover, comparatively to control microsites, in CM1 and CM2. Conversely, SSa distribution did not evidence charcoal effects, in CV and SA. This result was probably due to the finer and nutrient richer soils of these plantations, as well as the higher rainfall in the northern region.

Ash tended to be more abundant (cover and depth) in the SSa microsites than in the controls. Water retention in soil and water availability to plants can be improved, when ash is produced by combustion above 300ºC and then is incorporated into the soil (Stoof *et al.*, 2010). Bailey *et al.* (2012) found that juveniles of several *Eucalyptus* species have ash beds as preferential microsites. Moreover, *E. globulus* shows competitive advantage over other eucalypts on ash beds, having more even and rapid germination and higher initial growth rate (Florence, 1996). Therefore, ash may have favoured SSa establishment in studied plantations.

Soil tended to be less acidic in SSa microsites than in controls but differences only were significant in CM1 and CM2 plantations. Moreover, soil pH was positively correlated to ash cover of microsites ($r^2 = 0.24$; $P = 0.003$; $n = 147$). The soil pH increase may have been due to both ash deposition and soil heating (Raison, 1979; Khanna *et al.*, 1994). In terms of nutrient availability for plants, pH = 6.5 is generally considered as very favourable, while very acidic soils are frequently nutrient deficient (specially in P) and contain toxic levels of some other elements (*e. g.* Mn, Fe, Al) (Santos, 2014 ; Truog, 1947). Acid soils reduce activity of nitrifying bacteria reducing N availability for plants (Santos, 2014). Phosphorous content and growth of ectomycorrhized *E. globulus* seedlings are larger when soils have pH 6 rather than pH 5 (Thomson *et al.*, 1996). Our results match these ideas. Soil pH was positively correlated to soil K ($r^2 = 0.41$; $P < 0.001$; $n = 147$), and Mg ($r^2 = 0.77$; $P < 0.001$; $n = 147$). Importantly, many observed SSa were visibly ectomycorrhized. Additionally, CM1 and CM2 were the plantations which had their soil pH farther from the favourable range, and where SSa were present only in microsites with the least extreme soil pH. In the other plantations, soil pH did not look to affect SSa distribution because it was close from the favourable range in the two microsite types. Therefore, as far as soil pH improves plant nutrition, it can be a factor that favours wildling establishment.

The soils of SSa microsites tended to be richer in K available to plants than those of control microsites, but differences were significant only in plantations with lower K concentrations and less rainfall (CM1 and CM2). Additionally, soil K was also positively correlated with microsite ash cover ($r^2 = 0.23$; $P = 0.003$; $n = 155$). The main mineral nutrients in the above ground biomass of *E. globulus* are Ca, K, and N (Brañas *et al.*, 2000), whereas ashes of *Eucalyptus* spp. are dominated by Ca and K (Raison, 1979; Khanna *et al.*, 1994). Hence, K concentration in some soils increased due to the ash deposition. Potassium enhances plant

resistance to diseases and pests, as well as tolerance to drought, frost (Marschner, 1995). Notably, drought and frost are the main restrictions to *E. globulus* in Portugal (Ribeiro and Tomé, 2000). This species has relatively high water potential thresholds for stomatal closure (Stoneman, 1994), and it is able to make osmotic adjustment (Correia *et al.*, 1989), processes where K plays a fundamental role (Marschner, 1995). Though the K concentrations were medium for fertility standards (Santos, 2014), in the plantations with the poorest soils (CM1 and CM2); the higher concentrations in the SSa microsites may have helped wildlings to better cope with stressful hydric conditions. This may have been particularly useful to SSa which were next to their major competitor, the adult trees or their resprouting stumps, what happened in 90.0% of SSa microsites of those plantations. Additionally, K is involved in phloem translocation, cell expansion, and protein synthesis (Marschner, 1995). Foliar K content of 1-year old *E. globulus* has a strongly positive correlation with their growth in the following years (Judd *et al.*, 1996). Hence, K contributions for SSa water balance and growth may have improved their survival chances in CM1 and CM2. Conversely, soil K did not differentiated SSa microsites from controls in CV and SA, probably because those soils had very high content of available K (Santos, 2014) and events of water stress were less probable there.

Soil in SSa microsites tended also to be less severely and persistently hydrophobic than in controls. This result could be regarded as another fire effect, since fire can induce or change soil hydrophobicity (DeBano and Krammes, 1966; DeBano, 1981). However, neither severity nor persistence was correlated with the other fire-related traits of microsites. Instead, severity of hydrophobicity was positively correlated with litter cover (r^2 = 0.29; P < 0.001; n = 183), and wildling height (r^2 = 0.31; P = 0.002; n = 102). In *E. globulus* stands, fire does not reinforce the strong soil hydrophobicity that exists before fire (Doerr *et al.*, 1998). This hydrophobicity is most likely due to: the chemical composition of *E. globulus* litter (either under decomposition or burnt), which is produced in large amounts, and by the high evapotranspiration of *E. globulus* trees, which dries soils during summer (Doerr *et al.*, 1996). In fact, even hydrophilic soils become extremely hydrophobic two years after afforestation with *E. globulus* (Doerr *et al.*, 1998). This hydrophobicity may be extended along soil profile, down to 10–30 cm deep (Doerr *et al.*, 1996). Therefore, generally high soil hydrophobicity observed in our study plantations is likely to be both a legacy of hydrophobicity developed during the plantation aging before fire, as in Leighton-Boyce *et al.* (2005), and a byproduct of planted trees and wildlings after fire.

4.4.3. Size differences among wildlings

In general terms, the three types of wildling microsites (studied in CM1) were distinguishable among each other and had different degrees of similarity with the unoccupied control microsites (Fig. 4.3). Controls were statistically similar to Se microsites in all but one studied factors, while they differed from SSa microsites in a few factors and differed from TSa microsites in the great majority of factors. Additionally, SSa microsites usually had characteristics in-between TSa microsites and Se microsites. These results suggest a narrowing transition of niche that would gradually occur as wildlings age, as Quero *et al.* (2008) observed

in *Acer opalus*. However, according to our age estimates, an important event has separated the emergences of the first and second cohorts; it was salvage logging. This management operation causes major environmental changes in a forest, which also favour *Eucalyptus* regeneration. Therefore, the recruitment niche that was at the origin of the first cohort was certainly different to the one that allowed the emergence of the second.

Many of the features of Se microsites were typical of areas which were deeply disturbed by tree harvesting operations that destroyed or damaged existing vegetation, exposed mineral soil, and altered its profile (Ferreira *et al.*, 2000; McIver and Starr, 2000). When compared to sapling microsites, Se microsites were characterized by: shallow slope; short shelter; neighbouring plants being moderately close but generally not including resprouting stumps or small shrubs; large area of bare soil; reduced presence of ash; and relatively low soil hydrophobicity. *Eucalyptus* seedlings are very sensitive to competition and a substrate free of competition is required for their establishment (Kirkpatrick, 1977). They often grow up amongst unburnt slash, especially if the timing of tree harvesting coincides with the occurrence of ripe seed crops, suitable seedbed, and good germination conditions (Fagg, 2001). In fact, the observed Se were precisely in microsites which show joint evidences of logging disturbance and presence of seeds. Therefore, salvage logging occurred in CM1 when seeds were available, and its disturbance created a multitude of microsites that were good enough for germination and Se survival. In practice, Se microsites provided important conditions for Se survival through reduced competition, some protection from physical stresses, and potentially increased water availability (by infiltration).

Nevertheless, microsite suitability is time dynamic. A favourable microsite in one moment in time may become unfavourable in another, due to changes in weather or other conditions (Battaglia and Reid, 1993). *Eucalyptus globulus* seedlings are very sensitive to drought. The first summer drought is more lethal to *E. globulus* germinants from spring and summer than to those from the other seasons (Calviño-Cancela and Rubido-Bará, 2013; Fernandes *et al.*, 2017). The observed Se have presumably germinated in the 2014 spring. However, the summer months in 2014 had mean temperatures 0.2–2.0ºC below the average 1961–90 (data provided by Instituto Superior Técnico, Portugal). Therefore, salvage logging played a major role creating good microsites for germination in spring, which turned out to be safe enough for Se during an extraordinarily mild summer. Furthermore, fire-related traits were much less obvious in Se microsites than in sapling microsites. By the time the studied Se had emerged, the favourable microsites created by fire were already crowded by much larger plants. Asymmetrical competition is an important factor of suppression for young eucalypts, including *E. globulus* (Bowman and Kirkpatrick, 1986b; Tomé *et al.*, 1994). Therefore, microsites occupied by saplings of the first cohort were not favourable for germinants of the second cohort. These results highlight the context dependence of wildling recruitment and early survival.

The tall sapling microsites were the ones which contrasted the most with the Se microsites. Their features were: steep slopes; moderately wide and very tall shelters; very close neighbouring plants except resprouting stumps; small areas of bare soil; strong presence of

ash; and very high soil hydrophobicities. Microsites with SSa generally had intermediate characteristics between TSa microsites and Se microsites, but they were more similar to the latter than to the former. Most attributes shared by TSa and SSa microsites were either direct or indirect consequences of fire occurrence (*e. g.* ash, plant clusters). The share of such niche features reinforces the idea that TSa and SSa belong to the same cohort.

The only factors which were significantly divergent between the two types of sapling microsites were shelter maximum height and the occurrence of conspecific plants nearby. These factors probably have contributed to size differences between TSa and SSa. Firstly, TSa had taller neighbouring objects than SSa, and thus were the most protected from adverse conditions for the longest time, before they have reached the height of their shelter. This extra protection of TSa might have favoured their growth. Secondly, resprouting stumps were more common close to SSa than close to TSa. These stumps were complete adult trees until several months before sampling and they had complete root system and strong resprouts at sampling time. Therefore, they were fierce competitors for the youngsters (Potts, 1986). Competition, which largely suppresses growth rates of *Eucalyptus* seedlings and saplings beneath adult trees (Bowman and Kirkpatrick, 1986b), can be extended to distances equivalent to 1–6 crown diameters (Incoll, 1979; Rotheram, 1983; Bowman and Kirkpatrick, 1986a). Growth of *E. globulus* saplings is very sensitive to asymmetrical competition (Tomé *et al.*, 1994). Water and nutrients are the most likely resources under competition, between these saplings and adults (Bowman and Kirkpatrick, 1986a; Potts, 1986; Tomé *et al.*, 1994). Therefore, the occurrence of a very unbalanced competition between saplings and stumps, where the former are the weakest competitors, is a sufficient cause for the size differences found between SSa and TSa, which presumably have a similar age. Thirdly, wildling density around TSa was 3-fold higher than around SSa. Despite competition, high densities of many terrestrial plants (even of the same species) also have advantages, reducing the susceptibility of individuals to predators and to physical or physiological stresses, during vulnerable juvenile stages (Stachowicz, 2001). Although, *E. globulus* usually self-thins more rapidly than other eucalypts (Florence, 1996), TSa microsites could be richer in resources than SSa microsites. Moreover, competition does not need to be weak for a biotic interaction having a net positive outcome (Stachowicz, 2001). Indeed, our results indicate that the competition between siblings was overcome by the advantages of living together, at least up to TSa stage. Additionally, tree harvesters did not venture into areas occupied by dense clusters of vegetation, which were mainly composed by wildlings and did not have mature trees to cut. As a result, plants in those areas were safe from disturbance associated to harvesting. Comparable results can be found in studies focused on selective herbivory, where target plants within thicket or unpalatable vegetation are safer than similar plants within palatable vegetation or isolated (Callaway, 1995; García *et al.*, 2000; Quero *et al.*, 2008). Nevertheless, the favourable balance of dense sibling-clusters would not last for long. The increasing demand for resources of growing individuals would eventually result in competitive exclusion within those groups. In fact, the height of saplings was already heterogeneous within clusters at sampling time. Notably, Quero *et al.* (2008) also identified the

coexistence of a positive interaction between saplings and similar plants (facilitation) with a negative interaction between them and adult trees (asymmetrical competition).

Finally, although many differences have been found between Se and TSa microsites, we have found no evidences of niche shift in post-fire *E. globulus* regeneration, two years after fire. Other studies succeeded in the characterization of both recruitment and persistence niches and in the assessment of niche shifts following a methodology which was similar to ours (*e. g.* Quero *et al.*, 2008; Bailey *et al.*, 2012), however they were looking at a longer time frame. In addition, a major post-fire disturbance (salvage logging) made the dynamics of the studied populations more complex. Nevertheless, salvage logging is the most common management operation in reproductive industrial burnt plantations of *E. globulus* in Portugal. Therefore, the inclusion of this factor brought important information to this first attempt to know the microsite factors underlying the sexual regeneration of this species in burnt plantations. The clear-cut separation of fire effects from logging effects on *E. globulus* regeneration requires further studies, under experimental conditions and/or using a longitudinal (follow-up) approach.

4.5. Conclusions

Both fire and salvage logging create environmental conditions for the occurrence of *E. globulus* regeneration from seeds in burnt plantations. Each of these events enabled the emergence of a cohort of wildlings, as identified in the most recently logged of the studied plantations.

Eucalyptus globulus wildlings in burnt plantations are often associated with features that are consequences of fire. The age estimates of wildlings are compatible with the emergence of a large cohort short time after fire. Two years after fire, these wildlings were already established and had survived to logging disturbance. Microsites inhabited by them still evidenced several effects of fire (ash, charcoal, high soil pH, and nutrient richness), which are beneficial for plant growth and establishment. Established wildlings are often surrounded by objects that seem to have protective role against adverse conditions. Steep slope is associated with the occurrence of regeneration. Its relevance seems to be associated with the presence of neighbouring vegetation that can favour the retention of water, nutrients, and seeds from hillwash. The very frequent co-occurrence of established wildlings and other plants (wildlings or not) may be a result of resource-rich islands. The prevalence of facilitative relationships over competitive ones could also explain the aggregated plant distribution. However, competition between wildlings and adult trees or resprouting stumps is the only exception, clearly hindering wildling growth despite not preventing the establishment. While it was not possible to completely characterize the recruitment niche of the first cohort, fire effects on the physical and chemical environment were observed to have lasted at least two years. Fire-related features should have been important for wildling recruitment, as they have been for establishment, and still were for persistence of those plants two years after fire.

Salvage logging enabled the emergence of a second cohort of regeneration, by providing a supplementary input of seeds and exposing mineral soil. This cohort inhabited microsites where the effects of salvage logging were more evident to the detriment of fire related traits, showing also that its recruitment conditions were substantially different from those of first cohort. Nevertheless, water availability, reduced asymmetrical competition, and some shelter were crucial for these plants as for the others. In addition, the second cohort was much less abundant than the first, and it occupied microsites that solely differ from those without wildlings by having capsules more often. These facts indicate a seed limitation at the origin of the second cohort. Logging also affected the first cohort, by injuring many of its individuals. However, tree harvesters avoided sapling clusters, allowing them to persist in plantations. No evidences of ontogenic niche shifts were found in either cohort.

In conclusion, the heterogeneity of *E. globulus* wildling distribution in stands can be explained by microsite diversity and seed availability. Fire and salvage logging at reproductive stands can induce seed release and create safe microsites. Therefore, attention should be paid to the effects that wildfires and salvage logging have in enabling and promoting *E. globulus* regeneration from seeds, and thus presenting a potential weed risk in areas outside the native range.

4.6. References

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Chapter 5

Fire effects on litter chemistry and early development of *Eucalyptus globulus* Labill.

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5.1. Introduction

Wildland fires are common in many regions in the world. They strongly influence ecosystem dynamics (Naveh, 1974; Chandler *et al.,* 1983; Bond and van Wilgen, 2012). As a result, many plant taxa, which have evolved in fire-prone environments, have reproductive traits that allow for regeneration after fires have occurred (Keeley *et al.,* 2011; Pausas and Keeley, 2014). These traits may be vital for plants not only in their native range, but also in other territories. Exotic species face new challenges to thrive outside their native ranges and may challenge other species for survival (Hallett, 2006; Mitchell *et al.,* 2006; Alpert, 2006). Some of these challenges are related to allelopathy (Callaway and Aschehoug, 2000; Cummings *et al.,* 2012). Fire, which can cause profound chemical changes in organic matter (González-Pérez *et al.,* 2004), has the potential to alter the chemical interaction amongst plants (Bonanomi *et al.,* 2006), if allelochemicals are affected. Consequently, fire may bring about a shift on the probability of plant establishment. This point is especially relevant with reference to the increasing cultivation of exotic species worldwide and in regions where fire regimes are changing.

Plants may release chemical compounds that affect other plants, either negatively or positively (Rice, 1984). Such contrasting effects have been documented as far back as ancient Greece (Willis, 1985), and are included in the concept of allelopathy, as expressed by Rice (1984). Although allelopathy is a phenomenon which has been known for centuries, especially in agriculture, the understanding of its impact on natural plant populations and communities is in its early stages (Meiners *et al.,* 2012). Nevertheless, evidence shows that allelopathy affects species' capabilities of self-perpetuation and colonization, which influence succession dynamics (*e. g.* Bazzaz, 1979), plant diversity (Bonanomi *et al.,* 2005), and vegetation spatial-patterns (Cartenì *et al.,* 2012).

When plant species are introduced outside their native ranges, they interact with native species of recipient communities. In the medium- to long-term, new interactions can have different outcomes for the exotic species, ranging from introduction failure to invasion (Sakai *et al.,* 2001; Hallet, 2006; Mitchell *et al.,* 2006; Blackburn *et al.,* 2011). Allelopathy is considered in some of the hypotheses that explain these different outcomes (Catford *et al.,* 2009). In his seminal work, Rabotnov (1974) postulated that species which have coevolved are less likely to chemically interfere with each other, than those from different biogeographical regions. In fact, some decades later, this idea was used to formulate two complementary hypotheses: the 'novel weapons hypothesis' (Callaway and Aschehoug 2000; Callaway and Ridenour, 2004) and the 'homeland security hypothesis' (Cummings *et al.,* 2012). Some authors claim that the use of a biogeographical approach is important in understanding allelopathy's role on the level of success of exotic species (Hierro *et al.,* 2005; Inderjit *et al.,* 2008). This approach has been mostly used to test the allelopathic potential of exotic species on other species, in the recipient communities (*e. g.* Callaway and Aschehoug, 2000; Loydi *et al.,* 2015). However, as there have

been few studies testing the effects of recipient communities on exotic species (*e. g.* Christina *et al.,* 2015; Ning *et al.,* 2016), there is a need for further research on this topic.

Plant litter is an important source of allelochemicals in terrestrial ecosystems. In fact, inhibitory effects of litter have often been reported in agriculture, forestry (Rice, 1984; Souto *et al.,* 2001), and natural ecosystems (Rice, 1984). The chemical substances of litter and their degradation products affect plant-soil feedbacks in varied ways (Mazzoleni *et al.,* 2015). Litter degradation can be caused by soil biological activity (Schlesinger, 1977), photo-degradation (Austin and Vivanco 2006), or combustion caused by fire (Raison, 1979; Pyne *et al.,* 1996). As a result, litter can release organic compounds and mineral nutrients (Schlesinger, 1977; Attiwill and Adams, 1993). The effects of litter dynamics are observed on different scales: seed germination and seedling establishment; population demography and community structuring; and species evolution (Facelli and Pickett, 1991; Stinchcombe and Schmitt 2006). The effects of litter decomposition on living plants have received a great deal of attention (Facelli and Pickett, 1991; Reigosa *et al.,* 2006), while the effects of litter combustion on plants have not.

Fire alters the chemical properties of organic matter, as thermally-induced modifications occur during combustion (González-Pérez *et al.,* 2004). The effects of fire on soil organic molecules are complex and depend on fire-exposure time, fire temperature, and post-fire biological activity (reviews in González-Pérez *et al.,* 2004; Certini, 2005; Knicker, 2007). Fire can directly neutralize phytotoxicity, through: direct alteration of allelochemicals (McPherson and Muller, 1969); insolubilisation by condensation reactions, or sorption by pyrogenic char (Wardle *et al.,* 1998; Hille and den Ouden, 2005). In addition, some combustion products can break seed dormancy (Keeley and Fotheringham, 2000), stimulate germination, and regulate seedling growth (Nelson *et al.,* 2012). Conversely, others can inhibit germination (Nelson *et al.,* 2012). Fire-derived allelochemicals are present in both smoke and combustion residues in soil, where they can come in contact with plants (Nelson *et al.,* 2012). Additionally, the quality and magnitude of fire effects on allelopathic interactions can be highly variable, as they depend on heating temperature (Johnson, 1919; Bonanomi *et al.,* 2016) and are species specific (Johnson, 1919; Keeley *et al.,* 1985; Nelson *et al.,* 2012). Therefore, litter combustion can induce changes in the chemical environment of plants, thereby influencing their development.

The idea that fire can affect allelopathic interactions in ecosystems has been around for a long time (Johnson, 1919; Muller *et al.,* 1968), however, little is still known. Subsequent studies focused mainly on Californian chaparral species (*e. g.* McPherson and Muller, 1969; Christensen and Muller 1975), and rarely on other systems (*e. g.* Razanamandranto *et al.,* 2005). For instance, no relevant studies were conducted on *Eucalyptus*, a tree genus that deserves to be studied in this context for various reasons. Firstly, it has evolved in a fire-prone environment (Mount, 1969). Secondly, its regeneration from seeds seems to be fire-dependent (Mount, 1964; Kirkpatrick 1975). Indeed, spurts of regeneration have often been associated with the occurrence of severe fires (*e. g.* Mount, 1964; Florence, 1996). Thirdly, several species of this genus have allelopathic potential (May and Ash, 1990; Willis, 1999). Moreover, Mount (1969) suggests that the regeneration success of this *taxon* in burnt areas may be linked to the

removal of chemical inhibitors from the soil's surface. Nevertheless, only Florence and Crocker (1962) have tested the effect of soil heating on eucalypt development, but their test temperatures were low (T ≤ 160°C) compared to those reached on soil surface during a fire. Finally, *Eucalyptus* has a large distribution area both as a native and as an exotic genus. It is almost exclusively native to Australia, existing abundantly throughout the country (Mount, 1969; Rejmánek and Richardson, 2011), and is the second most cultivated tree genus in the world (≈ 20 x 10^6 ha, Rejmánek and Richardson, 2011). Furthermore, its naturalisation and spread from cultivation has been documented in several countries (Rejmánek and Richardson, 2011). As a result, *Eucalyptus* is a relevant plant genus to study the changes in allelopathic interactions that may underlie post-fire plant establishment.

Despite the numerous studies that have been conducted, allelopathic interactions remain difficult to disentangle from other ecological processes. Field-based investigations face a multitude of confounding factors, while bioassays, under controlled conditions, produce results whose extrapolation to field conditions is somewhat limited (May and Ash, 1990; Inderjit and Nilsen, 2003). However, if the latter are complemented by chemical analyses, not only will they allow for the separation of the effects of several influencing factors, but also for the identification of potentially involved substances (Inderjit and Dakshini, 1995; Inderjit and Nilsen, 2003). A useful analytic technique for this purpose is solid-state ^{13}C nuclear magnetic resonance spectroscopy (^{13}CNMR), as it is a powerful tool for studying the molecular composition of complex organic materials (Baldock and Smernik, 2002). Moreover, this technique has been successfully used for tracking organic-matter changes caused by heat and fire (*e. g.* Freitas *et al.,* 1999; Almendros *et al.,* 2003; Knicker *et al.,* 2005). Another important strategy employed to improve ecological relevance of bioassay results is the careful selection of the species to be tested (donors and targets). Besides model species, species that coexist in the field need to be used. However, most laboratorial studies only use model species as target species (Inderjit and Nilsen, 2003). As interspecific differences in sensitivity to allelochemicals are common (Inderjit and Nilsen, 2003; Meiners *et al.,* 2012), the ecological relevance of those studies is lessened. Furthermore, some studies have analysed the ecological impact of chemical changes caused by fire on litter (*e. g.* Guinto *et al.,* 1999; Campos *et al.,* 2012; Reyes *et al.,* 2015; Bonanomi *et al.,* 2016). However, these were exploratory, very divergent in their aims, and rarely examined interactions between species that actually coexist. Hence, bioassay results can be more meaningful in ecological terms, if the bioassays use donor and target species that coexist in the field, and if they are coupled with appropriate chemical analyses of the substrates.

This article focuses on allelopathic interactions in wildland fire context. It aims to study the potential of a fire-adapted species to persist in self-dominated forests or to colonize heterospecific communities. In particular, we have used bioassays under optimal environmental conditions to study the early development of *Eucalyptus globulus* Labill.. Substrates were dry and charred leaf litter, from either conspecific (*E. globulus*) or heterospecific plants (*Acacia dealbata* Link, *Pinus* pinaster Aiton, and *Quercus suber* L.). These species coexist with the target species in its native range (*A. dealbata*) and/or outside it (*A. dealbata*, *P. pinaster*, and *Q.*

suber). The allelopathic effects of the same substrates were also examined on a model species (*Lepidium sativum* L.). Litter biochemical quality was analysed by Cross Polarization Magic Angle Spinning ^{13}C NMR (^{13}C CPMAS NMR) and its relationship with plant development was assessed.

5.2. Material and methods

5.2.1. Study area and plant species

Study sites were located in the central massif of Serra de Monchique, in the hinterland of Algarve, SW Portugal. Sites´ latitude ranges between 37º18'N and 37º20'N, while longitude ranges between 8º29'W and 8º34'W. Altitude ranges between 293 m and 442 m a.s.l.. The local climate is classified as type Csb type according to Köppen-Geiger (Faria *et al.,* 1981; Peel *et al.,* 2007). The mean annual temperature is 15.5°C, and annual precipitation is 1348 mm (Faria *et al.,* 1981). The bedrock is a sienitic intrusion (Rock, 1983). Soil is a humic cambisol, with a sandy texture, a high water-holding capacity, and a high base saturation (Kopp *et al.,* 1989). Local forests are mainly plantations of *E. globulus*, but *P. pinaster* plantations and *Q. suber* woodlands are also of importance there. These forests tend to be pure, but different combinations of these species also exist. In addition, *A. dealbata* occurs both within these main forest types and in monospecific patches.

The four aforementioned species were selected for this study, not solely based on their importance for forest management and conservation in S Europe and their relevance in fire ecology (Silva *et al.,* 2009; Lorenzo *et al.,* 2010; ICNF 2013; Águas *et al.,* 2014), but also based on their allelopathic potential (del Moral and Muller, 1969; Gonçalves *et al.,* 2008; Lorenzo *et al.,* 2008; Amri *et al.,* 2013). In addition, we selected *Lepidium sativum* as a model target species because of its recognized sensitivity to phytotoxicity (Macías *et al.,* 2000; Bonanomi *et al.,* 2006). Hereafter, plant species will be referred to by their genus names. Species names will only be used if clarification is required.

5.2.2. Plant material collection

In the study region, different forest patches were selected by a stratified random design, with strata classified according to different dominant species. In May 2015, leaf litter and seeds were sampled. Recently abscised leaves were collected from under the canopies of 20 trees of the dominant species. These were randomly selected within each patch. With reference to *E. globulus*, which shows leaf heteroblasty, only adult leaves were collected. *Eucalyptus* seeds and litter were collected on the same day, from the same trees. Thirty ripened capsules were collected from each tree and were air dried. At the laboratory, leaf litter materials were also air dried at room temperature, until constant mass was reached. They were milled (particle size < 1 mm), mixed to a single composite sample per species, and stored at room temperature. Although milling could increase allelochemical bioavailability in litter samples (May and Ash, 1990; Inderjit and Nilsen, 2003), it was deemed necessary for homogeneous burning to occur,

due to the large inter-specific differences in both structural and morphological leaf traits. *Eucalyptus* seeds were gravitationally extracted from capsules and separated from the chaff. *Lepidium* seeds were obtained from a commercial seedlot.

5.2.3. Heat treatments

Treatments consisted of the heating of samples from each species at six different temperatures (100; 200; 300; 400; 500; and 600°C), for 30 minutes. In addition, one unheated sample from each litter species was used as the control for temperature. The crucible was a metal tray (21 × 19 × 4 cm). Litter samples were 100 g (dry mass), prior to heating. These samples were heated uncovered, in single batches, one litter species at a time, inside a pre-heated closed muffle furnace. As a result, 28 different litter types were obtained (4 litter species × 7 temperature levels).

5.2.4. Chemical characterization of litter materials

The 28 litter types were characterized by ^{13}C CPMAS NMR obtained in solid state, under the same conditions, thus enabling quantitative comparisons among spectra. The method followed Bonanomi *et al.,*(2011). A Bruker AV-300 spectrometer (Bruker Instrumental, Billerica, USA), equipped with a 4-mm-wide bore MAS (magic angle spinning) probe, was used for these analyses. The NMR spectra were obtained with a MAS of 13,000 Hz of rotor spin; a recycle time of 1 s; a contact time of 1 ms; an acquisition time of 20 ms; and 2000 scans. Samples were packed in 4-mm zirconium rotors with Kel-F™ caps (Wilmad ⁄ Lab Glass, Buena, USA). The pulse sequence was applied with a ^{1}H ramp to account for non-homogeneity of the Hartmann–Hahn condition at high spin rotor rates. Pre-processing consisted of an exponential multiplication, with a line broadening of 50.0 Hz, that was applied to the free induction decay, prior to Fourier transformation. All spectra were processed using the AMIX program (www.bruker.com), phased, and the baselines corrected manually, to calculate the area of the peaks in selected regions. The relationship between spectral regions and C types was drawn from reference studies (Almendros *et al.,* 2000; Kögel-Knabner 2002; Bonanomi *et al.,* 2011): 0–45 ppm = alkyl C; 46–60 ppm = methoxyl + *N*-alkyl C; 61–90 ppm = *O*-alkyl C; 91–110 ppm = di-*O*-alkyl C; 111–140 ppm = *H*,*C*-substituted aromatic C; 141–160 ppm = *O,N*-substituted aromatic C (phenolic C, *O*-aryl C, *N*-aryl C); and 161–190 ppm = carboxyl C.

All litter types were characterized for total C and N content in an elemental analyser (Primac SCN100, Skalar, Netherlands). Flash combustion at 1100°C of microsamples was followed by quantification of C by a near-infrared detector (NIRD) and quantification of N through thermal conductivity (Dumas method).

In addition, proximate cellulose and lignin content were quantified for unheated litter as the acid hydrolysable fraction and the acid non-hydrolysable materials, respectively (Gessner 2005). In short, proximate cellulose was determined as the hydrolysable fraction following a sulphuric acid treatment (loss upon 3 h, in H_2SO_4 at 72%). Proximate lignin was determined as

the non-hydrolysable fraction (loss upon ignition, after the aforementioned H_2SO_4 treatment). All carbon fractions were presented as ash-free dry mass.

5.2.5. Plant bioassays

Plant bioassays were aimed at assessing the effects of unheated and heated litters on *Eucalyptus* seed germination and root growth, compared to *Lepidium*'s. Ten seeds of either *Lepidium* or *Eucalyptus* were placed in 9-cm Petri dishes, over 2 sheets of sterile filter paper, with 0.2 g of litter, and wetted with 4 mL of distilled water. Subsequently, dishes were wrapped with plastic film to ensure air tightness. Controls for each target species were Petri dishes with seeds placed on water-wetted paper. Each treatment combination was replicated three times. A total of 1740 seeds were used in the experiment ((28 litter types + control) × 2 target species × 10 seeds × 3 replicates). All Petri dishes were arranged following a fully randomized design at the laboratory, and were kept at room temperature and under natural daylight (photoperiod of 12 h).

Germinated seeds were counted and seedling root lengths were measured after incubations periods of 3 and 5 days for *Lepidium* and *Eucalyptus*, respectively, based on the germination time of each species (ISTA, 2003). For both target species, seedlings were counted and roots were measured when the longest roots in the control assays reached a length of 2 cm. In order to ensure that statistical analyses could be run without missing data, the few replicates which had no germinations were considered as having a root growth equal to zero. This approach is unlikely to produce biased inference, considering that in the absence of germination, there is no seedling growth, nor plant establishment.

5.2.6. Data analysis

One-way ANOVAs were used to test the effect of heating temperature (7 levels) on litter chemical quality. The concentrations of different C types, assessed by ^{13}C NMR, were considered as the dependent variables. The tested null-hypotheses stated that litters heated at different temperatures contained an equal relative amount of each C type revealed in the respective spectrograms.

Species responses from the bioassays were expressed as percentage of the respective control and submitted to General Linear Models (GLM) analysis. Separate GLMs were used for modelling germination and root growth, taking into consideration main and interactive fixed effects of target species (2 levels), litter species (4 levels), and heating temperature (7 levels). Pairwise differences among treatments were tested using Tukey's HSD *post-hoc* test. The occurrence of either inhibitory or stimulatory effects of treatments on the target-species responses were assessed, using two-tailed t-tests which compared each treatment combination and the respective control. For these tests, $\alpha = 0.05/N$, with N being the number of multiple comparisons, according to the Bonferroni's method.

To analyse the relationship between the litter chemical composition and the bioassay results, seed germination and seedling root growth of each target species were tested for

correlation with litter N and C contents, C/N ratio, and amounts of the different C types assessed by ^{13}C NMR. Following this, the relationship between litter C and plant responses was assessed in greater detail. For that purpose, linear correlations were calculated between the peak of each ^{13}C NMR signal (width = 1 ppm; n = 200), recorded for the 28 litter types, and plant responses of the target species incubated on the same litters. Correlations were alculated separately for the two target species, as well as for germination and root growth. In order to control for type I statistical error, correlation was tested at $\alpha = 0.001$. This value was obtained using the false discovery rate method (Benjamini and Hochberg, 1995), for multiple comparisons correction.

Finally, litter spectral data were submitted to multivariate analyses, including Cluster Analysis (CA) and Principal Component Analysis (PCA), in order to provide a synthetic representation of litter chemistry changes across heating treatments and their effects on the early development of the target species. Ward's aggregation rule and Euclidean distance were used in CA to minimize the within-cluster variance (Podani, 2000). In the PCA, several supplementary variables were included (*i.e.* plotted in the multivariate space, but not used to calculate the principal components). This procedure followed the approach suggested by Legendre and Legendre (1998). The supplementary variables were: litter C and N contents; C/N ratio; and germination and root growth of *Eucalyptus* and *Lepidium* on the litter samples.

Statistica 7 software (StatSoft, USA) was used for all statistical analyses.

5.3. Results

5.3.1. Molecular changes in heated litters

The ^{13}C NMR spectra revealed consistent changes of litter quality defined by C types across the four species, with major chemical changes progressively occurring at increasing heating temperatures (Fig. 5.1, Fig. S1). In general, these spectra only showed significant changes in litter chemical composition at T ≥ 300°C (Fig. 5.1). Within this temperature range, concentrations of both methoxyl + *N*-alkyl C and *O*-alkyl C fell, the latter experiencing a much more pronounced decline (Fig. 5.1). The concentration of alkyl C increased slightly between 200°C and 300°C, and decreased substantially at higher temperatures (Fig. 5.1). In contrast, aromatic C (111–140 ppm) showed a steep increase when samples were burnt at T ≥ 300°C (Fig. 5.1).

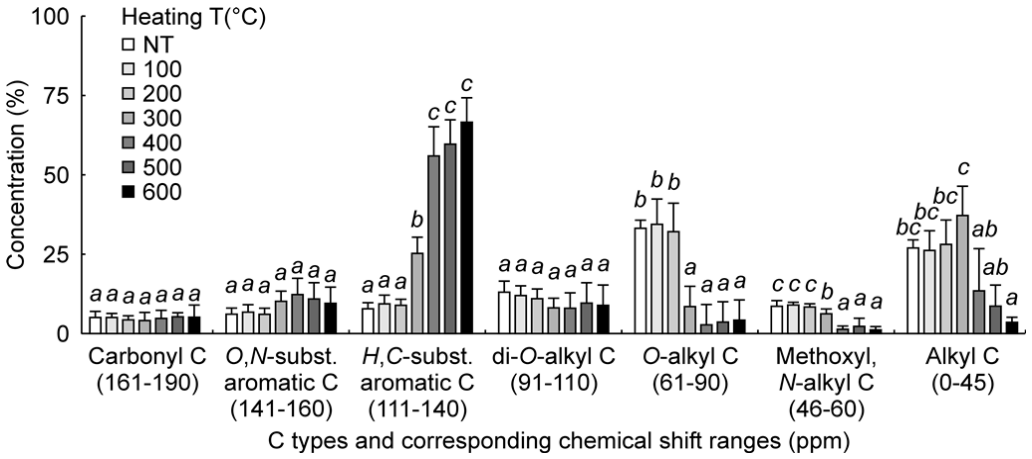


Fig. 5.1 – Concentrations of seven classes of organic C types and corresponding ^{13}C CPMAS NMR spectral regions in litter materials treated at different temperatures for 30 min.. Data refer to mean ± SD of litter species (n = 4). Different letters within each organic C class indicate temperature-dependent significant differences (Tukey's HSD *post-hoc* test from one-way ANOVA, $P < 0.05$). NT: unheated

Aside from the general pattern, interspecific differences among litter species were observed along the temperature gradient (Fig. 5.2 and Fig. S1). Among these, alkyl C was relatively more abundant in unheated litters of *Acacia* and even more so with respect to *Eucalyptus* litters. Above 300°C, the relative amount of this C type dropped more gradually in *Acacia* litter compared with the other litter species (Fig. 5.2). The relative amount of *O,N*-substituted aroma-tic C showed a significant increase when litters from *Eucalyptus*, *Pinus* and *Quercus* were heated at T ≥ 300°C (Fig. 5.2). However, this did not occur with *Acacia* litter. Finally, changes in the relative amounts of carbonyl C and di-*O*-alkyl presented a species-specific pattern at T ≥ 300°C (Fig. 5.2).

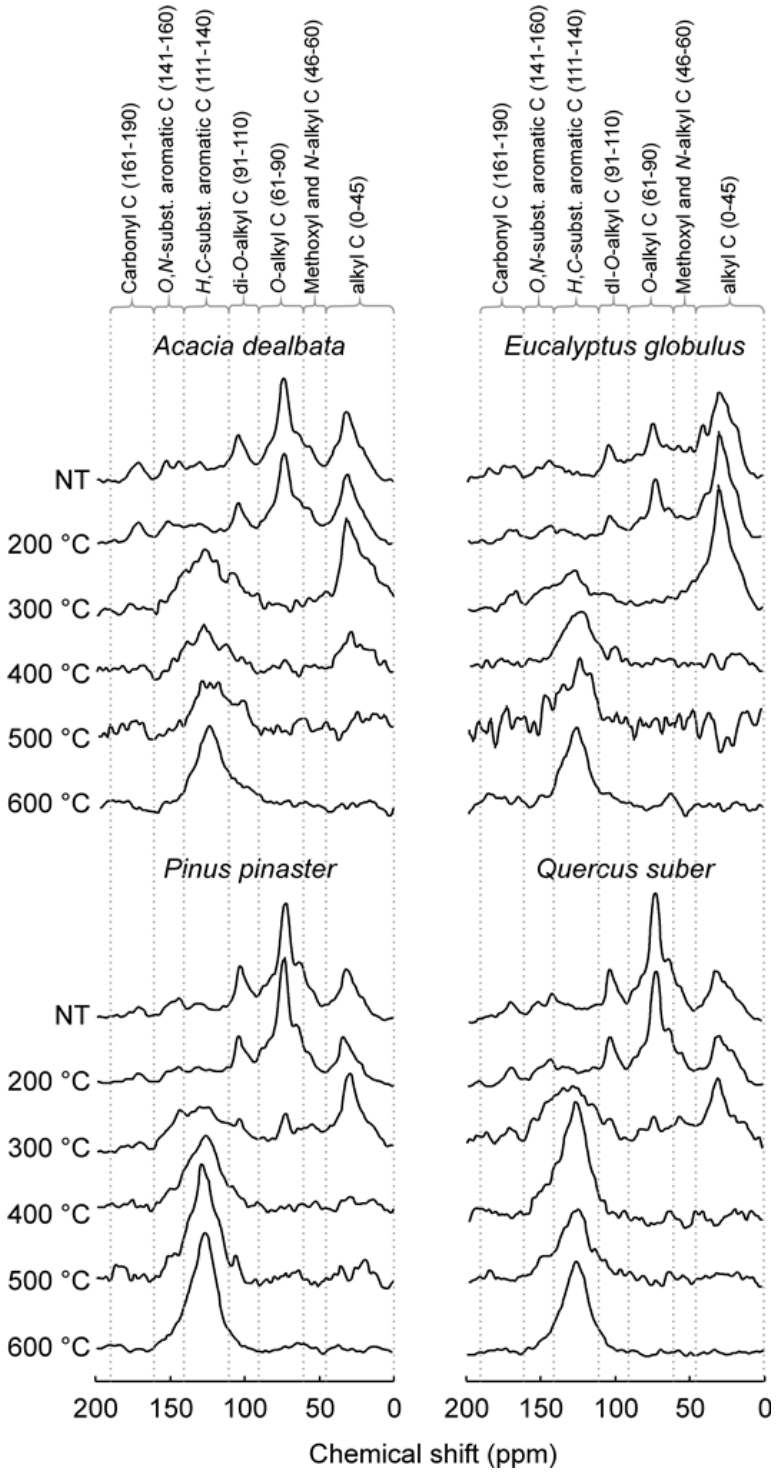


Fig. 5.2 - ^{13}C CPMAS NMR spectra of *Acacia dealbata*, *Eucalyptus globulus*, *Pinus pinaster* and *Quercus suber* litters, heated at different temperatures for 30 min.. Spectra obtained for 100ºC heated materials are not shown because they were not different from those of unheated materials (NT, 25ºC). Reference spectral regions and corresponding C types are reported on top of the panels, with chemical shift ranges indicated in brackets and by vertical dotted lines

5.3.2. Effects of heated litter on plant early development

Considering the overall effects of treatments in the bioassays (Table 5.1), results were consistent between germination and root growth response. Firstly, the main effects of temperature (T), and litter species (L) were highly significant (Table 5.1). Secondly, the thermal impact of temperature on litter was also highly significant for both responses, that is, species-specific differences induced by different litter species also varied with heating temperature (interactions L × T, Table 5.1). Thirdly, the effect of target species (S) was also significant on both germination and root growth (Table 5.1), indicating a remarkable species-specific sensitivity of seeds and roots to treatments. Such a trend was consistent across litter materials, as indicated by the non-significant interaction of target species and litter species for both response metrics (interaction S × L, Table 5.1). Finally, the effect of heating at particular temperatures differed between *Lepidium* and *Eucalyptus* both for germination ($P < 0.001$) and root growth ($P = 0.007$) (interactions S × T, Table 5.1). Although this effect had the same significance as the one of L × T interaction for germination, it was less significant for root growth.

Table 5.1 – Summary of the General Linear Models (GLM) testing for main and interactive effects of target species (fixed factor, two levels: *Eucalyptus globulus* and *Lepidium sativum*), litter species (fixed factor, four levels: *Acacia dealbata*, *E. globulus*, *Pinus pinaster*, and *Quercus suber*), and litter heating temperature (seven levels: 25; 100; 200; 300; 400; 500; and 600°C) on target species germination and root growth. For each effect, sum of squares (SS), degrees of freedom (df), mean of squares (MS), Fisher's statistic (F) and associated *P*-value are shown.

	SS	df	MS	*F*	*P*
Germination					
Target species (S)	28273.7	1	28273.7	83.0	< 0.001
Litter type (L)	9997.1	3	3332.4	9.8	< 0.001
Temperature (T)	82245.8	6	13707.6	40.3	< 0.001
S × L	1239.4	3	413.1	1.2	n.s.
S × T	35479.8	6	5913.3	17.4	< 0.001
L × T	28740.5	18	1596.7	4.7	< 0.001
S × L × T	25647.6	18	1424.9	1.6	n.s.
Residual	38137.8	112	340.5		
Root growth					
Target species (S)	41476.7	1	41476.7	46.9	< 0.001
Litter type (L)	65841.3	3	21947.1	24.8	< 0.001
Temperature (T)	133804.1	6	22300.7	25.2	< 0.001
S × L	3855.1	3	1285.0	1.5	n.s.
S × T	16674.0	6	2779.0	3.1	0.007
L × T	102154.8	18	5675.3	6.4	< 0.001
S × L × T	12033.9	18	668.5	1.9	0.018
Residual	99036.1	112	884.3		

Upon examining significant heating effects in greater detail, interesting patterns became evident (Fig. 5.3, Table S4). On the one hand, *Lepidium* germination was more sensitive to litter treatments than *Eucalyptus* (Fig. 5.3a–b, Table S4). The former was consistently inhibited by litter heated at T ≤ 200°C (Fig. 5.3a, Table S4), but the magnitude of inhibition depended on both temperature and litter species. In addition, litters heated at T ≥ 300°C had no effect on the germination of this species (Fig. 5.3a, Table S2). Meanwhile, *Eucalyptus* was inhibited only by litter of *Acacia*, *Eucalyptus* or *Quercus*, either unheated or heated at 100°C (Table S4). Remarkably, conspecific litter heated at 100°C was the substrate where *Eucalyptus* seeds experienced the worst performance (Fig. 5.3b). On the other hand, *Eucalyptus* performed better than *Lepidium* also in terms of root growth (in 19 out of 28 treatments, Table S4). However, in the case of root growth, differences were due not only to inhibition, but also to stimulation of plant development. Similarly to the germination results, *Acacia* and *Pinus* litters tended to inhibit less root growth than those of *Eucalyptus* and *Quercus*, when they were unheated or moderately heated. Conspecific litter heated at 100°C was the most inhibiting litter for *Eucalyptus* root growth (Fig. 5.3d), just as it was for germination (Fig. 5.3b, Table S4). Litters heated at T ≥ 300°C did not inhibit root growth (Fig. 5.3c–d, Table S4). In particular, *Lepidium* root growth was not significantly affected by those treatments (Fig. 5.3c, Table S4), whereas several types of charred litter stimulated *Eucalyptus* root growth (Fig. 5.3d, Table S4). Thus, there is a recurring pattern. Despite target species respond differently to different litter types, they usually develop better and more homogeneously on litters which were severely charred than on those which were not.

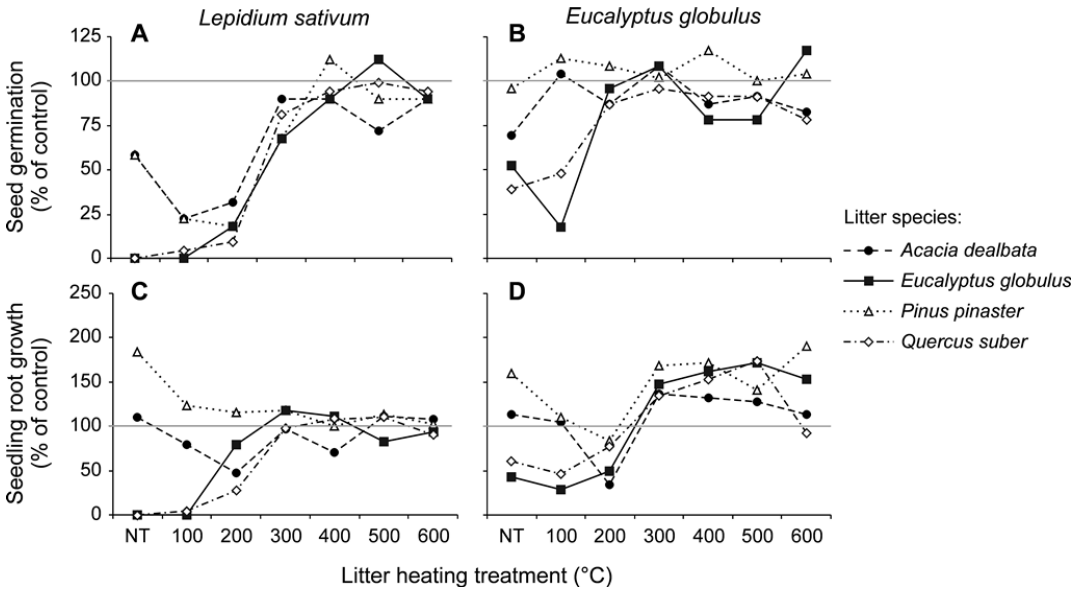


Fig. 5.3 – Responses of *Lepidium sativum* (a, c) and *Eucalyptus globulus* (b, d) exposed to plant litters either unheated (NT) or heated at six different temperatures for 30 min. Data refer to germination and seedling root growth, expressed as percentage of unexposed controls (=100). Values are means of three replicates; error bars are omitted to improve readability (results of testing for main and interactive effects of treatments are in Table 5.1; means, standard deviations, and results of pair-wise *post-hoc* comparisons among treatment combinations are in Table S4)

5.3.3. Litter quality and target species sensitivity

Target species sensitivity to litter quality had a specific pattern, which was evidenced by correlations between plant responses on litter samples and biochemical quality of the same litter materials, as expressed by ^{13}C NMR data from reference spectral regions (Table 5.2). Indeed, seed germination of *Lepidium* was positively associated with aromatic C regions and negatively with *O*-alkyl C and methoxyl + *N*-alkyl C regions, while in the case of *Eucalyptus* the correlation values presented an identical pattern but were not statistically significant (Table 5.2). The same pattern, in terms of correlation magnitude and direction, was also found in seedling root growth, but, contrarily to germination, the associations with ^{13}C NMR data were significant for *Eucalyptus*, but not for *Lepidium* (Table 5.2). Interestingly, plant responses were unrelated to litter total C and N contents, as well as to litter C/N ratio (Table 5.2).

Table 5.2 – Linear correlation (Pearson's r) between ^{13}C CPMAS NMR spectral data, describing biochemical quality of 28 litter types, and target species responses on those litter types. Pearson's r was separately calculated for germination and root growth of each target species (*Eucalyptus globulus* and *Lepidium sativum*). Correlations with litter content of N and C, and with C/N ratio are also reported. **Bold** indicates statistically significant r values ($P < 0.001$, after correction for multiple comparisons according to the false discovery rate method, Benjamini and Hochberg, 1995)

Litter quality parameter	Germination		Root growth	
	Lepidium	*Eucalyptus*	*Lepidium*	*Eucalyptus*
Carbonyl C (161–200 ppm)	0.168	0.131	-0.058	0.012
O,N-substituted aromatic C (141–160 ppm)	**0.599**	0.221	0.216	**0.578**
H,*C*-substituted aromatic C (111–140 ppm)	**0.809**	0.312	0.298	**0.622**
di-*O*-alkyl C (91–110 ppm)	-0.322	-0.241	0.008	-0.281
O-alkyl C (61–90 ppm)	**-0.837**	-0.345	-0.262	**-0.618**
Methoxyl + *N*-alkyl C (46–60 ppm)	**-0.735**	-0.374	-0.310	**-0.571**
Alkyl C (0–45 ppm)	-0.529	-0.138	-0.243	-0.439
C content (%)	0.083	0.168	0.137	0.174
N content (%)	0.255	0.085	-0.010	-0.010
C/N ratio	-0.273	0.073	0.212	0.042

The detailed correlation profiles of plant responses along the NMR spectrum provided further insight into the relationships between the molecular composition of litters and the early development of plants (Fig. 5.4). These profiles were qualitatively similar between the two target species, as well as between the two response metrics. However, different restricted spectral regions were significantly related with either inhibitory or stimulatory effects (Fig. 5.4). On the one hand, correlation profiles of germination *vs.* NMR spectral data were similar for both target species but significant correlations existed only in the case of *Lepidium*. Germination of this species (Fig. 5.4a) was negatively associated with the whole *O*-alkyl C region, as well as with different restricted intervals within the carboxyl C (169–172 ppm), di-*O* alkyl C (102–104 ppm), methoxyl + *N*-alkyl C (51– 60 ppm), and alkyl C (38–44 ppm and 31–34 ppm) regions. However,

it was positively correlated with several parts of the spectrum: the interval 108–150 ppm, which includes the whole *H*,*C*-substituted aromatic C region and two adjacent restricted parts of the di-*O*-alkyl C and *O,N*-substituted aromatic C regions; plus a restricted interval resonating at 193–195 ppm. Meanwhile, *Eucalyptus* germination followed a similar pattern of qualitative response in relation to spectral data, but correlations were not significant (Fig. 5.4b). On the other hand,

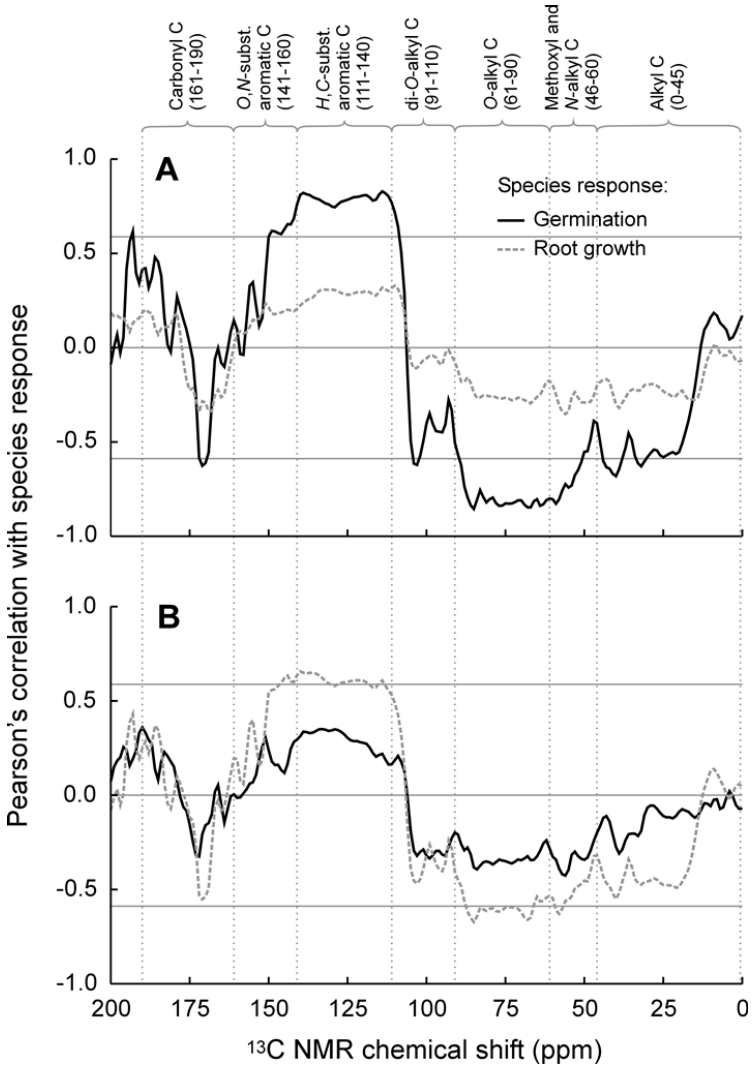


Fig. 5.4 – Profiles of correlation (Pearson's r) between ^{13}C CPMAS NMR signals and plant responses (either germination or root growth) when sown on burnt litter materials, reported separately for *Lepidium sativum* (**a**) and *Eucalyptus globulus* (**b**). Grey horizontal lines in each panel indicate threshold values of statistical significance for r according to sample size (n = 28, P = 0.001), after correction for multiple comparisons according to false discovery rate method (Benjamini and Hochberg 1995). Organic C types corresponding to reference spectral regions are indicated on the top of the panel

correlation profiles of root growth *vs.* NMR spectral data were qualitatively similar to those of germination but, in this case, significant correlations existed only for *Eucalyptus*. Root growth of this species was significantly correlated with several spectral regions (Fig. 5.4b). It was negatively correlated with most of the *O*-alkyl C region (66–87 ppm) and with a restricted interval of methoxyl + *N*-alkyl C region (57–59 ppm), while it was positively correlated with most

of the *H*,*C*-substituted aromatic C region (114–140 ppm) and with an adjacent restricted interval within the *O,N*-substituted aromatic C region (141–146 ppm). Meanwhile, *Lepidium* root growth had a similar qualitative correlation pattern with NMR spectral data, but correlations were not significant (Fig. 5.4a). Thus, the pattern of correlations between plant development and chemical quality of litter was qualitatively similar for the two development indicators and the two target species. Nevertheless, the litter quality had the strongest correlations with *Lepidium* germination and *Eucalyptus* root growth.

The CA results highlighted species-specific differences among unheated or lightly heated litters (up to 200°C) and confirmed that litters of different species treated at higher temperatures were chemically more similar (Fig. 5.5). Indeed, the dendrogram of litter samples, based on ^{13}C NMR spectra, presented two main clusters segregated at high Euclidean distance and including treatments either at T ≤ 300°C or at higher temperatures (Fig. 5.5). Moreover, within the low temperature cluster, samples heated below 300°C and belonging to the same litter species were consistently aggregated at the lowest distance levels, while within the high-temperature cluster sample aggregation was independent from litter species, and more related to temperature (Fig. 5.5).

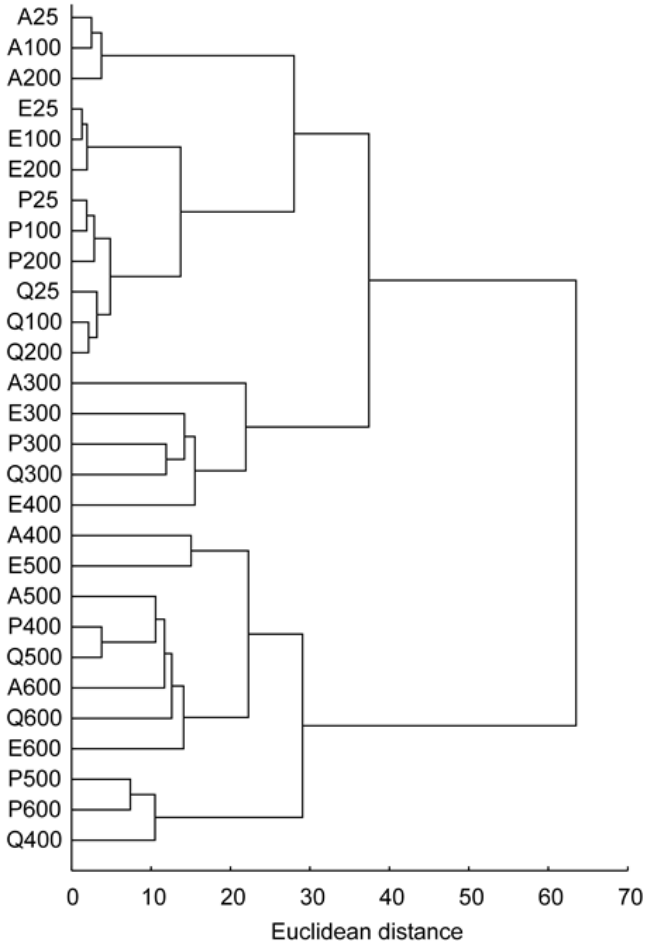
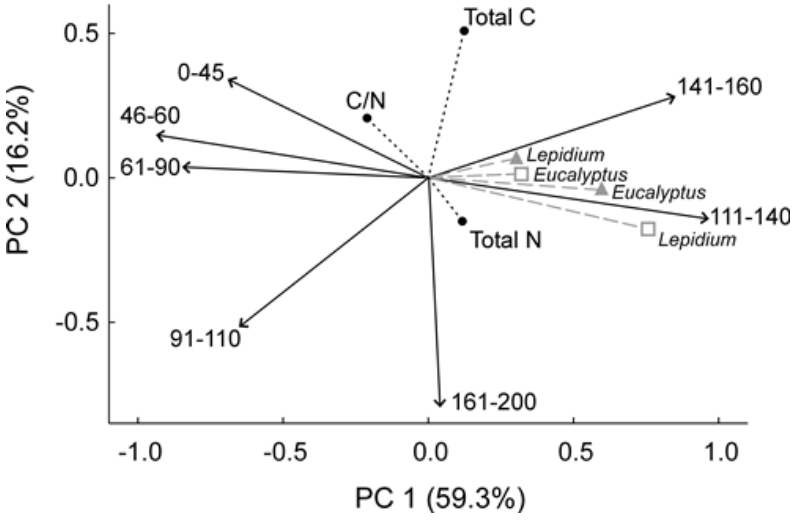


Fig. 5.5 – Dendrogram of litter materials, labelled by species initial (A, *Acacia dealbata*; E, *Eucalyptus globulus*; P, *Pinus pinaster*; and Q, *Quercus suber*) and temperature of heating treatment (25, 100, 200, 300, 400, 500, or 600°C).

The PCA provided a synthetic picture of the chemistry-dependent effects on the target species responses (Fig. 5.6), confirming the above-described relationships between the proportions of organic C types in the litters and *Eucalyptus* and *Lepidium* germination and root growth over such materials.



PCA ordination of the spectral regions in the litter materials. Data refer to loading vectors of the spectral regions (black arrows), labelled by chemical shift ranges (0–45 ppm: alkyl C; 46–60 ppm: methoxyl + *N*-alkyl C; 61–90 ppm: *O*-alkyl C; 91–110 ppm: di-*O*-alkyl C; 111–140 ppm: *H*,*C*-substituted aromatic C; 141– 160 ppm: *O,N*-substituted aromatic C; and 161–190 ppm: carbonyl C). Supplementary variables are also plotted, following Legendre and Legendre (1998): germination (grey dashed vectors with open squares); root growth (grey dashed vectors with solid triangles); C and N content, and C/N ratio in litter (dotted vectors)

5.4. Discussion

We assessed the allelopathic potential of unheated and heated litter, from both conspecific and heterospecific trees coexisting with *E. globulus* in the field. Burning, aside from altering litter molecular composition, generally affected the early stages of plant development on litter materials. The magnitude and direction of the effect decisively depended on the heating temperature, the litter species, and the target-species sensitivity.

5.4.1. Burning effects on litter chemistry

Original litter materials were chemically diverse among species. Those differences were kept in litters that were moderately heated. Cluster analysis showed that interspecific differences in the chemical composition of litter clearly prevailed over temperature effects on litter chemistry at T ≤ 200°C. However, heat treatments above 200°C homogenized the chemical composition of litter across species. In keeping with this, Gundale and DeLuca (2006) found that temperature has greater influence on chemical properties of severely burnt plant materials than do plant species. Therefore, the influence of heating temperature is more important than litter species, for determining the chemical composition of charred litter. Nevertheless, the opposite is true for non-charred litter.

The spectral regions of *O*-alkyl C, di-*O*-alkyl C, and alkyl C had a greater contribution to the molecular composition of unheated litters than the other regions. Firstly, *O*-alkyl C and di-*O*-alkyl C are related to polysaccharides (Kögel-Knabner, 2002), such as cellulose and hemicelluloses (Freitas *et al.,* 1999; Czimczik *et al.,* 2002). In fact, proximate cellulose content in litter samples ranged from 14.9% to 27.0%, and was consistently higher in samples with higher concentration of corresponding C types. Secondly, as expected, the amount of alkyl C present in unheated litters was also high, corresponding to the spectral region centred at 30 ppm. This region is associated with: polymethylene C; cutin (Kögel-Knabner, 2002); cutan (McKinney *et al.,* 1996; Knicker *et al.,* 2005); aminoacid residues and protein-like structures (Tinoco *et al.,* 2004; Knicker *et al.,* 2005); dehydroxylated lignin side-chains (Knicker *et al.,* 2005); and probably with other not yet described aliphatic biomacromolecules. Usually, this signal presents high intensity in cutin- and wax-rich litter (Preston *et al.,* 2009). Thus, peaks in this spectral region indicate a high content of waxes in *Eucalyptus* and *Acacia* samples, which ensure leaf surface hydrophobicity (Li *et al.,* 1997; Neinhuis and Barthlott, 1997). Regarding *Eucalyptus* samples, the peak could also indicate the existence of leaf essential oils such as cineole and eudesmol (Jacobs, 1979). As for the *Acacia* samples, the peak likely corresponded no only to lipids, but also to peptides, as this litter is very rich in nitrogen. Finally, it is worth mentioning that, although lignin shows relevant levels in litter samples (26.4–37.0%), it cannot be easily detected in ^{13}C NMR spectra. This difficulty arises from the fact that lignin carbon atoms resonate in several different spectral regions, overlapping with signals of other chemical compounds (Kögel-Knabner, 2002).

According to ^{13}C NMR data, all litter samples were chemically stable when heated up to 200°C. On the contrary, litter chemical changes were recorded at T > 200°C, namely: the decrease of *O*-alkyl C and methoxyl + *N*-alkyl C, and the increase of *H*,*C*-substituted aromatic C. These changes are consistent with recent observations on other litter species (Bonanomi *et al.,* 2016) and are further supported by literature concerning burning effects on specific molecular classes. Indeed, the decomposition of lignin and hemicelluloses accelerates at 200°C, and cellulose chemically dehydrates between 200°C and 280°C (Chandler *et al.,* 1983). Organic matter charring starts above 200°C (González-Pérez *et al.,* 2004) and progressively alters *O*-alkyl structures forming aromatic compounds (Baldock and Smernik, 2002; Czimczik *et al.,* 2002; Certini 2005), which replace carbohydrates in the range of 250°C to 310°C (Knicker, 2007), while a more destructive distillation occurs between 280°C and 320°C (Chandler *et al.,* 1983). Temperatures exceeding 300°C cause further structural changes, promoting an additional increase of the aromatic fraction by: dehydration; dealkylation; decarboxylation of six- and five-membered rings, alkyl chains and aminoacids; and, to a lesser extent, selective persistence of heat-resistant aromatics (Freitas *et al.,* 1999; González-Pérez *et al.,* 2004; Knicker *et al.,* 2005). In particular, signals of mono- and polycyclic aromatic hydrocarbons appear between 300°C and 600°C, while the methoxyl C signal of lignin disappears at T ≥ 400°C (Knicker, 2007). Notably, the chemical shifts that occurred in the litter materials, between 200°C and 400°C, corresponded to clearly observable variations in litter colour, which changed

from brown to black shades, as heating temperature increased. Therefore, litter composition changes dramatically, when heated between 200°C and 400°C.

Alkyl C showed a unimodal pattern along the temperature gradient, with the maximum at 300°C followed by an abrupt depletion. The spectral peak may have emerged from several concomitant processes. For example, thermal distillation is known to release lipidic compounds, at early heating stages of litter (González- Pérez *et al.,* 2004), and to lead to the accumulation of additional aliphatic C above 270°C (Knicker, 2007). Long-chain residual paraffins and methyl, methylene or alicyclic structures can remain embedded in the matrix of newly formed aromatics, while alkyl groups can be subsequently removed by distillation (Almendros *et al.,* 2003). In addition, during browning, the sugars react with proteins to produce melanoidins and other Maillard-type substances (Brands, 2002). A further decrease of alkyl C, at T > 300°C, can be explained by progressive polymerization, demethylation and volatilization of hydrocarbons and hydrophobic products (Savage, 1974; Freitas *et al.,* 1999). Interestingly, the temperature-dependent pattern observed for alkyl C is consistent with water repellency changes, which are reported for experimentally heated soils (DeBano and Krammes, 1966) and are often observed in burnt soils (DeBano, 1981; Doerr *et al.,* 2000).

Carbonyl C content showed species-specific changes at T ≥ 300°C. Different chemical compounds in the litter could contribute to this signal, namely: hemicelluloses; uronic acids; biopolyesters; wax esters, possessing free carboxylic or ester groups; and also lignoproteins and melanoidins, possessing amide groups. At such temperature, organic molecules can undergo decarboxylation (Freitas *et al.,* 1999; González- Pérez *et al.,* 2004), often leading to the loss of carboxyl C in charred litter (Bonanomi *et al.,* 2016). This is consistent with our results for *Pinus* litter, but not for the remaining litter species, nor for all pooled data. These results might be explained by species-specific net effects, emerging from the balance of several chemical transformations related to litter charring. For instance, carboxyl C may persist at high temperatures, bonded to aromatic rings which are formed after the breakdown of aliphatic and polycyclic structures (Almendros *et al.,* 2003; González-Pérez *et al.,* 2004). This is confirmed by the relatively high amount of carboxyl C in the soluble fraction of char (Knicker, 2007). Furthermore, the non-association of carbonyl C content variations with heating temperature, in *Q. suber* samples, is consistent with a recent report by Bonanomi *et al.* (2016) on *Quercus* ilex, an oak with leaf traits that are closely related. An increase of carbonyl C with temperature, as we observed for *Eucalyptus* litter, was also previously reported. Knicker *et al.* (2005) observed higher content of carboxyl C in burnt forest soils than in unburnt forest. As changes of carbonyl C content were very diverse in our litter samples at T > 300°C, no generalizations can be inferred.

PCA results outlined the general chemical composition of litter samples and its changes with temperature quite well, though they concealed interspecific differences of litter composition. PCA highlighted two opposing groups of C types: substituted and unsubstituted alkyl C *vs.* substituted and unsubstituted aromatic C. The former dominated the composition of uncharred litters, while the latter dominated that of charred litters. Chemical processes responsible for this shift mainly occurred at temperatures between 200°C and 400°C.

5.4.2. Effects of litter chemistry on germination and root growth

The associations of germination and root growth with ^{13}C NMR data were qualitatively similar in the two target species. These species showed a consistent pattern of inhibition or stimulation. However, the magnitude of the effects was different in the two target species. In particular, both germination and root growth were negatively correlated with the litter content of *O*-alkyl C and methoxyl + *N*-alkyl C, and positively correlated with the content of aromatic C. These correlations confirm recent observations made by Bonanomi *et al.* (2016) and support an old idea by Johnson (1919), suggesting that the same property can negatively affect both germination and growth. This pattern may be explained by the phylogenetically widespread sensitivity to bioactive combustion products (Nelson *et al.,* 2012). Developmental mechanisms in angiosperms have emerged early in their evolution (Taiz and Zeiger, 2002). Inhibitions of germination and early root growth are often mutually associated (Evenari, 1949), probably because these developmental stages share similar basic processes of morphogenesis and size increase in higher plants. Cell division and inherently intense metabolic activity may be affected by known phytotoxins, targeting respiration, protein synthesis, and mitosis (Rice, 1984). On the other hand, species-specific differences in the magnitude of litter effects clearly indicate target-species differ in their sensitivity to litter quality. This because *Lepidium* germination and *Eucalyptus* root growth were the most affected by the same interfering C types.

Finally, the species responses were not related with either litter total C or N contents or with C/N ratio. The N available to seeds and seedlings in differently heated litters most likely represented different fractions of the total N because combustion has a mineralizing effect. Nevertheless, our results suggest that the early plant-development was independent from external nutrient supply, being sustained by seed resources (Ingestad and Lund, 1986).

5.4.3. Unheated litter and allelopathic interactions

Unheated litter had species-specific effects. Differences in molecular composition among the tested materials could contribute to explain these results.

Eucalyptus globulus litter prevented *Lepidium* germination and strongly inhibited conspecific root growth. Its allelopathic potential, as well as that of its congenerics, has been known for a long time (del Moral and Muller, 1969; May and Ash 1990; Espinosa-García, 1996; Willis, 1999). It is more intense in spring (Souto *et al.,* 2001) and relies on several leaf compounds, such as: essential oils, mainly terpenes; phenolics; flavonoids; and ellagitannins (Hillis, 1966; del Moral and Muller, 1969; Rice, 1984; Souto *et al.,* 1994). Allelopathic effects of this species on germination and/or root growth were previously assessed using: different plant parts, either fresh or abscised; their leachates or macerates; throughfall; stemflow; litter; and beneath-canopy soils (del Moral and Muller, 1969; May and Ash, 1990; Molina *et al.,* 1991; Souto *et al.,* 2001). Consistent with our findings, these studies found that interspecific effects range from strong inhibition to neutral. Hence, phytotoxicity might contribute to explain the reduced biodiversity and the low densities of herbs in plantations of this species, observed by other authors outside its native range (del Moral and Muller, 1969; Basanta *et al.,* 1989).

Actually, phytotoxicity involving species from different biogeographical regions is an important mechanism to enhance the success of species outside their native ranges (novel weapons hypothesis) (Rabotnov, 1974; Hierro *et al.,* 2005). In addition, *Eucalyptus* autotoxic effects were also reported by del Moral and Muller (1969). Autotoxicity might also help to explain why *E. globulus* wildlings are usually absent or sparse in unburnt conspecific plantations (Larcombe *et al.,* 2013; Fernandes *et al.,* 2016; Águas *et al.,* 2017). Furthermore, considering that other eucalypt species have autotoxic potential (Willis, 1999), it is likely that similar phenomena happen in unburnt natural forests, where the failure of natural regeneration is apparently related to litter presence (Mount, 1964). Autotoxicity has ecological significance in the self-regulation of populations in space and time, inhibiting plant development where and when conspecific plants coexist (Singh *et al.,* 1999). Therefore, *Eucalyptus* populations might be self-controlled through allelopathy.

Quercus suber litter prevented *Lepidium* germination and inhibited *Eucalyptus* development, affecting the germination of the latter more than its root growth. Previous tests on *Lactuca sativa* reported low phytotoxic potential on both seeds (Gonçalves *et al.,* 2008) and roots (Domínguez 1994). Nevertheless, *Q. suber* contains significant amounts of allelochemicals in phellogen, cork and leaves, including: flavonoids; gallic; ellagic, *p*-coumaric, caffeic, and ferulic acids; and juglone (Carvalho, 1992). Therefore, species-specific sensitivity of plants to different allelochemicals might explain our results on different target species, as well as differences between our results and those from the aforementioned studies. Meiners *et al.* (2012) postulate that interspecific differences in sensitivity to phytotoxic substances might result from selective pressures. Indeed, they are common, even among standard target species or related species (Perez, 1990; Haugland and Brandsaeter, 1996; Macías *et al.,* 2000). In addition, differences in results among studies may also come from differences in the methods used in those studies (Haugland and Brandsaeter, 1996). It is noteworthy that *Eucalyptus* germination was inhibited by *Quercus*. This result is consistent with field observations (pers. obs.) and supports the 'homeland security hypothesis' (Cummings *et al.,* 2012). In fact, it is a very relevant result as it points to the potential resistance of native forests, as *Q. suber* forests, to invasion by exotic species, as *E. globulus.*

Acacia dealbata litter showed phytotoxic effects on germination, but apparently did not affect seedling root growth. Its leaves contain at least three allelochemicals (resorcinol, moretenone, and maculosin; Aguilera *et al.,* 2015). Interestingly, our sampling period is compatible with the seasonal peak of phytotoxicity previously observed in Spain (Carballeira and Reigosa, 1999). Our results are similar to those from other studies, as both germination inhibition (Carballeira and Reigosa, 1999; Lorenzo *et al.,* 2008) and the neutral effects on root growth (Lorenzo *et al.,* 2010) have already been observed in other species. The absence of inhibition on root growth could be related to short-lived phytotoxic compounds (up to few days; Rice, 1984), which may have been lost through decomposition.

Although *P. pinaster* litter inhibited germination of *Lepidium*, but not *Eucalyptus*'s, it stimulated root growth of both target species. Essential oils of *P. pinaster* leaves are well-known

weedicides. They have high contents of important phytotoxics, as α-pinene (31.4%) and β-pinene (4.2%), as well as the antifungal (*Z*)-caryophyllene (28.1%) (Amri *et al.,* 2013). Furthermore, plantations of this species lay over soils rich in phenol derivatives, including vanillic, benzoic, salicylic, syringic, *p*-coumaric, and gentisic acids (Muscolo *et al.,* 2005), which can act as plant development inhibitors (Evenari, 1949; Rice, 1984). Such soils also contain protocatechuic acid, which protects plants from fungal infection (Rice, 1984). On the other hand, some allelochemicals are known for changing effect magnitude and direction along a gradient of concentration, being inhibitory at high concentrations and stimulatory at low concentrations (Evenari 1949; Leather and Einhellig, 1988). Souto *et al.* (1994) observed an effect shift from inhibition to stimulation of plant development by *Pinus radiata* and *Acacia melanoxylon* litters, after seven days of decomposition. Therefore, if the concentrations of allelopathic substances decreased with incubation time due to litter decay, those substances could have firstly inhibited germination and then stimulated growth. Moreover, antifungal substances were probably more relevant for plant development as time went by. Notably, fungi were not observed in any assay with *Pinus* litter, however they were observed in several assays with unheated or slightly heated litters of either *Eucalyptus* or *Quercus*. Fungi control might have contributed for root growth on *Pinus* litter, the strongest among the four litter species. These results are consistent with the occurrence of *E. globulus* in at least 13.7% of the forests dominated by *P. pinaster* in Portugal (AFN, 2010), a country where such a species combination does not usually result from human decision.

5.4.4. Heated litter and allelopathic interactions

Interestingly, both target species had very poor performances on moderately heated materials, either at 100°C or at 200°C. Most of these performances were even poorer than those on the correspondent unheated litters. This result is consistent with those from previous studies. Several explanations were previously proposed for inhibition of plant development by soils heated at similar temperatures: an unknown water-soluble substance (Attiwill, 1962; Wilkinson *et al.,* 1993), ammonia (Johnson, 1919), or manganese released during combustion (Chambers and Attiwill, 1994). In our bioassays, the inhibition of germination and root growth, for both target species, was not due to ammonia (Britto and Kronzucker, 2002) nor manganese (Reichman, 2002) toxicity, as no other typical symptoms of such toxicities were present. Nevertheless, the apparent chemical stability of litter heated up to 200°C, revealed by ^{13}C NMR, may hide some physiological changes. For example, the break-up of biological tissues can start at temperatures between 40°C and 70°C (Knicker, 2007), and leaf cells left intact by the grinding alone may then release their content. This release would include phytotoxic compounds, which would then increase their concentrations around both seeds and seedling roots. In line with this, Chou and Muller (1972) observed that phytotoxic substances from *Arctostaphylos glandulosa* became more leachable after litter heating up to 140°C. Moreover, those concentration differences among aqueous extracts had a close correspondence to root growth inhibition in *Bromus rigidus* (Chou and Muller, 1972). It is noteworthy that leaf litter of *A.*

glandulosa shares several phenolic allelochemicals with the *Eucalyptus*, *Pinus*, and *Quercus* we have studied (Hillis 1966; del Moral and Muller, 1969; Chou and Muller, 1972; Carvalho 1992; Muscolo *et al.,* 2005).

Remarkably, the strongest inhibition of *Eucalyptus* development was on conspecific litter heated at 100°C. The specificity of these effects might indicate another underlying phenomenon, possibly related to the ability of self-DNA to inhibit plant development, similar to what Mazzoleni *et al.* (2015) have found in bioassays with decomposed conspecific litter. Indeed, we can speculate that litter heating might cause an increased concentration of degraded fragments of DNA, which would be able to induce the observed self-inhibition. This hypothesis will require further investigation.

However, the inhibition of plant development completely disappeared at either T ≥ 200°C or T ≥ 300°C, and plant responses were much more similar across litter species at those heating temperatures than at lower ones. Our results are consistent with previous findings by Bonanomi *et al.* (2016) and Chou and Muller (1972), who observed that 200°C is the temperature turning point above which litter heating results in the complete loss of root growth inhibition. Moreover, the disappearance of inhibitory capacity occurred across all litter species, somewhat in parallel with the deep chemical changes caused by severe heating in those litters.

The results, originating from our severely charred litter bioassays, generally suggest that molecular transformations have occurred at high temperatures, causing not only the disappearance or the neutralization of inhibitory compounds, but also the production of new stimulatory substances. Data from ^{13}C NMR summarized by PCA support such inference. In fact, charring of litters significantly decreased concentrations of the substances which were negatively correlated with plant development. This was evident for methoxyl + *N*-alkyl C (46–60 ppm) and even more so for *O*-alkyl C (61–90 ppm). Conversely, the same charring strongly increased aromatic C concentration (111–140 ppm) in litters, as revealed by a very sharp and typical peak at 128 ppm (Almendros *et al.,* 2003; Knicker 2007). And this was precisely the C type that had the strongest positive correlation with plant development. Research from Bonanomi *et al.* (2016) seems to corroborate these ideas. In addition, Chou and Muller (1972) found that phytotoxic phenolics substantially reduce their concentrations in litter aqueous extracts, after heating litter at T ≈ 180°C, and completely disappear, when litter is heated at T > 200°C. Therefore, our results and literature suggest three complementary hypotheses to explain the observed inhibition release.

First, bioactive compounds which existed in unheated litter have been affected by important physicochemical changes, which occurred in organic matter. Severe heating may have destroyed them (McPherson and Muller, 1969; Chou and Muller, 1972), or turned them insoluble, condensed, or chemically bonded to other macromolecules (Brodowski *et al.,* 2005).

Second, as char is able to adsorb and deactivate phytotoxic compounds (Bonanomi *et al.* 2015), it might have enabled seed germination and seedling growth that otherwise would be inhibited (Wardle *et al.,* 1998; Hille and den Ouden, 2005). This way, combustion-derived

inhibitory compounds, as phenolics (Campos *et al.,* 2012; Nelson *et al.,* 2012), might have been neutralized by char, which kept their concentration low in the bioassay.

Third, litter combustion produced new substances, as discussed before, and some of them might have stimulatory effects on plants. Residues of plant combustion can stimulate germination and seedling growth, as shown for shrub species of chaparral (Nelson *et al.,* 2012). Few are known, as cyanohydrins and karrikins (Kochanek *et al.,* 2016, Flematti *et al.,* 2011, 2015), but there are others whose identity is still unknown (Downes *et al.,* 2013, Baldos *et al.,* 2015). Each of these substances is produced at a particular temperature range and becomes unstable when heated at a higher range (Flematti *et al.,* 2015).

In addition, allelochemicals can interfere differently with the several components of plant development, because they have specific targets in plant physiology (Lotina-Hennsen *et al.,* 2006). Similarly, interspecific differences exist in plant sensitivity to the various combustion-derived compounds (Downes *et al.,* 2013, Baldos *et al.,* 2015). These facts may explain why *Eucalyptus* germination was the only response variable that turned up at 200°C and not at 300°C. Therefore, plant development on heated litters is influenced by the balance between active stimulants and inhibitors that result from combustion (Baldos *et al.,* 2015).

Bioassay results also suggest that heating litter, at T ≥ 300°C, substantially homogenized the litter-species effects on the early development of plants. The dramatic chemical changes shared by all litter species, between 200°C and 400°C, resulted in a chemical homogenisation across all litter species, as demonstrated by CA. Aromatic C (111–160 ppm) became absolutely dominant in all litters after charring. Heat directly homogenised chemical composition across litter species, as it promoted chemical reactions which destroyed or inactivated different substances and produced new ones. Char is a direct product of combustion and is very rich in aromatic C (Certini, 2005), which positively correlated with plant development of both target species. Since char is able to sorb and inactivate bioactive compounds, it can make the several litter species functionally similar, even if they contain different arrays of stimulants and inhibitors. Therefore, severe heating of different litter species nullifies many chemical differences among them, through both direct and indirect processes. As a consequence, their capacity to interfere with plant development becomes similar.

Our study bears great important and significant implications for fire ecology. *Eucalyptus globulus* saplings frequently occur in burnt areas, as in the cases of forests dominated by *P. pinaster* or *E. globulus* or mixed forests of both species in Portugal (Águas *et al.,* 2014). Similarly, saplings from many eucalypt species abound in burnt Australian forests, but are not nearly as prevalent in the unburnt forests (Mount, 1964, 1969; Florence, 1996). Our results suggest that the depletion or deactivation of chemical inhibitors caused by fire passage, as well as the production of stimulatory molecules by combustion, could play a role in *E. globulus* success in recently burnt areas, along with other fire-related factors reported in literature. Importantly, inhibition caused by *Quercus suber* litter, a species from *Eucalyptus* exotic range, was lost by severe combustion. This fact might ease the spread of *Eucalyptus* into forests dominated by *Quercus.*

5.5. Conclusions

Burning litter of tree species coexisting with *E. globulus* may affect the early development of this species. The potential effects are species-specific, ranging from prevention of seed germination to stimulation of seedling growth, and greatly depend on the severity of litter burning. Differences in plant responses are associated with litter molecular composition. In *E. globulus*, early root growth is more sensitive than germination to thermally induced changes of litter quality. Mild heating exacerbates the inhibition of plant development by litter, especially in the case of conspecific material. Conversely, severe litter burning results in the general disappearance of inhibition of both germination and root growth and, in some cases, in the appearance of stimulation of root growth. This effect shift corresponds to litter chemical transformations related to combustion, namely a decrease of *O*-alkyl C and methoxyl + *N*-alkyl C contents, along with an increase of aromatic C content. Severe heating of litter releases *E. globulus* germination and allows roots to grow longer in the resultant seedlings. Therefore, burning litter seems to improve recruitment and establishment of plants of this species. Hence, a window of opportunity may open for (re)colonization of forests by this species right after fire. Since *E. globulus* is widely planted in fire-prone regions outside its native range, our results are extremely relevant for forest management and conservation.

5.6. References

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5.7. Supplementary Material

Supplementary Table S1 – Total-C and total-N content, C/N ratio, protein, cellulose and lignin content in unheated dry litter of four plant species. Values refer to means of three replicates ± standard deviation. Lignin content is expressed as ash free relative value of total structure.

Litter species	C (%)	N (%)	C/N ratio	Protein (%)	Cellulose (%)	Lignin (%)
Acacia dealbata	45.2	1.7	26.1	10.6	14.9	37.9
Eucalyptus globulus	45.3	0.6	73.7	3.8	15.6	27.5
Pinus pinaster	46.0	0.4	117.4	2.5	27.0	34.2
Quercus suber	45.7	0.8	55.5	5.0	19.6	26.4

Supplementary Table S2 – Mass loss in heated litters comparatively to non-heated litters (%) of four plant species.

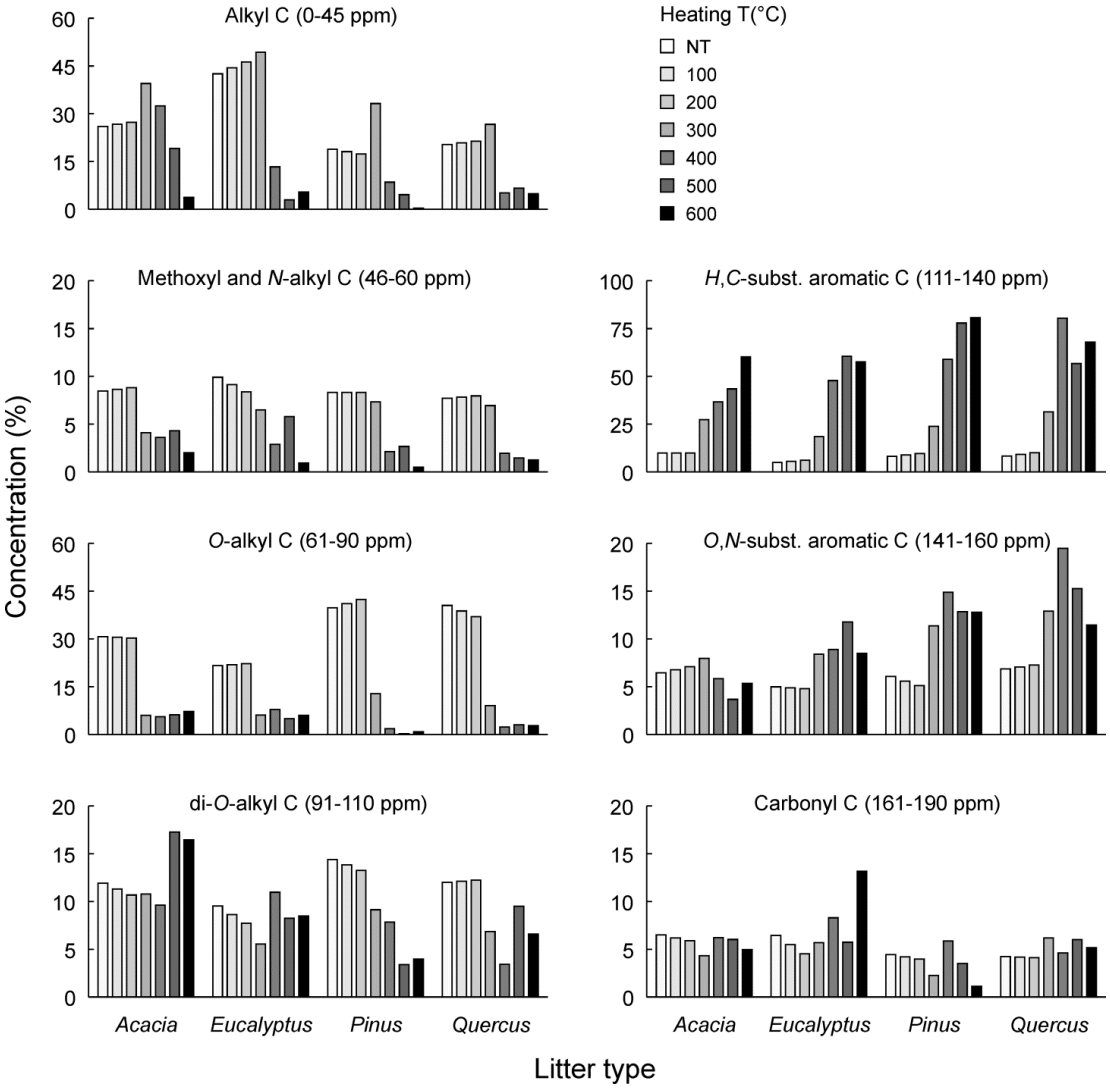
Litter species	Mass loss (%)					
	Heat treatments (ºC, 30 min)					
	100	200	300	400	500	600
Acacia dealbata	7.7	11.3	48.5	56.5	65.3	68.6
Eucalyptus globulus	4.5	13.2	42.6	69.3	71.9	74.9
Pinus pinaster	6.8	8.4	46.0	69.9	71.6	76.5
Quercus suber	5.1	10.0	46.1	62.2	65.7	72.1

Supplementary Table S3 – Total-N content (%) in heated litters of four plant species.

Litter species	Total-N (%)					
	Heat treatments (ºC, 30 min)					
	100	200	300	400	500	600
Acacia dealbata	1.8	1.8	2.2	2.1	1.9	1.8
Eucalyptus globulus	0.6	0.7	0.8	0.9	0.9	0.9
Pinus pinaster	0.4	0.4	0.6	0.7	0.6	0.6
Quercus suber	0.8	0.9	1.2	1.2	1.2	1.1

Supplementary Table S4 – Results of *post-hoc* tests for assessing the interactive effects of litter type and heating treatment from GLMs in Table 1. Data refer to mean and standard deviation of each treatment combination. Different letters indicate significant differences for pairs-wise comparisons among treatment combinations (Tukey's HSD test, $P < 0.05$). Values significantly higher or lower than the control, as resulting from two-tailed *t*-tests after Bonferroni's correction, are marked in **bold** or *underlined italic*, respectively. Germination and root-growth results were analysed separately.

Litter species	T (°C)	Germination				Root growth			
		Lepidium sativum		*Eucalyptus globulus*		*Lepidium sativum*		*Eucalyptus globulus*	
Acacia dealbata	25	*59.1±7.9*	fg	*69.6±7.5*	h	110.5±18.8	kl	113.8±32.6	kl
	100	*22.7±7.9*	cd	104.3±13.0	i	79.2±14.8	hi	**104.1±1.2**	jk
	200	*31.8±15.7*	de	87.0±7.5	h	47.1±45.7	def	*33.9±19.1*	cd
	300	90.9±15.7	hi	108.7±19.9	i	97.0±3.5	j	136.7±28.7	m
	400	90.9±20.8	hi	87.0±19.9	h	70.2±18.1	fgh	132.3±37.0	m
	500	72.7±28.4	gh	91.3±13.0	hi	111.1±42.7	kl	127.8±21.5	lm
	600	90.9±7.9	hi	82.6±27.2	h	108.1±7.3	kl	114.0±30.5	kl
Eucalyptus globulus	25	*0.0±0.0*	a	52.2±26.1	fgh	*0.0±0.0*	a	*42.5±7.9*	de
	100	*0.0±0.0*	a	*17.4±15.1*	bc	*0.0±0.0*	a	*28.7±24.8*	bc
	200	*18.2±20.8*	bc	95.7±7.5	hi	78.8±68.5	fghi	*49.3±14.7*	def
	300	68.2±27.3	gh	108.7±15.1	i	117.6±7.1	kl	147.7±47.0	mn
	400	90.9±31.5	hi	78.3±13.0	gh	111.2±42.0	kl	**162.3±8.7**	n
	500	113.6±20.8	i	78.3±22.6	gh	82.5±12.8	hij	171.5±37.8	no
	600	90.9±7.9	h	117.4±13.0	i	93.7±28.3	hij	**153.0±18.6**	mn
Pinus pinaster	25	*59.1±7.9*	fg	95.7±19.9	hi	**183.7±2.5**	no	**159.9±8.4**	n
	100	*22.7±20.8*	cd	113.0±15.1	i	123.0±111.0	lm	109.7±24.0	kl
	200	*18.2±7.9*	bc	108.7±19.9	i	115.7±10.5	kl	83.8±17.4	hij
	300	68.2±13.6	fg	102.9±15.1	hi	117.6±23.8	kl	168.5±45.0	no
	400	113.6±7.9	i	117.4±13.0	i	100.5±22.3	ij	171.6±44.0	no
	500	90.9±43.8	hi	100.0±19.9	hi	113.0±7.8	jkl	141.3±31.0	mn
	600	90.9±15.7	hi	104.3±0.0	i	102.4±36.2	ijk	**190.8±19.2**	o
Quercus suber	25	*0.0±0.0*	a	*39.1±13.0*	ef	*0.0±0.0*	a	60.8±16.1	efg
	100	*4.5±7.9*	a	47.8±39.8	efg	*4.1±7.2*	ab	45.8±23.5	de
	200	*9.1±7.9*	ab	87.0±19.9	h	27.3±34.3	bc	76.8±16.8	hi
	300	81.8±36.1	h	95.7±7.5	hi	98.4±9.7	j	**134.1±8.8**	m
	400	95.5±23.6	hi	91.3±0.0	hi	108.0±7.4	kl	**153.3±19.7**	mn
	500	100.0±20.8	hi	91.3±26.1	hi	109.6±14.1	kl	174.3±34.4	no
	600	95.5±13.6	hi	78.3±13.0	h	90.1±20.2	hij	92.1±37.0	ij



Supplementary Fig. S1 – Variation of seven main classes of organic C assessed by ^{13}C CPMAS NMR spectroscopy in four plant litter species (*Acacia dealbata*, *Eucalypts globulus*, *Pinus pinaster*, and *Quercus suber*), either unheated (NT) or treated at six different temperatures (100; 200; 300; 400; 500; or 600ºC), for 30 minutes. Note different scales on y-axes.

Section III – General discussion and conclusions

1. General discussion

Human activities have transported species all over the globe. Every exotic species depends on humans to go beyond their native territory (Richardson *et al.*, 2000). Many exotic species do not thrive in the arrival territories, some are able to survive if nurtured, and others are able to live and even reproduce in the wild (Mack, 2000; Blackburn *et al.*, 2011; Richardson and Pyšek, 2012). The independence from human care may exist at arrival time or may be acquired later on. Naturalization is the crucial step in which these species become released from human support and may interact freely within the ecosystems they live in, for better or worse (Mack, 2000; Richardson *et al.*, 2000).

Eucalyptus globulus Labill., a species with a very restricted natural distribution , is native to SE Australia, Tasmania, and adjacent islands (Kirkpatrick, 1975). However, it is cultivated in many regions of the world nowadays, due to the high quality of its pulp for paper production (Jacobs, 1979). In the Iberian Peninsula, its arrival happened in mid-19th century, but its planted area has only been substantially expanded in the last six decades (Pimentel, 1876; Radich, 1994; Federico *et al.*, 2008). The western Iberian Peninsula currently has the largest planted area of this species worldwide.

Until recently, the natural regeneration of *E. globulus* from seeds in Portugal has been overlooked by scientists and undervalued by professional managers, but used by countryside people to establish new stands. Recently, more attention has been drawn to this issue, as a consequence of news about *E. globulus* invasive potential and consequent discussions, which often misuse terminology generating noise in communication (*e. g.* Camargo, 2013). The *media* have very recently published and broadcasted news about flushes of post-fire regeneration from seeds, in central Portugal (Lusa, 2018). Despite mounting evidence that the occurrence of spontaneous seminal regeneration in Portugal exists, only a few short references about naturalization are published in scientific or technical literature (Goes, 1962; Almeida and Freitas, 2006), and a few quantitative studies exist. In contrast, several detailed studies about its invasive potential were recently led by P. Fernandes (Fernandes *et al.*, 2016; Fernandes *et al.*, 2017; Fernandes *et al.*, 2018), and M. Calviño (Calviño-Cancela and Rubido-Bará, 2013; Calviño-Cancela *et al.*, 2018). In addition, a broad-scale wildling assessment was performed by Catry *et al.* (2015) (summarized in chapter 1, App. 1).

All these studies are very valuable but, before plants can invade any habitat, they have to be able to produce offspring *in situ*. Naturalization is prior to invasion (Richardson *et al.*, 2000; Blackburn *et al.*, 2011; Richardson and Pyšek, 2012). Knowledge on naturalization is required, to better understand what follows. Some studies were conducted, concerning isolated specific factors that affect seed availability, as related to: pollination (Calviño-Cancela and Neumann, 2015); seed predation (Deus *et al.*, 2018); and protection of seeds from fire by capsules (*cf.* App. 2) (Silva *et al.*, 2016). However, these fragmentary studies are unable to provide a holistic understanding of biological phenomena, as ecological processes occur on

several spatial and temporal scales, and biological systems have emergent properties which result from their organization (Levin, 1992; Chave, 2013). A multiscale approach is required to better understand both naturalization and invasion, as well as the effects of ecological factors, like fire, on those processes (Moritz *et al.*, 2005; Brown *et al.*, 2008). The work presented in this thesis was the first to address the issue of naturalization of *E. globulus* in an exotic territory, using a multiscale approach.

Mainland Portugal which is adjacent to the Mediterranean Basin has a climate strong Mediterranean influence (Ribeiro *et al.*, 2001). Fire is an ecological factor which is often present in Mediterranean ecosystems (Naveh, 1975). Considering that eucalypts are quite flammable and are from a region where fire has been an important selective agent (Florence, 1996), we have hypothesized that fire occurrence may be advantageous to *E. globulus* in face of other species in Portugal. Fire may play an important role as a facilitator of *E. globulus* regeneration from seeds, providing opportunities for this species to occupy spaces previously occupied by other species or by parent trees (Kirkpatrick, 1975). The researcher has tried to investigate some of the fire-related mechanisms by which *E. globulus* can have an advantage over other existing species.

1.1. *Eucalyptus globulus* natural regeneration from seeds, as observed on different scales

The exploratory work (Apps. 3–4) and the work by Catry *et al.* (2015) (App. 1), both summarized in chapter 1, have assessed regeneration of *E. globulus* in the entire Portuguese mainland, an assessment scale which was never used before for this species. This assessment was achieved by two different methods: (1) the screening of databases of the 4th and 5th Portuguese National Forest Inventories (NFI4 and NFI5, respectively); and (2) a direct survey of roadsides. Results obtained by the two methods were consistent in terms of the geographical distribution of regeneration. Both showed that regeneration of *E. globulus*, including natural seminal regeneration, is widespread in the Portuguese mainland. Trees of this species are able to produce offspring in all the natural regions of this territory. However, the geographical distribution is not uniform. Regeneration occurs more often associated to stands where *E. globulus* is the most common species or the second most common. No evidence of long-distance dispersal was found. This result is in agreement with previous studies which referred to the limited dispersal capacity of this species (Cremer, 1977; Calviño-Cancela and Rubido-Bará, 2013; Fernandes *et al.*, 2016). The occurrence of regeneration is more frequent in regions where the climate and soil are favourable for the growth of individuals of this species. Moreover, densities of wildlings are the highest precisely in those regions. The environmental envelope of wildlings in Portugal generally coincides with that of the native territory (Kirkpatrick, 1975; Jacobs, 1979; Jovanovic and Booth, 2002). Wildling occurrence is limited in the inland and highlands, when temperatures go beyond its tolerance limit. Drought is also likely to limit wildling density, especially in the lowlands of southern Portugal. These results concur with previous findings of frost and drought as the main limiting factors of *E. globulus* growth in

Portuguese plantations (Almeida, 1993; Almeida *et al.*, 1994; Ribeiro and Tomé, 2000; Alves *et al.*, 2012). Some soil types may also exclude wildlings, especially when they are thin and/or calcareous, similarly to what has been found in other countries (Jacobs, 1979; Turnbull and Pryor, 1984). However, in terms of magnitude, climate influence overrides soil influence on wildling geographical distribution across mainland Portugal, just as in other countries (Jacobs, 1979). The massive densities of wildlings, observed at many disturbed sites in the coastal half of northern and central Portugal (chapters 1–4; Apps. 1, 3–5; Catry *et al*., 2015), indicate the very high capacity *E. globulus* has to produce propagules and offspring. In fact, biomass productivity models for this species consider that this region has high to very high productivity potential (Tomé *et al.*, 1998; Ribeiro and Tomé, 2000; Tomé and Ribeiro, 2000; Tomé *et al.*, 2001; Tomé *et al.*, 2006). Moreover, this region and NW Spain are said to be the ideal exotic territories for this species (Jacobs, 1979). It is no coincidence that a large proportion of the *E. globulus* stands are located there. Hence, geographical distribution of *E. globulus* regeneration, in mainland Portugal, is mostly determined by the existence of parent trees and suitable climate, and to a much lesser extent by soil type.

The work presented in chapter 2 compared several biogeographical regions where *E. globulus* is planted: one region in central Portugal; six regions in the south of Australia and Tasmania, where the species is exotic but the genus is native; and one region where the species is native. Wildlings found at the borders of Australian industrial plantations generally had much lower densities than in central Portugal (southwestern part of Tagus River valley; *cf.* chapter 1; App. 1; Catry *et al*., 2015). Notably, the latter region had moderate densities in the Portuguese context. These large differences are not related to climate, which is usually a determinant factor in the biogeographical distribution of species (Milbau *et al.*, 2009). Climate is suitable enough for *E. globulus* survival and reproduction in all the sampled regions in Portugal and Australia. However, the annual precipitation in the Portuguese region is strongly seasonal and close to the lower limit for the species (Jovanovic and Booth, 2002). Mean annual temperature is rather high and summers are warm for the species (Jovanovic and Booth, 2002). Under similar climatic conditions, Australian plantations have the lowest wildling densities. Hence, other factors override the effects of climate on wildling densities on a biogeographical scale. The causes of the differences between Portugal and the various Australian regions are varied. Differences between the native region in Australia and Portugal are compatible with the enemy release hypothesis (Keane and Crawley, 2002), while the differences between Portugal and the other exotic Australian regions might be related to introduction history and biogeographical factors. Firstly, the native provenances which were used in the Portuguese and Australian plantations seem to have been different (Lopez *et al.*, 2001; Freeman *et al.*, 2007; Potts *et al.*, 2014). The ones that were most likely used in Portugal tend to produce larger seeds and in greater number, having better chances of seedling establishment (McGowen *et al.*, 2004; Simberloff, 2009). Secondly, the residence time of the species differs amongst the sampled territories, being much longer in Portugal than in any other one. This longer residence time concerned, not only each region as a whole, but also the sampled sites in those regions. In

Portugal, *E. globulus* began being used in forestry a few years after its introduction, in other words, more than 150 years ago (Pimentel, 1876), while massive planting started more than six decades ago (Radich, 1994). Conversely, the dates of introduction of this species into the various Australian exotic regions have yet to be determined. However, in 1976, Australian plantations of *E. globulus* complex (*sensu* Kirkpatrick, 1974) were restricted to Victoria and Tasmania (825 ha), the Australian states where the native range of this *taxon* is located (Turnbull and Pryor, 1984). Massive planting of *E. globulus* at the sampled Australian regions only began after 1996 (Gavran and Parsons, 2011). In local terms, over 95% of Portuguese sampled sites had replantations, whereas only two Australian regions had sites with replantations but in a much lower proportion (13% in Albany and 49% in Manjimup). Thirdly, natural and artificial selections may also have played their role in the observed differences in wildling abundance. Evidence exists that natural selection has improved drought and frost tolerance of *E. globulus* in the Iberian Peninsula (Turnbull and Pryor, 1984; Almeida, 1993; Toro *et al.*, 1998), which may have enhanced the likelihood of establishment. Moreover, observed planted trees bore capsules more frequently in Portugal than in Australia (Barbour *et al.*, 2008; Larcombe *et al.*, 2014), and a few wildlings shorter than 1.30 m had reproductive structures in Portugal but not in Australia. Artificial selection could explain part of flowering precocity and fecundity observed in Portugal, where breeding programmes have existed since 1966 (Borralho et al., 2007; Leal, pers. com.). Fourthly, all the Australian regions were inhabited by other native species of *Eucalyptus*. Thus, *E. globulus* had much more opportunities for interspecific relationships in Australia, whether beneficial or detrimental to itself (Strauss, 2001). For instance, pathogens, predators, symbionts, and pollinators might be shared with other *Eucalyptus* species (Strauss, 2001). Competition is likely to be stronger in Australia, as ecological niche overlaps are more probable. Finally, the hybridization of *E. globulus* with other species is very unlikely in Portugal because individuals of phylogenetically close species are scarce. On the contrary, this can easily happen in Australia, where congeneric individuals abound and may reduce the reproductive performance of the species (Larcombe *et al.*, 2014). On reflection, it may be said that biogeographical factors may be at least as important as introduction history but no objective data were found to confirm this idea.

Site-scale drivers of *E. globulus* regeneration from seeds were also analysed in the work presented in chapter 2. Factors related to site and plantation conditions explained much of the regional variation of wildling density in central Portugal. This result is consistent with Fernandes *et al.* (2018), who have identified site factors and plantation characteristics as very influential for wildling densities, close to plantations. In the current study, aspect was identified as the most important factor: wildling densities were the highest at western and northern aspects, which are the coolest and the moistest aspects in the region. The same was observed by Fernandes *et al.* (2018), in sites all over the Portuguese mainland, but with less significance. A similar result was also found in an initial model with data from the whole Portuguese mainland (App. 1; Catry *et al.*, 2015). However, this factor was just marginally relevant to explain wildling density on a mainland scale (App. 1; Catry *et al.*, 2015), whereas it was the most relevant factor

on a regional scale (chapter 2). The different magnitude of aspect effect on wildling density, on these two scales, is therefore understandable. Plantations sampled for the work in chapter 2 were all located in the southern part of central Portugal, a region with fairly hot dry summers and a very large area of very permeable soils (Cardoso *et al.*, 1971; Ribeiro *et al.*, 2001). Contrariwise, sites sampled by Catry *et al.* (2015) (App.1) and by Fernandes *et al.* (2018) were located in many different regions and part of them have no drought restrictions to *E. globulus* development (Ribeiro and Tomé, 2000). Site quality was also identified as influential: wildlings were found at high densities in intermediate quality sites. Low densities were probably related to either poor conditions, in low quality sites, or to suppression by planted trees, in high quality sites where competition is stronger (Tomé *et al.*, 1994; Florence, 1996). In addition, propagule pressure also had its role, as higher rotations and older stems of planted trees were associated with higher wildling densities. Notably, rotation and stem age were significant factors in the comparative model of wildling densities in Portugal *vs.* Australia. Moreover, the effects of coppicing and 'country' (Portugal *vs.* Australia) on wildling densities had the same order of magnitude, placing the relevance of residence time and propagule pressure into a wider spatial framework. Therefore, regarding site scale, wildling densities at plantation borders are influenced by local abiotic factors, as topography and site quality, and plantation-related factors, as coppicing and stem age of planted trees.

In the Portuguese mainland, stands dominated by either *E. globulus* or *P. pinaster* had *E. globulus* regeneration more often (chapter 1; Apps. 3–4). These are two of the most fire-prone forest types in this territory (Silva *et al.*, 2009). Considering the relevance of fire in *Eucalyptus* evolution, as well as the traits that enable reproduction and survival of its individuals after fire (Pryor, 1976; Florence, 1996; Gill, 1997), another aim was established by the researcher. Hence, another focus of this work had to do with understanding how fire affects *E. globulus* natural regeneration from seeds in Portugal. To study this issue, several spatial scales were used (Fig. 1 in section I): a regional scale (chapter 3); a site scale (chapter 3); a microsite scale (chapters 4–5); and a molecular scale (chapter 5). The researcher also collaborated in a study on a microsite scale (Santos *et al.*, 2015) (App 2).

In the work described in chapter 3, the sampled stands were: pure stands of either *E. globulus* or *P. pinaster*, and mixed stands with both species. A total of 284 stands were sampled across central and northern Portugal. Sampling was done 5–7 years after fire, in sites as close as possible to NFI5 plots. The probability of wildling occurrence in burnt *E. globulus* pure stands and in those mixed with *P. pinaster* was above 90%. Moreover, 99.5% of the occurrences were near parent trees. When comparing these results, with those obtained by screening NFI databases (chapter 1; Apps. 3–4), it became evident that wildling occurrence was much more frequent in burnt stands than inside or close to unburnt stands. The primary importance of close seed sources was confirmed, as reproductively mature trees were nearly always detected next to wildlings. Once again, climate and soil factors were influential on wildling cohort. Just as it was observed in the exploratory work and in the work led by F. Catry (chapter 1; Apps.1, 3, and 4; Catry *et al.*, 2015), wildlings occurred much more frequently in the high and medium regions

than in the low productivity ones, according to a classification based on climatic and edaphic criteria (Ribeiro and Tomé, 2000; Tomé *et al.*, 2001). When comparing burnt stands of the high and medium productivity regions, the former had the highest wildling densities and the tallest post-fire wildlings. This result apparently contrasts with the one obtained in chapter 2, in which wildling densities were the highest in plantations with intermediate site quality indexes. However, plantations sampled in the work of chapter 2 were all from a single region with moderate productivity (*sensu* Ribeiro and Tomé, 2000), and nearly all of them were unburnt (97.2%). The identified reasons for that result were related to wildling suppression by cultivated trees, which is much more reduced in burnt plantations, for some months following fire. Moreover, the different scales used in the two studies influenced the results, as regional patterns of wildling distribution may hide landscape heterogeneity (Milbau *et al.*, 2009). Finally, three forest management operations were identified as influencing wildlings. Understorey removal favoured wildling occurrence, while salvage logging favoured wildling density. *Eucalyptus globulus* juveniles are very sensitive to competition, especially if it is asymmetrical (Stoneman, 1994; Tomé *et al.*, 1994). Thus, removal of competitors have probably potentiated the establishment of wildlings. Seeds from slash have presumably contributed to the existence of extra recruits (Skolmen and Ledig, 1990; Fagg, 2001), enhancing wildling density. Conversely, tillage was very detrimental for both wildling density and height. This result might have been both a direct effect of tillage, damaging or destroying wildlings (Catry *et al.*, 2010), as well as an indirect effect, mediated by soil nutrient decrease and erosion, caused by tillage (Madeira *et al.*, 1989; Shakesby *et al.*, 1996; Carneiro *et al.*, 2008). Thus, the different outcomes of the several management operations were related to the capacity each one had to release wildlings from competitors or, in reverse, to destroy or damage wildlings. Considering the characteristics of the post-fire cohort of wildlings, observed 5–7 years after fire, one can say that the favourable conditions for the natural persistence of *E. globulus* were probably met in a multitude of burnt stands in Portugal, mostly the pure *E. globulus* stands and the stands where this species is mixed with *P. pinaster.* This work also identified several factors that affect post-fire seminal regeneration. To begin with, the presence of adult trees was vital for supplying seeds. Secondly, favourable conditions for growth, in terms of soil and climate, were very important for wildlings, as they are for planted trees. Finally, stand management might have either benefited or harmed the post-fire wildlings.

The work in chapter 3 demonstrated that wildlings are able to emerge from seeds, grow, and become established in burnt stands. However, it raised some questions that required further investigation. The work presented in chapter 4 was an attempt to answer such queries. Pure *E*. *globulus* burnt plantations were sampled in high and moderate productivity regions (*sensu* Ribeiro and Tomé, 2000). Those plantations had no management operations other than salvage logging since they were burnt. This assessment was conducted on a single occasion, two years after fire, just a few months after logging. In the plantations, microsites of wildlings of different sizes, as well as other microsites with no wildlings, were sampled. Results showed that two recruitment events were possible, in the post-fire environment of stands with *E. globulus*

trees, at the reproductive stage: a major event just after fire and a minor event after salvage logging. The former event was probably caused by massive seed release from the canopies of burnt trees a short-time after fire, as described by Santos *et al.* (2015) (App. 2). The latter recruitment event was probably originated by seed release from slash, after logging (Fagg, 2001). The identification of this second cohort confirms the previously obtained result (chapter 3), which indicated that salvage logging favours wildling density. Wildlings of either cohort had different spatial distributions and occupied microsites with different characteristics.

Wildlings recruited just after fire were very abundant and heterogeneously distributed (densities: 0–9.9 plants m^{-2}) and were in microsites which had strong evidence of fire. Comparatively to other microsites, these had: higher amounts of ash and charcoal, and soil with higher pH and K content. Substrates with these features are generally more favourable to plant development than unburnt substrates. Charcoal can improve soil water retention and adsorb/deactivate phytotoxic substances in soils (Glaser *et al.*, 2002; Hille and den Ouden, 2005). Ashes of *Eucalyptus* are alkaline and rich in K (Raison, 1979; Khanna *et al.*, 1994). Increased pH improves fertility of previously acidic soils (Truog, 1947). *Eucalyptus globulus* juveniles grow more when nutrient conditions in soil are improved, enhancing their chance of establishment (Judd *et al.*, 1996). Moreover, K is important for osmoregulation. It improves the ability of plants to cope with drought, frost, pests, and pathogens (Marschner, 1995). Thus, areas with ashes and charcoal are favourable to *E. globulus* growth and may enhance wildling survival in several types of stressful conditions. Additionally, sheltering objects and small shrubs also tended to be more frequently closer to plants of the first cohort than those of the second cohort. Shelter is important for *Eucalyptus* seedlings, as it mitigates the effects of harsh weather (Battaglia and Reid, 1993). Although shrubs may compete with wildlings, the nursing effects of shrubs on juvenile woody plants, including *E. globulus*, have been observed in Mediterranean ecosystems (Callaway, 1995; Quero *et al.*, 2008; Fernandes *et al.*, 2017; Fernandes *et al.*, 2018). Therefore, the wildlings of the first cohort which were originated by the massive seed release from the canopies of burnt trees; benefitted from both the soil amelioration, caused by fire, and the protection from surrounding objects and shrubs. Indeed, the largest wildlings of the first cohort were in more crowded microsites (with more siblings and small shrubs), apparently sharing the favourable soil and shelter conditions or taking advantage of mutual protection. Meanwhile, the smallest wildlings of first cohort grew very close to adult tree stumps, and their short size evidenced the sensitivity of *E. globulus* juveniles to asymmetrical competition (Tomé *et al.*, 1994). This result supports the idea that favourable sites for germination are not always good for further stages of development (Eriksson and Ehrlén, 1992). In short, the results evidenced that fire effects on the physical and chemical environment can last at least two years and are crucial for wildlings recruited just after fire. Two other facts worth mentioning are: the suppression of wildling growth by cultivated trees; and the clusters of first cohort wildlings which were left undisturbed by logging operations.

Conversely, wildlings recruited after salvage logging were much rarer than those of the first cohort. They were also at more exposed microsites and where soil mechanical disturbance

was more evident. The association of spontaneous recruitment with disturbed soil and low ground cover is common, in literature about eucalypts (Florence, 1996; Pennington *et al.*, 2001). This is also true to *E. globulus* (Fernandes *et al.*, 2017). Indeed, readily accessible mineral soil is very valuable for germinants originating from small seeds with little reserves, as those of *Eucalyptus*, because they need to access water and nutrients from soil very quickly (Rejmánek and Richardson, 2011). At a first glance, this result would seem contradictory with the one presented in chapter 3, but it is not. In fact, results of chapter 3 show that tillage was detrimental to wildling density and height, but not to occurrence; while those of chapter 4 show that the youngest and shortest wildlings occurred at the most disturbed soil and were rarer than the elderly. Results of both chapters can be explained by timing of soil disturbance. Tillage may injure or destroy wildlings that already exist (chapters 3–4), but enables the recruitment of new ones (chapter 4). Moreover, the lack of competitors should have been also an advantage, as *E. globulus* seedlings are very sensitive to competition (Stoneman, 1994). Finally, microsites of second cohort wildlings only differed from those without wildlings in having capsules much more frequently. This difference was an indicator of a likely seed limitation in the second recruitment event. The cause of seed limitation may lay on the fact that burnt trees had little time to produce a new seed crop (Goes, 1977), after the exhaustion of the canopy seed bank by the fire-induced dehiscence of capsules (App. 2; Santos *et al.*, 2015). Therefore, salvage logging enabled a second opportunity for *E. globulus* recruitment, creating favourable microsites and inducing seed release. However, two major disturbances, in less than two years, were a too high disturbance frequency which has limited the magnitude of the second recruitment event (Eriksson and Ehrlén, 1992).

The association of post-fire recruitment with microsites where ashes and other combustion residuals had a strong presence (chapter 4) led to the exploration of an interesting idea of interdependence between fire and eucalypts, concerning allelopathy, which was postulated by Mount (1964). A laboratory bioassay was established to test an allelopathy hypothesis (chapter 5; App. 6). *Eucalyptus globulus* seeds were sown and allowed to develop on the leaf litter of four tree species (*E. globulus* and three others which coexist with it in Portugal and/or in the native territory). Litters were previously heated (temperature range: 25–600ºC) and then cooled. Bioassay results showed that burning litter did affect the early development of *E. globulus.* The effects depended on the litter species and its heating temperature. They ranged from germination prevention to rootlet growth stimulation. *Eucalyptus globulus* rootlet growth was more sensitive to litter than germination was. Different responses were associated to litter composition. As expected, this composition varied across species when litters were unheated. Interspecific differences in litter composition were reduced after heating at very high temperatures. Combustion of organic matter consists of a set of chemical reactions that occur at different temperatures (Pyne *et al.*, 1996). Consequently, the type and amount of combustion products depend on several factors, including temperature and the original composition of materials. Heating at 100–200ºC enhanced the inhibiting effect of litters of some species, while heating at temperatures 300–600ºC eliminated all the inhibiting effects and even

enabled the stimulation of rootlet growth on *E. globulus* germinants. Heating biological tissues at the former temperature range may lead to cell disruption, permitting the leak of cell content, including phytotoxic substances (Chou and Muller, 1972; Knicker, 2007). Conversely, when organic matter is charred, substances that inhibit plant development may be destroyed or modified (McPherson and Muller, 1969; Chou and Muller, 1972; Brodowski *et al.*, 2005; Bonanomi *et al.*, 2015), whereas stimulatory substances may be produced (Flematti *et al.*, 2011; Nelson *et al.*, 2012; Kochanek *et al.*, 2016). These results may have important ecological consequences, because the chemical effects of either unburnt or burnt litter may determine the success of recruitment and early growth of *E. globulus* wildlings. The seeds of this species are very small (Krugman and Whitesell, 2008), and consequently lack reserves for feeding germinants for more than a couple of days (Rejmánek and Richardson, 2011). For this reason, rapid rootlet growth is crucial for survival of the very young wildlings. Therefore, burning litter at high temperatures is important for this early development, whereas the existence of unburnt litter may even prevent the very beginning of recruitment, the germination. These results help to explain why wildlings recruited just after fire tend to be in microsites where combustion residues are abundant (chapter 4), and the so frequent and sometimes dense post-fire cohorts of wildlings observed in burnt stands (chapter 3). Importantly, the unheated litters that completely inhibited germination were of *Q. suber* and *E. globulus* itself. This result is relevant, because native forests of *Q. suber* may become very favourable for seminal regeneration of *E. globulus* after fire, provided seeds are available. Observations in *Q. robur* forests in NW Spain seem to support this result as they are one of the most resistant habitats to colonization by *E. globulus,* but they lose that resistance after wildfires (Calviño-Cancela and Rubido-Bará, 2013; Calviño-Cancela *et al.*, 2018). *Eucalyptus globulus* plantations also become more favourable sites for wildling recruitment and establishment (chapters 1–2 *vs.* chapters 3–4). Additionally, the results also help to explain why regeneration from seeds is more frequent in pinewoods than in other unburnt native forests in Portugal and in NW Spain (chapter 1; Apps. 3–4) (Calviño-Cancela and Rubido-Bará, 2013). Finally, this work supports Mount's (1964) ideas that, in eucalypt forests, regeneration from seeds is enabled through the destruction of conspecific litter by fire.

1.2. Naturalization of *Eucalyptus globulus* Labill. in Portugal´s mainland

1.2.1. The Portuguese population(s)

Eucalyptus globulus is an exotic species cultivated in every natural region of mainland Portugal. The survival of individuals in a territory, outside their species native range, is just an early step for the integration of an exotic species, in that particular arrival territory. *Eucalyptus globulus* has taken this step just after being introduced. Furthermore, the long time since introduction (almost 170 years) and cultivation have enabled the species to experience a wide variety of environments in mainland Portugal, to become adapted to some of them, and to establish several types of biotic interactions there (*cf.* section I). Naturalization is a further step for its integration in this arrival territory.

Naturalization is a population process that requires time. It results from recurrent reproduction, which allows the species to sustain a population in an area, outside the native territory, regardless of any human intervention. A population is usually defined as a group of individuals from the same species occupying a given area (Odum, 1997). This definition assumes the possibility of breeding within the group, under natural conditions, *i.e.*, regardless human intervention. However, no indications are given about the spatial scale of the area occupied by the conspecific group of individuals. In some cases, the boundaries of a population are not evident; and researchers must use rational criteria to limit the populations, according their purpose or convenience (Begon *et al.*, 2009).

Although *E. globulus* exists in every natural region of mainland Portugal, its distribution is not continuous (Fig. 1.1a; chapter 1). Moreover, the distances between the disjunct areas are often long enough to be natural barriers for pollination and propagule dispersal. Consequently, the Portuguese mainland does not have a single population of *E. globulus*, but rather many, though some do not have conspicuous boundaries. Therefore, naturalization status of the species should be identified for particular populations and not for the complete set of individuals in Portugal. Nonetheless, general patterns may be described, contributing to the understanding of the *E. globulus* naturalization process in the entire territory of mainland Portugal.

1.2.2. From cultivated trees to reproductive wildlings

Although naturalization is a population process, it depends on the individual's reproduction capacity. The reproductive capacity exists frequently in *E. globulus* forests of the Portuguese mainland. All the reproduction stages have been observed occurring naturally in the field. The coppicing system used to exploit *E. globulus* in Portugal has rotation lengths which are compatible with the attainment of reproductively mature individuals (chapters 1–4; Apps. 1, 3–5; Catry *et al*., 2015). Cultivated trees are able to produce fruits with viable seeds, which, when naturally dispersed, can germinate under field conditions (chapters 1–4; Apps. 1, 3–5; Catry *et al*., 2015). Seminal regeneration of this species is spontaneous in all its natural regions, but it spatially uneven (chapters 1–4; Apps. 1, 3–5; Catry *et al*., 2015). The occurrence of this regeneration is primarily determined by the existence of close seed sources, namely reproductively mature stems of cultivated trees (chapters 1–4; Apps. 1, 3–5; Catry *et al*., 2015), evidencing the limited dispersal capacity of the species. In summation, these facts show that many plantations are clearly able to naturally produce offspring in the Portuguese mainland.

Where seed supply is ensured, *E. globulus* seminal regeneration is mainly driven by the favourable environment for the development, survival, and establishment of the offspring. The several environmental factors operate on different spatial scales. On a biogeographical scale, introduction history and propagule pressure were found to be the most determinant factors for wildling density, when plantations in Portugal and exotic Australian regions are compared. The effects of these factors override those of climate, as long as the climate is suitable for the species. Other biogeographical factors were hard to assess objectively. Considering both the Portuguese mainland as whole and its individual regions, soils and specially climate are the

determinant factors. On national (mainland) and regional scales (chapters 1 and 3; Apps. 1, 3–4; Catry *et al*., 2015), topography seems to be a surrogate of climate and soil conditions which exist at different elevations or aspects. On a local scale, the most relevant factors are generally forestry and fire (chapters 2–4; App. 5). However, topography may play a crucial role on this scale. In regions where the climate is close to the tolerance limits of the species, site topography may either mitigate or exacerbate adverse conditions, determining the fate of natural regeneration. On a microsite scale, drivers were shelter, nutrients, disturbance (either fire or mechanical), and biotic interactions (chapter 4). Additionally, direct effects of fire on *E. globulus* reproduction were also detected on a microsite scale to an individual scale, in the collaboration with Santos *et al.* (2015) (App. 2). Finally, potential interactions have also been detected between tree leaf litter and the early development of *E. globulus*, which may occur between individual organisms, on a microsite scale, and are mediated by biochemical substances, on a molecular scale (chapter 5; App. 6). Therefore, many factors influence the spontaneous seminal regeneration of *E. globulus* and operate on different spatial scales, in an organized fashion. Some of those factors are only influent on one specific scale, while others are influent on two or more contiguous scales, following a hierarchical rule in which factors operating on smaller scales are subordinate to others from larger scales. For instance, seed supply and suitable climate are of primary importance and operate on the largest scales. Once these conditions are met, others may influence the reproductive output. Moreover, some single factors operating on several scales may have apparently contradictory effects (in magnitude or direction) on the different spatial scales, depending on the interactions that occur with other factors on each scale. Therefore, the results of the current work fit a general pattern which was theoretically identified, by Milbau *et al.* (2009), through a meta-analysis of multiple studies on exotic species.

Living long enough to become established is mandatory to attain maturity and become reproductive. A favourable environment is ensured at many sites in Portugal. Clearly established wildlings persist for at least seven years inside Portuguese forests (chapters 3; App. 5). These plants have the capacity for enduring a long time, waiting for an opportunity to grow more and to reproduce. Wildlings were also observed bearing flowers and/or fruits with fertile seeds, proving that the reproductive capacity was also attained in the wild part of some populations (chapter 2). The observed frequency of established wildlings, in very different parts of mainland Portugal, leaves no doubts: naturalization is in progress and it is a widespread process in many areas of the Portuguese mainland (chapters 1–4; Apps. 1, 3–5; Catry *et al*., 2015).

1.2.3. The role of fire

Fire was proved to be a facilitating factor for natural regeneration to occur and succeed (chapters 3–5). Its effects are evident from a site scale to a molecular scale. Seeds of this species are protected from fire, inside heat insulating capsules, in tree canopies (Santos *et al.*, 2015; Silva *et al.*, 2016) (App. 2). Branch scorching induces capsule dehiscence and enhances

the seed release in the following weeks (Santos *et al*., 2015; App. 2). Fire destroys phytotoxic substances of litter that hinder or prevent early development of *E. globulus* and may even produce others that stimulate it (chapter 5). Fire also increases nutrient availability and changes other chemical properties of soil which favour plant growth (chapter 4). Moreover, as fire kills and damages plants, it reduces competition for the survivors and the post-fire recruits. As a result, the fire-induced seed-rain falls onto burnt forest floor, where seeds have better chances to germinate and their germinants have better chances to develop and become established, as compared to those seeds that land on unburnt litter and the respective germinants. These effects are more evident when fires are intense (chapter 4–5; Apps. 2, 6; Santos *et al.,* 2015). Maximum densities (measured) are impressive: 9.9 plants m^{-2} (2 years after fire) and 4.6 plants m^{-2} (5–7 years after fire). Fire clearly opens a window of opportunity for *E. globulus* to increase their representation in communities, even where pre-fire recruitment was absent (chapter 4). This window gradually closes as time goes by, due to the environmental changes in burnt areas, typical of secondary succession, reducing the chances of successful recruitment for species with a pioneer character, as *E. globulus* (chapter 4). However, the increased abundance of this species is probably long-lasting in burnt areas. Individuals of post-fire cohort(s) establish and are resilient to disturbance due to their lignotuber (chapters 3–4; App. 5). They also live long enough to attain the age of sexual maturity (chapter 3; App. 5). Moreover, fire seasonality in Portugal is associated with other factors that enhance the probabilities of *E. globulus* seminal regeneration to occur and succeed. The so called 'fire season' in Portugal is compatible with the existence of the yearly crops of mature seeds in *E. globulus* canopies (Goes, 1977; Pereira *et al.*, 2006). Fire-induced seed rain is sometimes followed by favourable weather for germination and growth of *E. globulus*, namely when it occurs at the end of summer, and the following autumn happens to have early rain and mild temperatures (chapter 4) (Goes, 1977). Notably, late season wildfires also tend to be more intense than early season ones, due to the progressive drying of fuels during summer. Furthermore, fire intervals in Portugal (Pereira *et al.*, 2006) are compatible with the attainment of reproductive maturity of *E. globulus* individuals, and allow for at least one recruitment event during the normal lifespan of these individuals. These are precisely the necessary conditions for the persistence of eucalypt species, at site level, which are referred by several eminent authors when they speak about the interdependence of fire and flammable forests of eucalypts, including *E. globulus* forests (Mount, 1964; Mount, 1969; Kirkpatrick, 1975; Attiwill, 1994; Gill, 1997).

1.2.4. The current situation

At present, the natural regeneration of *E. globulus* from seeds occurs frequently in Portuguese forests. Moreover, it evidences regional patterns, being mostly located in regions where climate and soils are more favourable, namely: the western part of central and northern Portugal; the Tagus River valley; and a cluster in SW Portugal (chapter 1; Apps. 1, 3–4; Catry *et al*., 2015). In addition, this regeneration is more frequent at elevations where climate is

favourable and less frequent in thin and calcareous soils (chapters 1–2; Apps. 3–4). Despite its wide geographical distribution, this regeneration was nearly always observed at less than a few tens of meters from potential seed sources (mature trees). At site level, this regeneration is more abundant in: coppiced stands with reproductively mature tree-stems; sites with moderate quality indexes; non-tilled soils; and aspects that mitigate climate extremes (chapters 2–3; App. 5). On a microsite level, wildlings tend to occur: in fairly protected microsites; in areas where soils were either mechanically disturbed, before their recruitment, or were left undisturbed, since they were recruited; close to mature *E. globulus* trees, especially the very old ones (age >>> 12 years); and at plantation borders (chapter 2–4; App. 5). In burnt stands, wildlings are mainly located at severely burnt areas. Wildling height is strongly conditioned by tillage and by competition with cultivated trees.

Nowadays, most forests which have abundance of *E. globulus* trees under cultivation have low wildling density, although high wildling densities are reached in some sites (chapters 2–4). Such high densities may be an ecological problem on a local scale, because they may reduce biodiversity, increase fire risk, and increase competition with species of interest (either for production or conservation). This is not a new issue, despite just starting to be perceived by society, including foresters and scientists (*cf*. Fig. 5 of section I; Goes, 1962). The combination of increased planted area, increased yearly burnt area, and increased residence time just have made this situation evident and spatially more extensive in Portugal.

Furthermore, although wildlings exist inside forest stands, their development is negatively affected by cultivated trees. These trees are very strong competitors and some species may even chemically interfere with wildling development (chapters 3–5; App. 6). Wildlings exist outside *E. globulus* dominated stands or even in habitats other than forests (chapters 1–3; Apps. 1, 3–5; Catry *et al*., 2015). This fact was the subject of several previously cited studies led by P. Fernandes and M. Calviño-Cancela. These studies have shown that some habitats other than *E. globulus* plantations are more favourable for seminal regeneration of this species, while others are more adverse. Several factors contribute to that outcome. Competition and potential allelopathic interactions might be among them (chapter 3–5; App. 6). Moreover, fire occurrence also creates new habitats, where competition is temporarily greatly reduced, producing a chemically favourable environment for *E. globulus* recruitment, growth, and establishment (chapter 4 and 5; App. 6). Therefore, several habitats exist in the Iberian Peninsula that are potentially more favourable than the unburnt *E. globulus* plantations, for the growth and establishment of wildlings. If seeds are available in abundance, these habitats may work as hubs for naturalization of this species.

In the current study, numerous industrial plantations of *E. globulus* were sampled. Wildling densities were much lower at their borders than those observed at roadside surveys next to the same type of plantations (chapters 1–2; App. 1; Catry *et al.,* 2015). In reality, the difference between intensively managed plantations and unmanaged disturbed areas should be much larger for three main reasons: (1) plantations whose borders were sampled were all at the reproductive stage while those nearby roadsides could be at any life stage; (2) the trees at

plantation borders are generally more prolific than those deep inside the plantations; and (3) plantation borders are areas with more disturbance than the inner parts of plantations and where management operations are not fully developed. Furthermore, the work presented in chapter 3 shows that the least managed stand type (mixed stands) had wildlings even more frequently than the pure *E. globulus* stands (plantations) where parent trees were more abundant. It also shows the strongly detrimental effect of tillage on both wildling density and height. Therefore, the intensive management of *E. globulus* stands hampers wildling recruitment, growth, and establishment.

Finally, it should be highlighted that, although the occurrence of wildlings is frequent inside forests where *E. globulus* is a common species, the high wildling densities are fairly rare. Moreover, high densities are not very frequent even in situations where or when propagule pressure is high and competition is relatively small, as at plantation borders (median = 2.5 x 10^{-3} plants m^{-2}; max. = 0.4 plants m^{-2}) (chapter 2) or inside recently burnt forests (median = 0.2 plants m^{-2}; max. = 4.6 plants m^{-2}) (chapter 3). Very high densities are mostly restricted to very specific conditions: areas with slash accumulation; drainage channels; vicinity of old trees; and severely burnt areas. Therefore, the general pattern is the frequent occurrence of wildlings at low densities.

1.2.5. The foreseeable future

The dynamics of the naturalization process should be highlighted. Species can move along the introduction-naturalization-invasion continuum in either direction and the speed of these movements may change with time (Gurevitch *et al.*, 2011). Under the current setting of climate change, the predicted climate for Portugal's mainland will be harsher than now for *E. globulus*, especially in southern and central Portugal (Jovanovic and Booth, 2002; Santos *et al.*, 2002). Conversely, the current changes in fire regimes and their predicted trends in Portugal, make the opportunities for *E. globulus* regeneration more frequent, in time and space (Santos *et al.*, 2002; Oliveira *et al.*, 2017). In addition, land use is also changing. The area occupied by *E. globulus* forests has increased in the last decades and future trends are highly dependent of socioeconomic interests and policy (Oliveira *et al.*, 2017), which may change rapidly. The trend observed for land abandonment after fires is also relevant in this context (Silva *et al.*, 2011). The combination of the changes in climate, fire regime, and land-use will surely affect the *E. globulus* regeneration from seeds and the species persistence in sites. However, the direction and magnitude of these effects is largely unpredictable under the current knowledge.

The hierarchical framework of factor effects is helpful to obtain some insight into the future. The factors that were identified as determinant for natural regeneration on different spatial scales will probably remain influent. However, their relative importance may change, as well as the interactions among them. Climate, land use, and disturbance operate on several scales. The scale ranges of these factors partially overlap, making interactions among them possible (Milbau *et al.*, 2009). Climate affects phenology, growth, and survival from a biogeographical to regional scale. Land use is determinant for the availability of seed sources

from a national to site scale (chapter 1; App. 3–4). Judicious and active forest management may enhance cultivated trees productivity and reduce weedery, invasion, and fire risks from a local to microsite scale. Intense fire events promote recruitment and improve the chances of *E. globulus* establishment from a site to microsite scale (chapters 4–5; Apps. 2, 6). However, these effects will only happen where and when climate will be suitable for the species. It is important to bear in mind that fire regimes respond very rapidly to climate changes (Flannigan *et al.*, 2009), and this response may overshadow the direct effects of climate change on species distribution (Dale *et al.*, 2001). Moreover, humans can change the geographical distribution of planted areas, at their own convenience, enabling the species to live under the best climate conditions (Alves *et al.*, 2012). Cultivation may also keep plants (crops and weeds) protected from environmental stochasticity and may mitigate adverse effects of climate (Mack, 2000; Lockwood *et al.*, 2005; Wilson *et al.*, 2009). Finally, evolution can also play its role in the future, improving the tolerance of populations to the environmental conditions of the Portuguese territory, as it did in the past (Lopez *et al.*, 2001; Potts *et al.*, 2004; Borralho *et al.*, 2007). Therefore, the future of the *E. globulus* naturalization process in mainland Portugal cannot be accurately predicted and is highly context dependent. Nevertheless, under the current settings, this species is likely to have a place on its own in the flora of several Portuguese regions, in the foreseeable future.

2. Management recommendations

During the current work, it was observed that the production of wildlings in forest stands with adult trees of this species is common in mainland Portugal. Moreover, in some circumstances, it may reach high densities, causing an ecological problem. During this work, several factors that influence the natural regeneration of this species from seeds have been identified. Some of the produced information may be useful for detecting and controlling problematic situations.

Eucalyptus globulus is able to regenerate both sexually and vegetatively. Generally speaking, eucalypt species do not have a lasting soil seed bank, making local eradication of undesired plants an achievable goal (Rejmánek and Richardson, 2011). Conversely, resprouting makes control more difficult (Rejmánek and Richardson, 2011). The high mortality rate of seedlings reduces the effect of prolific seed production (Calviño-Cancela and Rubido-Bará, 2013) and the usually small dispersal capacity does not allow a fast spread (Cremer, 1977; Booth, 2017). As the wildlings get older their survival probability increases (Calviño-Cancela and Rubido-Bará, 2013). Considering the difficulties in destroying lignotuberous wildlings (Florence, 1996), early detection and control are the best options to avoid future problems. The relevance of fire as a facilitator of natural regeneration from seeds should be taken into account.

2.1. Prevention

Coppicing and rotation length

This species is usually exploited in a coppicing system in Portugal, with rotations that last from 10 to 12 years (Turnbull and Pryor, 1984; Soares *et al.*, 2007). This management is important to limit the reproductive output of planted trees. This way, stems are periodically restarting their development and are up to six years producing few or no seeds (chapter 2). Moreover, they never become old enough to reach the maximum reproductive capacity (Fernandes, 2016). However, the reproductive output of trees beyond six years old is already substantial. If the cultivation purposes of this species change in the future, the possibility of coppicing at shorter rotations would be useful for preventing sexual reproduction in plantations.

2.2. Monitoring

The spontaneous seminal regeneration from seeds is influenced by ecological factors, which operate on different spatial scales and work as a hierarchical set of filters (Milbau *et al.*, 2009). The spatially hierarchical framework is useful for monitoring.

Wide scale monitoring might be expensive and time-consuming, however, some tools might be useful. Firstly, NFI databases are of uppermost importance to have up-to-date information about the location of seed sources at the national level. Secondly, the screening of those databases proved to be useful as a first approach (chapter 1; Apps. 2–3) to identify the national and regional patterns of the occurrence of *E. globulus* regeneration. Furthermore, it provided systematic and periodic information concerning all Portuguese forests. Its effectiveness would be improved, if field data collection included the origin of the regeneration (artificial/natural, sexual/vegetative).

In the regions with the highest probabilities of wildling occurrence, monitoring should focus mainly on land uses where *E. globulus* trees are common and also on their vicinities. Burnt areas deserve special attention. In reverse, the high-elevated areas in these regions do not need so much attention, as climate and soils might be less favourable for wildlings. On this scale, the car surveys along stand borders might be a feasible assessment method with bearable costs, in terms of time and resources, whereas in the burnt areas, it is recommended to conduct surveys during the first spring or summer following a fire.

On a local scale, direct observations are recommended and may be done during ordinary management interventions. These observations are more important in the stands where *E. globulus* is one of the two most abundant species and in the vicinities of those stands. Amongst *E. globulus* dominated forests, those which have trees with stems older than six years deserve more attention, especially if they were already coppiced. The recently harvested stands, as well as the burnt stands deserve special attention. The latter should be inspected during spring, when post-fire wildlings become more conspicuous.

Inside stands, the priority areas of inspection should be: the periphery; places of slash accumulation; the areas along drainage channels; the northern and western aspects (especially

in dry regions); the areas around trees with stems aged well over the standard rotation length; and the severely burnt areas.

2.3. Control

2.3.1. General

As soon as high densities of wildlings are detected, their control should be planned. The window of opportunity for efficient control is probably narrower than one year. It begins after recruitment and ends when lignotuber starts to develop, because wildlings have much less chances to regenerate vegetatively prior to lignotuber development. During the first year after recruitment, wildling mortality is very high. It is especially intense during winter, in regions where frost frequently occurs, and in summer, in dry regions (Calviño-Cancela and Rubido-Bará, 2013; Fernandes *et al.*, 2018). Some 8-month old wildlings already have a visible small lignotuber (pers. observ.). Just before they turn two years old, they all have a lignotuber (chapter 4). Therefore, control interventions should occur during the first year of a wildling's life, preferably soon after their first harsh season (or, at most, the second). At that time, natural mortality has already thinned the recently recruited cohort and the need for any control intervention should be reevaluated. This timing saves resources, as most wildlings that would naturally die are not there anymore, and the survivors are still easy to destroy. Looking at the results of the current work, tillage would be the recommended action to take in order to control wildlings because simple cutting is less effective in killing wildlings, and may even improve conditions for wildlings survival (chapter 3; App. 5). If tillage is performed in the first year after recruitment, its depth may be shallow, because wildlings do not have deep roots yet. However, the risk of erosion should always be taken into account. The other option to control wildlings would be pulling them out manually, which is easy in the first year but very time-consuming if densities are high.

2.3.2. Specific cases

Salvage logging

Salvage logging proved to have a beneficial effect for *E. globulus* regeneration from seeds. It may, not only temporarily release post-fire wildlings from their strongest competitors, but also promote a second recruitment event, through a second release of seeds (from slash this time) (chapter 4). Hence, it is recommended to salvage log burnt forests within a short enough time period, in order to prevent the production of more seeds, *i. e.* between the fire event and up to 6–10 months after the first post-fire blooming (Goes, 1977).

Moreover, the researcher observed evidence that tree harvesters avoided the areas where wildlings were the tallest and those where the wildling densities were the highest, within burnt stands. These areas were either spots with several square meters scattered inside stands or strips along the drainage channels. This procedure was obviously due to the fact that these areas did not have trees to cut or were hard to access. However, it had a detrimental side effect, as it allowed the most vigorous wildlings to thrive. Hence, the need to control post-fire

wildlings should be evaluated prior to or during salvage logging, because those wildlings which survive to logging disturbance have a second chance to keep growing in an environment with temporarily reduced competition. Using wildlings for production purposes is another option that might be considered.

Drainage channels

Drainage channels are critical topographical features in terms of *E. globulus* seminal regeneration. Slash is often thrown to downslope positions and sometimes ends up in these channels. The researcher observed that planted trees growing in gullies are sometimes left unharvested, because harvesting them is time-consuming and dangerous for harvesters. Thus, the stems of these trees may reach very old age and, consequently, higher reproductive capacity. Moreover, Portuguese law strongly limits interventions along drainage channels and its sides because they are areas of public hydrologic interest and have a high erosion risk (DSVPF, 2003). Both the official 'Best forestry practices manual' and the official 'Recommendations for management in burnt areas' impose severe limits to interventions along drainage channels (DSVPF, 2003; MADRP *et al.*, 2005). However, while they say that the fuel build-up should be avoided to prevent these strips to be flammable corridors, they recommend the promotion of natural regeneration, not specifying the species. Moreover, they limit revegetation interventions to situations where all vegetation has been destroyed or where invasion or weedery are occurring. Although wildlings inside a conspecific plantation cannot be considered invaders and many would claim that they are also not weeds, the researcher believes that *E. globulus* wildlings should be removed from drainage channels inside and outside *E. globulus* stands. Firstly, because wildlings are preventing the regrowth of natural vegetation, which is usually less flammable than *E. globulus* in these channels. Secondly, because such topographic features enable long-distance dispersal of eucalypts (Rejmánek and Richardson, 2011). Finally, because temporary floods and erosion turn these channels and their banks into favourable habitats for wildling establishment (Kirkpatrick, 1977; Rejmánek and Richardson, 2011). Nevertheless, this type of intervention probably requires changes in the regulations or, at least, the reinterpretation of the current regulations. When these interventions are allowed in drainage channels, care should be taken in terms of erosion. Thus, revegetation should be considered (MADRP *et al.*, 2005) and the choice of timing for wildling removal should be judicious.

3. Future research

The current work was able to answer several questions about the natural regeneration of *E. globulus* and the naturalization process of this exotic species in Portugal's mainland. However, many questions related to these issues still exist and new ones were raised by observations made during this work.

The movement of exotic species along the introduction-naturalization-invasion continuum is affected by a multitude of factors interacting on several scales. The use of an integrated approach, dedicated to the natural regeneration of single species, allowed the identification of the factors that influence the natural regeneration of this species on the various spatial scales. Concomitantly, it allowed the observation of: the range of scales in which each factor acts; the variation of the effect magnitude of several factors across scales; and the interactions that occur between some factors on the different scales. This way, in what concerns *E. globulus* naturalization, some critical situations which deserve special attention were identified. However, many pieces are still missing in this puzzle. If future studies consider this hierarchical framework, a more integrated picture of naturalization process will be achieved. Moreover, a similar framework for timescales would probably be very useful too.

The role of individual biogeographical factors in the naturalization of Portuguese population could not be completely clarified during the current work. Such a complex subject with numerous factors and interactions, requires further studies dedicated to the various factors, within an integrative framework. This type of approach may be insightful for the understanding of processes associated to the introductions of other species, including other Australian exotics that already exist in Portugal.

The NFI databases proved to be interesting tools to systematically assess the occurrence of natural regeneration of tree species (chapter 1; App. 3-4). On a national scale, they also enabled the researcher to verify whether changes occurred on this regeneration, for nearly a decade. However, the data were not collected with that purpose in mind. Consequently, the distinctions of sexual/vegetative and artificial/natural regenerations had accuracy problems. Based on her experience, the researcher thinks it would be feasible to obtain quite accurate information regarding the origin of regeneration of tree species, with little effort, in the field. Therefore, the researcher recommends the inclusion of this procedure in the field protocol for data collection of future NFIs. This information would be valuable to assess the fitness of different species, either native or exotic, in Portuguese forests. Moreover, in the specific case of exotic species, this type of information would be crucial for the long-term follow-up of their movements along the continuum introduction-naturalization-invasion. The assessment of the reproductive self-sustainability of woody species populations requires decadal to centennial scale monitoring and the NFIs work on these scales.

Seminal regeneration is the keystone of naturalization, but it occurs at a shorter timescale. Although a follow-up study was planned to directly compare burnt and unburnt pure *E. globulus* stands, it was not feasible during the time period in which this thesis was developed. The field work of the current thesis was done during a time period in which wildfires had an average frequency. During this work, difficulties were faced to find comparable plantations having wildlings and just differing in fire occurrence (recently burnt/unburnt); and to collect the fire induced seed-rain. Therefore, a field-based follow-up study about this process with an appropriate control is still missing. This type of study would be very useful to understand the changes that occur in both cultivated plants (seed sources) and wildlings, throughout time, after

fire. It would provide more information about the factors that affect the different stages of wildling development from seeds, and the magnitude of their effects. This would be the best way to study the temporal dimension of natural seminal regeneration and its relation to fire.

To assess if naturalization is attained by a certain population, it is required to know if that population produces a substantial number of generations by natural means. To understand the population structure, in terms of generations, it is necessary to know the kinship between the individuals that compose the population. *Eucalyptus globulus* wildlings and planted trees have been observed in forest stands. These two plant types are easy to distinguish, when they only belong to two different generations recruited at very different moments and/or are within plantations whose cultivated trees were planted in regular spacing. The researcher is convinced that the plants which were identified as wildlings were the first generation of offspring (F1), produced by the planted trees that were visible in the sampled forests. However, distinction might become much more difficult or impossible when more than two generations coexist, or when the aim is to distinguish several cohorts of wildlings resulting from recruitment events very close in time. The use of genetic markers for parental analyses could solve this problem with precision and would be very useful in studies in sites where recruitment is fairly continuous, where several generations coexist, or in metapopulations.

Eucalyptus globulus does not have a soil seed bank, its natural seminal regeneration relies on the canopy seed bank. Its importance was evident in fire-induced seed rain (chapter 4; App. 2). However, very little is known about this seed bank. Its dimensions are still unknown: proportion of serotinous *vs.* non-serotinous capsules, in each crop; the time serotinous capsules can keep their seeds; and the longevity of seeds in serotinous capsules. No information exists about which mechanisms lead the serotinous capsules to keep their seeds during the normal dehiscence season and which factors, aside from fire, trigger their dehiscence. These pieces of information may be useful to predict which conditions can induce extra recruitments of wildlings. They might also help to explain why burnt plantations had different post-fire recruitment patterns at apparently very similar conditions (chapter 4).

In what concerns dispersal mechanisms, no studies have addressed particular mechanisms of dispersal at long distances in *E. globulus*. Some of these mechanisms are difficult to study because they depend on stochastic events, which last for a short time, such as gales. However, hydrocoric dispersal is easier to study, because it directly depends on topography, rainfall, and water flow, having a more continuous and predictable behaviour. Moreover, drainage channels and their sides are favourable areas for wildling establishment (chapter 4). In fact, the researcher has observed evidence of hydrocory, both inside and outside plantations during the field work.

Although evidence of capsule hoarding by vertebrates was occasionally observed during the field work, no information about this behaviour for *E. globulus* exists. As fruit hoarding may influence seed dispersal and seed availability for germination, it would be interesting to know which animals hoard *E. globulus* capsules, as well as to find out more about their hoarding behaviour.

Coppicing *E. globulus* trees at rotations of 10-12 years reduces their reproductive output comparatively to longer rotations. However, this output is already substantial when stems are older than six years (chapter 2). Thus, it would be interesting to consider the selection of genotypes associated to small reproductive capacity or to late sexual maturity, in future breeding programmes. Reducing the reproduction of plantations would be an effective way to control the occurrence and the number of wildlings. Postponing reproduction could also be effective to prevent it, if combined with the appropriate rotation length, to avoid seed production.

4. General conclusions

Eucalyptus globulus is an exotic species widely cultivated in the Portuguese mainland. Time since introduction and the cultivation practices have enabled the species to experience a wide variety of environments in mainland Portugal, and its individuals to attain reproductive maturity in many sites. The ability of cultivated trees to produce seeds and release them to the soil has clearly already been attained. The capacity of seeds to germinate and originate wildlings has also been achieved. Although the seminal regeneration of this species occurs in every natural region of mainland Portugal, its geographical distribution is not uniform. It is primarily determined by the existence of seed sources, namely trees with stems at a reproductive age. In the field work, wildlings were nearly always observed next to trees with adult stems. Provided the existence of seeds, the main drivers of seminal regeneration were good environmental conditions for survival, growth, and establishment. Among these environmental conditions, soils and mostly climate were the preponderant factors on the wide spatial scales (national or regional). Locally, the most relevant factors were forest management and fire. At a microsite level, the factors were shelter, nutrients, and disturbance (either mechanical disturbance or fire) and interactions with other organisms. On a biochemical scale, interactions were detected between litter chemistry and the early development of *E. globulus.* Importantly, heating litter within the temperature range of wildfires destroys compounds that inhibit this development and may even result in a stimulating effect.

Surviving and growing long enough to become established are mandatory steps towards maturity and reproduction. The environmental conditions and seed availability are ensured in many sites in Portugal. Wildlings that are clearly established and persist for at least seven years were observed, in Portuguese forests. Their endurance enables them to wait for an opportunity to grow more and reproduce. The existence of wildlings with flowers or fruits containing viable seeds indicates that these plants are reproductive. The observed frequency of established wildlings in very different parts of mainland Portugal, leaves no doubts that naturalization is in progress and it is a widespread process. During the current research, there was no opportunity to verify if wildlings were able to produce enough recruits that become established and reproductive. If that final step is confirmed by future studies, the ‘wild’ population(s) of *E. globulus* in Portugal’s mainland will ensure self-perpetuation, *i. e.*, they will be completely naturalized.

Fire was proven to be a facilitating factor for the occurrence and the success of spontaneous seminal regeneration of *E. globulus*. Its effects are evident from a site scale to a molecular scale. Considering the changes in fire regimes that are being observed across Portugal (Oliveira *et al.*, 2017), the probabilities of naturalization are increasing for this species.

Wildlings occur in most forests which have abundant adult trees and they are generally at low densities. However, very high wildling densities, which are reached in some sites in Portugal, are already an ecological problem. The species may have a weedy behaviour and increase the risk of fire, in some sites. Moreover, wildling occurrence outside plantations should be monitored to avoid the spread of these plants. The usually reduced dispersal capacity does not allow a fast spread. Notably, several factors that affect the natural regeneration of *E. globulus* from seeds are still human dependent, namely those associated with wildfires and forest management. Considering the difficulties in destroying lignotuberous wildlings, early detection and control are the best options to avoid future problems. Attention should also be paid to the effects of forest management operations, as they may be either detrimental or beneficial to wildling development, from recruitment to establishment. No management is not an option, since the species is already able to regenerate from seeds without any human support.

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Appendices

Appendix 1

Assessing the extent and the environmental drivers of *Eucalyptus globulus* wildling establishment in Portugal: results from a countrywide survey

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Note:

We have participated in this work but we have not leaded it. We have searched and provided the information about the ecology and reproductive biology of *E. globulus*. We have participated in the very initial planning of this work. We have written the draft of introduction. We have participated in the discussion of results. We have collaborated in the review of the manuscript.

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ORIGINAL PAPER

Assessing the extent and the environmental drivers of *Eucalyptus globulus* wildling establishment in Portugal: results from a countrywide survey

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Abstract Tasmanian blue gum (*Eucalyptus globulus*) has been increasingly used in forestry outside its native range, and is nowadays one of the most important pulpwood species in the world. *E. globulus* has great economic importance in many countries, and in Portugal it has recently become the most widespread tree species. However, there is also an increasing concern about the potential ability of eucalypts to naturally establish from seed (wildling establishment), because of negative ecological and economic impacts this could cause. The natural establishment of this fast-growing exotic species may have undesirable consequences, but little is known about its distribution, or which are the factors influencing its occurrence. In order to investigate these issues, we characterized wildling occurrence and abundance along 3111 roadside transects adjacent to eucalypt plantations distributed throughout continental Portugal. Eucalypt wildlings were found in 60 % of the sampled transects and across all natural regions, with densities ranging from 0 to 10,000 plants ha^{-1} (mean = 277 plants ha^{-1}). The potential influence of environmental variables on wildling establishment from plantations was assessed using boosted regression trees. The abundance of wildlings was found to be primarily affected by precipitation and distance from the sea (used as a surrogate of thermal amplitude), although topography, frost occurrence and soil type also played a significant role. Plant density peaked at around 1500 mm of annual precipitation and it decreased with both decreasing and increasing precipitation, reaching the lowest values below 800 mm

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and above 2400 mm. Eucalypt wildlings were also more abundant in areas with milder temperatures, namely closer to the sea (with lower thermal amplitude) and with lower number of frost days. Finally, plant density also seemed to be favoured in areas with intermediate elevation, higher slope and with certain soil types (namely Cambisols and Podzols). Knowing the regions with higher wildling density and understanding the factors influencing plant establishment may help managers to establish and prioritize eventual control plans in regions with higher probability of recruitment.

Keywords *Eucalyptus globulus* · Wildling · Seed regeneration · Natural establishment · Management

Introduction

Planted forests are used by people for many purposes and have a great socioeconomic importance worldwide. The area of planted forest globally is increasing and currently accounts for c. 7 % (264 million ha) of the total forest area, a quarter of which consists of introduced (alien) species (FAO 2010). Alien trees play a very important role in the economies of many countries, but there are also important environmental and economic costs associated with their widespread use in forestry, namely on the biodiversity, water and soil resources (Richardson 1998). One particular concern is the one associated with the potential invasive spread of cultivated trees, which may cause large impacts on a wide range of ecosystem properties and function (Richardson 1998), and cause serious management problems. For these reasons, it is important to study and monitor plantations of alien species, particularly those that have been reported as naturalized and potentially invasive. Generally, the species that cause the greatest problems are those that have been planted most widely and for the longest time (Richardson 1998).

Species of eucalypts are among the most widely planted taxa outside their natural ranges, namely in temperate, tropical and subtropical regions, where they cover c. 20 million ha (Rejmánek and Richardson 2011). *Eucalyptus globulus* Labill. (Tasmanian blue gum), native to southeast Australia, was discovered by the Europeans in 1792 and soon it was spread throughout the world (Potts et al. 2004). It was introduced in all continents (except Antarctica) and is nowadays one of the most important pulpwood plantation species in the world (among other uses), having great economic importance (Potts et al. 2004). In 2004 there were estimated to be over 2.5 million ha of *E. globulus* planted worldwide, with the main concentration of plantations occurring in the Iberian Peninsula (Potts et al. 2004).

The exact date when *E. globulus* was firstly introduced in Portugal is unknown, but it probably occurred by 1820-30, and in 1850 it was already common as an ornamental species (Doughty 2000; Goes 1962). However, the expansion of eucalypt plantations in the country only occurred since the mid-20th century, closely followed by the development of the pulp and paper industries (Alves et al. 2007). Portuguese stands are managed in a coppice system, usually with an average cutting cycle of 10–12 years (Soares et al. 2007). Nowadays plantations dominated by eucalypts cover c. 812,000 ha and represent 26 % of the whole forested area in continental Portugal (ICNF 2013). *E. globulus* is the only eucalypt species with a wide distribution in the country (>95 % of the eucalypt forests; Borralho et al. 2007), and it has recently become the tree species covering the largest forest area (ICNF 2013). This species is adaptable to a wide range of environmental conditions (e.g. Jacobs 1979; Kirkpatrick 1975), but as a widely cultivated alien tree, its current distribution is mainly determined by human activity.

Given the extent of cultivation (propagule pressure), eucalypts are generally viewed as markedly less invasive than several other widely cultivated trees (e.g. pines; Rejmánek and Richardson 2011). However, it is known that different species of the same genera (or cultivars, hybrids and trangenics used in forestry), may show very different levels of invasiveness (Richardson and Rejmánek 2011). One of the best predictors of invasiveness of alien species is whether they have invaded in other parts of the world (Richardson and Rejmánek 2011). According to Rejmánek and Richardson (2013) *E. globulus* is one of the 8 invasive alien *Eucalyptus* species in the world; it was reported as invasive in seven of the 15 broad geographical regions considered by these authors (North, Central and South America, Europe, New Zealand, Pacific Islands and Indian Ocean Islands). However, classification of invasive alien species is not

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always based on the same criteria and may have different levels. The invasion risk of *E. globulus* was assessed in several publications based on the Australian Weed Risk Assessment (Pheloung et al. 1999), and it was found to be "High" in the Mediterranean Basin (Gassó et al. 2010), in Portugal (Marchante et al. 2014), in the USA (Gordon et al. 2012) and in Hawaii and other Pacific Islands (Daehler et al. 2004). In California, *E. globulus* is considered invasive but rated with a "moderate" score in terms of the species' negative ecological impact in the region (Cal-IPC 2006). In Australia it has been reported as a weed in several regions outside its natural range (Lazarides et al. 1997), and as a species with a low risk of invasion (Larcombe et al. 2013). In Spain it was reported as invasive, posing high danger to natural and semi-natural ecosystems, although with local dispersion (Sanz-Elorza et al. 2001; 2004). In spite of these classifications, weed risk assessments are mainly based on expert knowledge and are rarely based on the known distribution of the species in the invaded range; indeed, field-based quantitative information about the abundance and geographical extent of *E. globulus* wildlings (natural regeneration from seed) is scarce (Águas et al. 2014; Calviño-Cancela and Rubido-Bará 2013; Larcombe et al. 2013).

The processes implicated in biological invasions are conceptualized as occurring along an introduction–naturalization–invasion continuum, thus naturalization is a critical stage of the invasion process (Richardson and Pyšek 2012). The first reference to naturalization of *E. globulus* in Portugal dates from 1943 (Almeida and Freitas 2006), and recently Águas et al. (2014) suggested that the naturalization process (sensu Richardson et al. 2000) is undergoing. However, quantitative information at the national level is still inexistent.

Although the natural establishment of this fast-growing alien species may have undesirable consequences (e.g. FAO 1985; Rejmánek and Richardson 2011), little is known about where and how often it occurs, or which are the factors influencing its occurrence. *E. globulus* has a marked ecological plasticity (e.g. Jacobs 1979). Precipitation, temperature and soil characteristics are often referred to as the most important factors affecting *E. globulus* (Boland et al. 2006; Kirkpatrick 1975; Jacobs 1979). Climatic conditions are said to be ideal where mean annual precipitation is above 900 mm, there is no severe dry season, and minimum temperatures do not drop below −7 °C (Jacobs 1979). Most studies report a rainfall requirement of 900–1400 mm, but drier climatic conditions can be tolerated, provided a sufficient level of soil moisture can be maintained (Jacobs 1979). Frost is particularly damaging to seedlings and sprouts (Boland et al. 2006; Jacobs 1979). The principal limiting soil factors are insufficient depth, poor drainage, salinity, strong alkalinity, and the presence of high content of assimilable carbonates (Kirkpatrick 1975; Jacobs 1979). Given the Mediterranean character of most of the Portuguese territory, with associated water scarcity during the summer months, we hypothesise that wildling establishment and abundance are mainly determined by water availability, thought thermal amplitude and other edaphoclimatic and topographic conditions may also play an important role. Given the wide distribution of *E. globulus* in Portugal and the existing knowledge gaps, we performed a countrywide survey which enabled gathering for the first time a large dataset of wildling establishment in a wide range of environmental conditions outside the continent of origin. Our main objectives in the present paper are: (1) to identify the areas where *E. globulus* wildlings occur at a national scale, and to map the areas where wildling establishment is more abundant; (2) to identify the main environmental drivers affecting wildling abundance.

Methods

Study species

Sexual reproduction in *E. globulus* plantations first occurs at 3–4 years of age, although flowering levels are usually reduced in closed plantations and the age of first flowering and reproductive output are genetically variable both between and within races/subraces (Potts et al. 2008). *E. globulus* is a prolific species: each tree may produce hundreds of fruits (woody capsules) per year and each fruit may contain 5–50 viable seeds (more frequently 10–30; Goes 1977). Seeds are small (but relatively large for eucalypts) and have no specialized dispersal mechanism, thus in most cases dispersal distances seem to be limited to 10–15 m (Calviño-Cancela et al. 2012; Larcombe et al. 2013). Since most seeds show no dormancy, any substantial seed storage should occur in the canopy but not in the

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soil; seedling establishment is thus likely to take place within a year after seed dispersal (fruits fall all year round with a maximum during winter; Calviño-Cancela et al. 2012).

E. globulus can tolerate a wide range of environmental conditions. As an exotic plantation tree, although sometimes considered to be a species of great plasticity with regard to climate, the major successes have been attained largely in mild, temperate climates and at high elevations in cool tropical climates; success in other climates is rare, except where moderating conditions occur (Jacobs 1979; Kirkpatrick 1975). Soil factors are generally considered as less important than climate in limiting its development (Jacobs 1979). However, adult trees and young plants can be differentially affected by the environmental factors, and as a result, some sites that have adult trees are not necessarily appropriate for natural seedling establishment and survival. The active selection of genotypes with desirable characteristics (such as frost and drought resistance) also means that variations in ecological limitations are likely to occur.

Study area and wildling density assessment

The study area in this paper was continental Portugal. In order to investigate the natural establishment of *E. globulus* throughout continental Portugal we performed car-based surveys aiming to detect and quantify the occurrence of wildlings (i.e. natural regeneration from seed) on roadsides in the boundaries of eucalypt plantations. Most eucalypt wildlings detected in this study were taller than 30–40 cm, as smaller seedlings were difficult to see (occasionally larger and already reproductive saplings, up to 5 m high, were also spotted). Other studies used similar approaches to detect alien species on roadsides (Abella et al. 2009; Milton and Dean 1998; Shuster et al. 2005; Wilson et al. 1992). This method was chosen because it allows a fast assessment of large areas, facilitated in this case by the conspicuous nature of eucalypt wildlings (bluish colour), and because roadsides are public domain and not cultivated, which strongly increases the confidence that plants are wildlings (naturally established).

Routes for car surveys were previously prepared using geographic information systems (GIS; ESRI 2012), by overlaying a road map and a forest map. Highways were excluded from the survey because it is not allowed to drive there under 50 km h^{-1} and because roadsides are frequently managed. All other kinds of asphalt roads were eligible. Road sections that crossed large areas of eucalypt-dominated stands were selected. Our sampling units were roadside transects 100 m long, established every 1000 m along eucalypt stands. If no eligible stand was found after 1000 m, the next transect was established in the closest eligible stand found along the car route. A roadside was defined as a non-cultivated strip of land, between the asphalt road and the eucalypt stand (see example in Fig. A1 in appendix of supplementary material), and included a range of typologies (e.g. excavated and non-excavated) and widths (large and narrow). In order to prevent difficulties in wildling detection due to excessive distance, roadside width was restricted to a maximum of 10 m. Transect length was shortened to less than 100 m whenever visual conditions for detection were not adequate or if there was no roadside available for detection at some extension. Also, when we found evidence of recent roadside management, the managed section was subtracted from the roadside width/length or, when all the width/length had been managed, the transect was discarded.

Initially we aimed to survey c. 3000 transects, regionally distributed according to the area of *E. globulus* plantations. This goal was achieved and in total 3111 road transects were assessed across the whole Portuguese mainland. These were proportionally distributed throughout the 12 Portuguese natural regions according to the area of eucalypt stands in each region (Fig. A2 and Table A1 in appendix of supplementary material). The Portuguese natural regions map (APA 2013) divides the territory in ecologically homogeneous regions, according to climatic, geological and orographic characteristics.

Data was collected along 6 months (December 2013–May 2014), and all transects were surveyed by the same observer. The car driver assured constant speed (30–40 km h^{-1}) along each transect, while the observer counted and registered the number of eucalypt wildlings on the roadside. The roadside width and the eligible transect length were visually estimated by the observer. Mean roadside width was 2.7 m (SD = 2.2) and mean transect length was 77.0 m (SD = 25.0). At the end of each transect, geographic coordinates were recorded with a global positioning system (GPS) device for location and further use in GIS.

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The observer had a training period prior to the car surveys. After the survey we also performed accuracy tests to assess the likely error in wildling counts, estimation of the roadside width and estimation of the transect length. These tests were performed in four types of sampling areas as a result of the combination of two factors: average width of the roadside (larger or shorter than 3 m) and transect length (100 m or shorter). Each combination was replicated five times, with a total of 20 transects. The observations were first performed on foot to assure the highest possible accuracy and afterwards by car, simulating the original survey procedure. The car observer did not participate in the assessment on foot.

In order to complement our car survey dataset, we used the GPS coordinates of each transect to survey again the same transects using Google Street View (GSV). GSV has also been used in other studies to assess species distribution (Rousselet et al. 2013). Our goal was to obtain the maximum possible count (from both approaches) reflecting the site potential for wildling establishment, in order to minimize the influence of temporal factors such as roadside management. All GSV images were obtained by Google between May 2009 and October 2010, i.e., about 4–5 years before car surveys. We were able to locate and survey 2418 (77.7 %) transects using this procedure (the remaining were not available on GSV or not eligible for some reason). The final wildling abundance database consisted of the maximum count obtained by comparing the two procedures, for each transect. This resulted in 16 % of car transect data being replaced by GSV data (GSV replacements being well distributed throughout the territory).

Environmental variables

Based on the existing knowledge about the ecological conditions affecting *E. globulus* occurrence (e.g. Boland et al. 2006; Jacobs 1979; Kirkpatrick 1975; Stoneman 1994), we selected a set of environmental variables related to climatic, topographic and edaphic conditions as potential explanatory variables influencing wildling establishment (see Table 1). Additionally, we also calculated the transects' linear distance from the sea as a surrogate for continentality and thermal amplitude, because we did not have access to detailed (maximum and minimum) temperature data to analyse directly or to compute a continentality index. Topographic maps (elevation, slope and aspect) were obtained from a raster map with 30 m resolution (METI and NASA 2011). Annual precipitation was provided as a raster map with 1 km resolution (Nicolau 2002). All remaining climatic and edaphic variables were provided as vector maps by the Portuguese Atlas of Environment (APA 2013) at scale 1:1,100,000. The geographic centre of each transect was calculated and overlaid with each environmental map, thus associating information from each variable to each transect and respective wildling density. For topographic variables, we used the bilinear interpolation method in ArcGis software (ESRI 2012) to get an average value for each transect.

Data analysis

Wildling abundance in each transect was computed as the observed number of eucalypt plants per hectare, considering both the transect length and the roadside width. To visualize the main patterns of relative wildling abundance across the country, interpolation of wildling density data was performed in ArcGis using the inverse distance weighting (IDW) method (Geostatistical wizard, default options). The final map was restricted to the areas where eucalypt forest plantations are more representative, i.e. we used a 2 km grid covering the Portuguese mainland and presented density predictions only for grid cells where eucalypt stands occupy at least 10 ha.

Accuracy tests have shown that errors occurred while carrying out wildling density estimates from a moving car (when compared to field assessments). These were associated to the estimation of the number of plants (mean of c. 40 %), estimation of transect length (mean of 10 %) and roadside width (mean of 6 %). This resulted in a mean error of c. 30 % in estimating wildling density. Main errors consisted of an underestimation of wildling density in transects with higher plant density (due to overlooking plants while carrying out estimates from inside the car). To minimize the effect of these errors on subsequent data analyses, density estimates were recoded to a more robust abundance category estimator using an ordinal variable coded as: 0 = no wildlings detected; 1 = 1–99 plants ha^{-1}; 2 = 100–299 plants ha^{-1}; 3 = 300–499 plants ha^{-1}; 4 = 500–699 plants ha^{-1}; 5 = 700–1499 plants ha^{-1}; 6 = over 1499 plants ha^{-1}. All following statistical analyses were carried out using

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Table 1 Description and summary statistics of the variables used to assess the effect of environmental factors on the abundance of eucalypt wildlings in continental Portugal. Means and range are provided for a total of 3111 sampling transects

Variable	Description (units)	Mean (SD)	Range
Alkaline soil	Predominantly alkaline soil (2 cat.)[a]	0.03 (0.18)	0–1
Aspect	Terrain orientation (4 cat.)[b]	–	–
Calcareous soil	Predominantly calcareous soil (2 cat.)[a]	0.02 (0.13)	0–1
Calcareous and alkaline	Calcareous and alkaline soil (2 cat.)[a]	0.01 (0.07)	0–1
Distance from sea	Distance from the Atlantic Ocean (km)	48.21 (34.29)	1.2–167.9
Elevation	Elevation above sea level (m)	259.31 (172.89)	9–930
Evapotranspiration	Real evapotranspiration (mm)	618.19 (92.58)	375–850
Frost days	Mean annual number of frost days (days)	22.03 (13.75)	0–65
Solar radiation	Mean annual global radiation (kcal/cm^2)	2577.42 (199.78)	1950-3150
Precipitation	Mean annual precipitation (mm)	1180.06 (426.827)	392–2679
Slope	Terrain slope (°)	9.87 (6.66)	0–43
Soil type	Major soil type (5 cat.)[c]	–	–
Temperature	Mean annual temperature (°C)	14.19 (1.91)	8.75–18.25
Wet days	Mean annual number of days with precipitation $\geq$1 mm (days)	88.66 (17.78)	37.5–112.5

[a] 2 categories: 1 (yes), or 0 (no)

[b] 4 categories: North, East, South, West

[c] 5 categories: Cambisol, Lithosol, Luvisol, Podzol, Others

the R language, version 3.1.1 (R Development Core Team 2014).

The potential occurrence of spatial autocorrelation in the estimator of wildling abundance, for different distance classes, was tested using the Moran's I (Fortin and Dale 2005). A lag distance of 5 km was considered and a spatial correlogram was estimated using package 'ncf' (BjØrnstad and Falck 2001). Significance of the autocorrelation estimated for each distance class was tested using a permutation test (500 permutations), followed by a sequential Bonferroni correction (Fortin and Dale 2005).

Correlations and collinearity between explanatory variables were checked using Spearman correlations and variance inflation factors (vif; Table A2 in appendix of supplementary material), estimated using package 'AED' (Zuur et al. 2009). More correlated variables included precipitation, insolation, evapotranspiration and number of precipitation days (all $|r| > 0.70$). After the removal of the latter three variables, vif values were all under 3 and pairwise correlations <0.60.

For exploring the main drivers of wildling abundance we used boosted regression trees (BRT) (De'ath 2007). Rather than seeking to fit the single most parsimonious model that best describes the relationship between a response variable and some set of predictors, BRT is a model-averaging method that fits a large number of relatively simple models whose predictions are then combined to give more robust estimates of the response (Elith et al. 2008). In BRT each of the individual models consists of a simple classification or regression tree (De'ath and Fabricius 2000), i.e. a rule-based classifier that partitions observations into groups having similar values for the response variable, based on a series of binary rules (splits) constructed from the predictor variables (Hastie et al. 2001). A BRT model can therefore be seen as a regression model in which each of the individual model terms is a simple regression tree (Elith et al. 2008). Advantages offered by a BRT model include its ability to accommodate both different types of predictor variables and missing values, its immunity to the effects of extreme outliers and the inclusion of irrelevant predictors, and its facility for fitting interactions between predictors (Leathwick et al. 2006). To fit BRT we followed Elith et al. (2008) and used the 'gbm' library (Ridgeway 2009), supplemented with functions provided by Elith et al. (2008).

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The predictive performance of the BRT models depends on two parameters that must be provided in advance, the learning rate (lr) and tree complexity (tc). The lr determines the contribution of each tree to the growing model, and tc determines the complexity of variable interactions that may be fitted. All BRT models had a tc of 5 and were optimized for their lr so that a minimum of 1000 trees was fitted for each model (Elith et al. 2008). To improve accuracy and reduce overfitting, stochasticity was introduced using a bag fraction of 0.5 (at each iteration, 50 % of the data are drawn at random). Each model was built with a default tenfold cross-validation (using gbm.step). Model simplification was performed (using gbm.simplify) in order to discard unimportant variables that do not affect predictive performance.

Following Buston and Elith (2011), model performance was assessed on the training data and on predictions to test data (points that were withheld during cross-validation), enabling the evaluation of the model ability to explain the observed data and its ability to predict left-out data. Both the explained deviance (as a percentage of the null deviance) and the correlation were used for this purpose. Cross-validated standard errors were used to derive 95 % confidence intervals. Spatial autocorrelation in model residuals was checked using Moran's I.

To assess the relative importance of each variable to the BRT models, its contribution was based on how often the predictor was selected for splitting, and the improvement to the model as the result of a variable being selected (Buston and Elith 2011). The relative contribution of each variable is scaled so that the sum adds to 100, with higher numbers indicating stronger influence.

To visualize the fitted functions from the BRT model, partial dependence plots were used (obtained using gbm.plot), which show the effect of a variable on the response, while controlling for the average effect of all other variables in the model. Interactions between predictors are modelled automatically in BRT, because the structure of a tree means that the response to one predictor variable depends on values of predictors higher in the tree (Buston and Elith 2011). The most important interactions between predictor variables were visualized using gbm.interaction.

To take into account existing spatial autocorrelation in model residuals, we used the Residuals Autocovariate (RAC) approach (Crase et al. 2012). RAC is an extension of the autologistic approach, which consists of including an additional term in the model (the autocovariate) to represent the influence of neighbouring observations (Augustin et al. 1996). Rather than being based on the original response variable, RAC is estimated based on model residuals. The advantage of the RAC approach over the autologistic approach is that the explanatory variables are fitted first and have an opportunity to account for autocorrelation in wildling abundances (Crase et al. 2012). RAC was based on BRT model residuals and calculated using the autocov_dist function of the package 'spdep' (Bivand and Piras 2015). The neighbourhood radius was set at 50 km, roughly corresponding to the maximum distance to the nearest neighbour, and each point within the neighbourhood was weighted by the squared inverse distance to the sampled point. Once the autocovariate was estimated, the BRT model was ran again including this new variable. Spatial autocorrelation in the residuals of this final model was tested using the Moran's I, using the same method as described above for the original response variable.

Results

Wildling presence and density

Eucalypt wildlings were found in all Portuguese natural regions and in 60 % of the sampled transects (Fig. 1; Tables A1 and A3 in appendix of supplementary material). A total of 10,592 wildlings were counted in the 3111 transects surveyed, ranging between 0 and 66 plants per transect.

The mean (± SD) eucalypt wildling density in the 3111 transects surveyed was 277 (± 655) plants per hectare, ranging from 0 to 10,000 plants ha^{-1} per transect. Among the 12 natural regions mean (± SD) wildling density ranged between 39 (± 149) and 524 (± 678) plants ha^{-1} (Table A3 in appendix of supplementary material). However, considering only transects where wildlings were present (n = 1863), the mean (± SD) densities were quite higher (463 ± 794 plants ha^{-1} at the national level, and ranging between 156 ± 205 and 690 ± 701 across regions; Table A3 in appendix of supplementary material).

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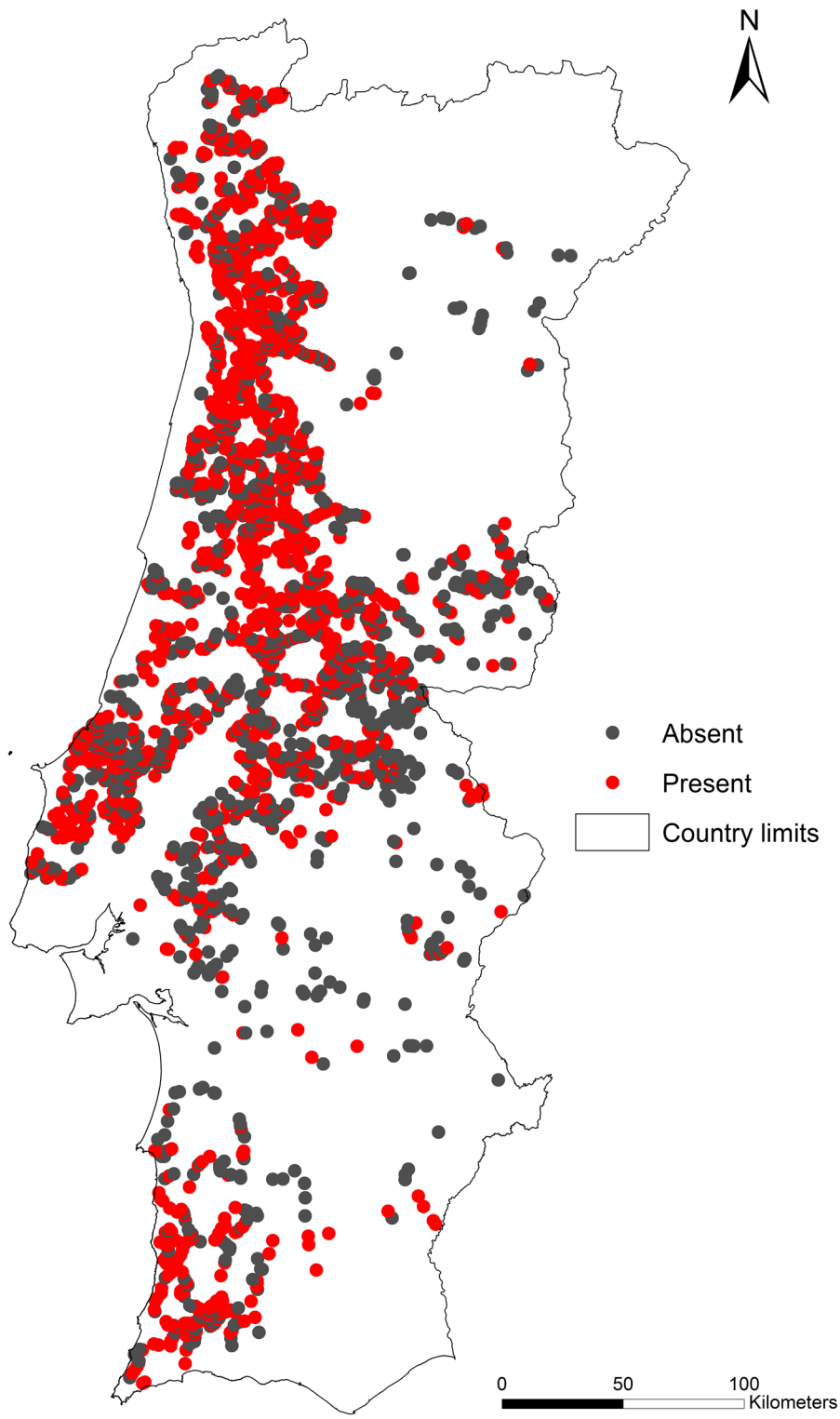

Fig. 1 Presence-absence of eucalypt wildlings in each transect in continental Portugal

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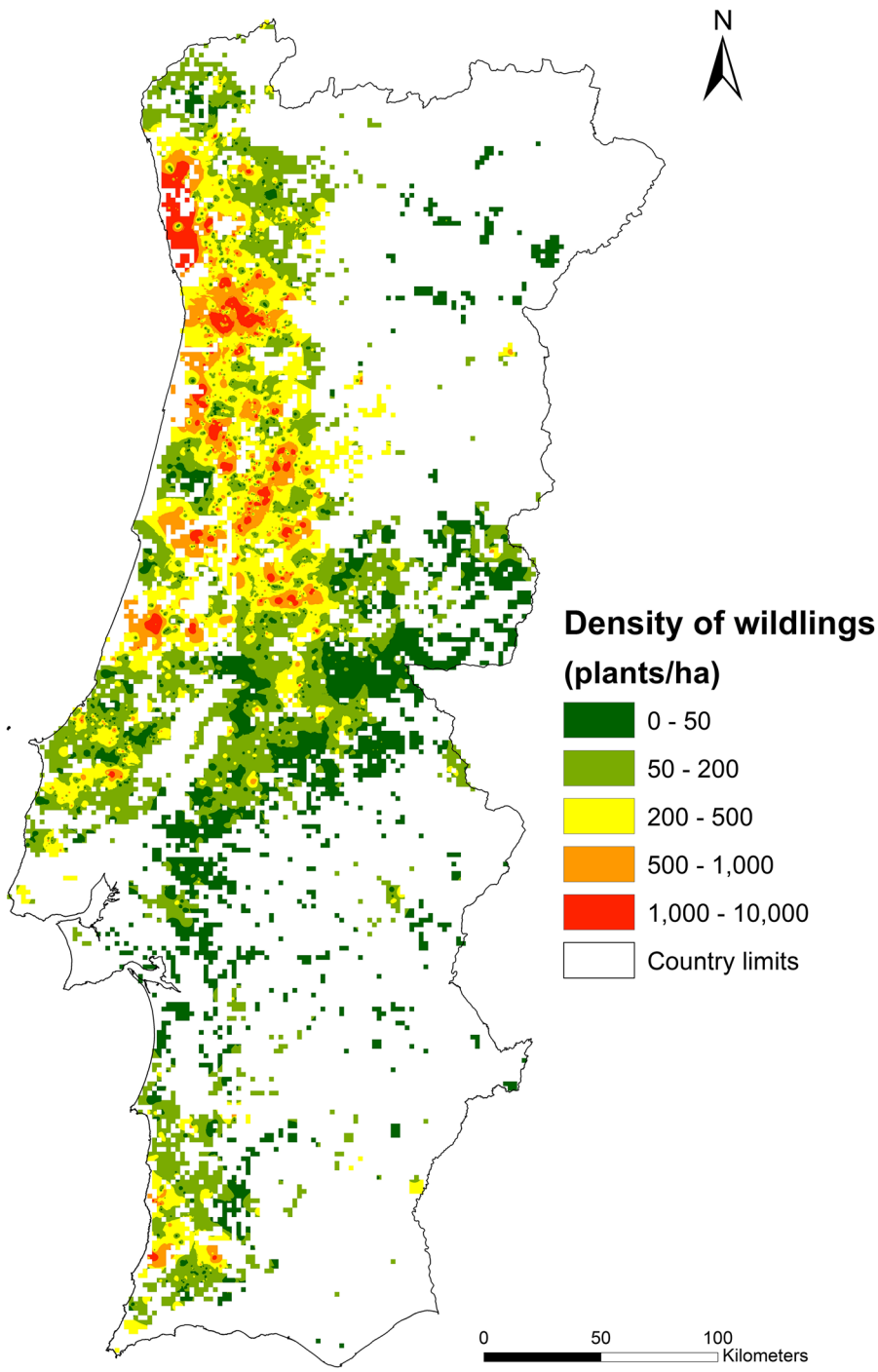

Fig. 2 Density of eucalypt wildlings in continental Portugal. Map resulting from the spatial interpolation (IDW) of wildling density in areas with major eucalypt forest plantations (grid resolution of 2 × 2 km where only the cells with at least 10 ha of eucalypt plantations are represented; this map was based on roadside transects only, thus the interpolation and spatialization of wildling densities beyond roadsides is a simplification which aims showing relative plant density at the country-level)

Spatial patterns and environmental drivers

The results from the spatial interpolation of eucalypt wildlings density in continental Portugal for the areas with major eucalypt forest plantations are shown in Fig. 2. It is possible to see that the areas with higher density of eucalypt wildlings are located mainly in the north and central coast regions.

Spatial autocorrelation in wildling abundance was present, with maximum values for nearby plots (up to

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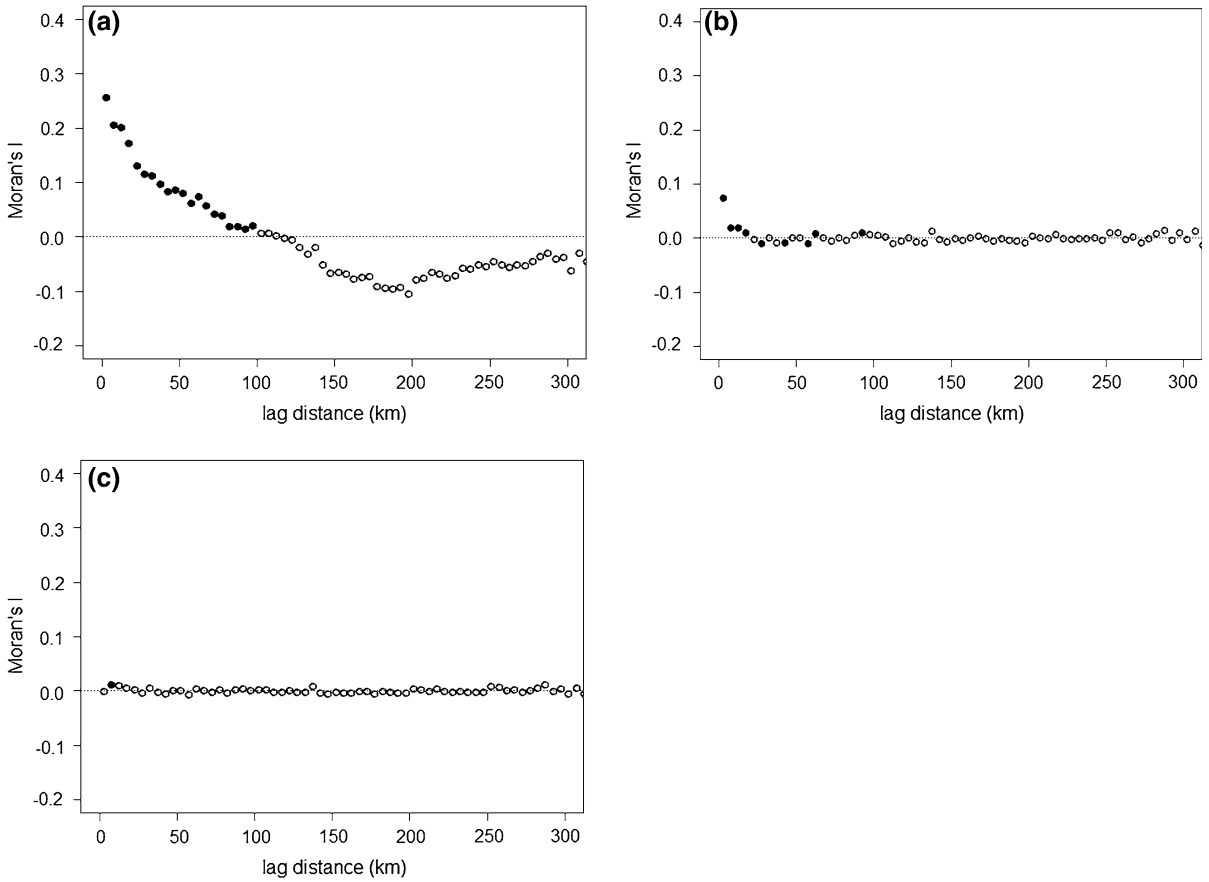

Fig. 3 Moran's I spatial correlograms of: **a** wildling abundance, **b** BRT model without Residual Autocovariate (RAC), **c** BRT model with RAC

10–15 km) and a declining correlation value (but still statistically significant) up to almost 100 km away (Fig. 3a).

The initial Boosted Regression Tree (BRT) model (without autocovariate) explained 25.6 % of the deviance using training data and 13–19 % using cross-validated data. The most important variables determining wildling abundance were precipitation (47 %) and distance from sea (25 %), though elevation, slope, frost, soil type, temperature and aspect also had some importance (Table 2; Fig. A3 in appendix of supplementary material). There was also one important interaction between predictor variables, with the effect of precipitation being more important closer to the sea (Fig. A4a in appendix of supplementary material).

In the initial BRT model environmental variables explained most of the spatial autocorrelation in wildling abundance, but significant autocorrelation was still present in the model residuals, mainly at shorter distances (Fig. 3b). Therefore, the model was re-run but adding the Residual Autocovariate (RAC). This new variable improved model fit (27.7 % of the deviance using training data and 17–22 % using cross-validated data) and ranked second in importance (Table 2), at the expense of the relative importance of all other variables. In any case, the order of importance for the remaining variables did not change. Two variables of marginal importance (temperature and aspect) were dropped from the model. One important interaction occurred between predictor variables, with the effect of the autocovariate being more important as transects were located closer to the sea (Fig. A4b in appendix of supplementary material). Spatial autocorrelation in this model residuals was negligible, with only one significant value (Fig. 3c) that was not considered biologically significant given the large sample size (Koenig 1999).

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Table 2 Relative importance (%) of explanatory variables used to model wildling abundance across Portugal, for BRT models without and with residual autocovariate (RAC). Explained deviance and correlation (with 95 % confidence intervals for cross-validated data) are also shown

Variable	Model without RAC	Model with RAC
Precipitation	46.6	38.9
Distance from sea	25.1	19.7
Elevation	10.0	5.9
Slope	6.0	4.8
Frost days	4.9	3.3
Soil type	3.3	2.1
Temperature	2.1	–
Aspect	2.0	–
RAC	–	25.0
Explained deviance (%)	25.6 (13.4–19.2)	27.7 (16.5–21.8)
Correlation	0.52 (0.38–0.43)	0.53 (0.41–0.47)

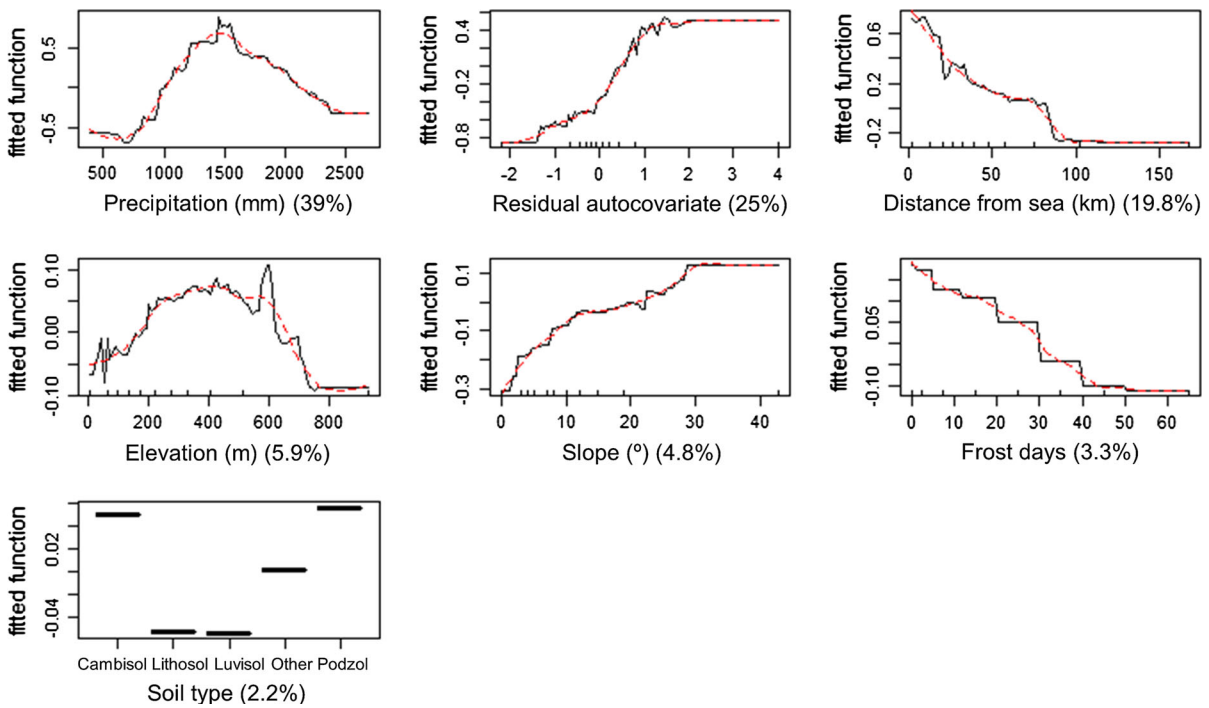

Fig. 4 Partial dependence plots for the variables influencing eucalypt wildling abundance in Portugal. A smoothed version of the fitted function is also shown (dashed line). The relative importance of the variables is shown as %. Ticks across the bottom of each plot show the distribution of deciles for each predictor variable

Fitted functions of the final model (Fig. 4) showed that the most important environmental variables affecting wildling abundance were precipitation and distance from sea (c. 39 and 20 %, respectively). Maximum wildling abundance peaked in areas with precipitation of 1300–1700 mm, with densities decreasing if precipitation was higher or lower than this range. Wildling density decreased monotonically with increasing distance from sea. Less important variables affecting wildling abundance included elevation (6 %), with higher densities occurring at 200–600 m, slope (5 %), with higher densities occurring at higher slopes, the number of frost days (3 %), with a negative influence on densities, and soil type (2 %), with lower densities registered in Lithosols and Luvisols.

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Discussion

Eucalypt wildlings distribution and abundance

The existence of a widespread natural establishment of *E. globulus* plants in Portugal has been recently suggested by Águas et al. (2014) whose results showed that seed regeneration was present in more than 90 % of sampled plots inside burned eucalypt stands in central and northern Portugal. In the present work we found wildlings in nearly 60 % of the 3111 transects assessed and in all natural regions of continental Portugal, suggesting that *E. globulus* is likely on the way to becoming naturalized (sensu Richardson et al. 2000) in a wide range of ecological conditions in Portugal, and that the species is able to colonize areas adjacent to plantations in a consistent manner.

The average density of naturally established plants found in our study (277 plants ha^{-1}) is much higher than the value found by Larcombe et al. (2013) in a similar study along plantation edges in Australia (8 plants ha^{-1}); indeed, this difference could be even higher since the transects surveyed by these authors also included the inside of plantations (10 m each side of the plantation edge), where they found a significantly higher density of wildlings compared to the outside. The eucalypt wildlings detected during our survey had in most cases more than 30–40 cm (smaller seedlings were hardly detected from the road). If this necessarily means an underestimation of real plant densities, it has the great advantage of ensuring that the observed densities correspond to plants that survived the initial stages of development and have a much lower probability of dying (Calviño-Cancela and Rubido-Bará 2013).

Another important result from our study was the finding of considerable regional differences in terms of wildling density, with a higher likelihood of eucalypt wildling occurrence in certain regions, particularly in coastal areas of central and northern Portugal.

Environmental factors influencing wildling abundance

Mean annual precipitation was clearly the most important factor affecting the abundance of eucalypt wildlings in continental Portugal. Model results show that wildling density was the lowest below c. 600–700 mm of annual precipitation and then it sharply increased up to around 1500 mm. However, precipitation levels above 1500 mm had a detrimental effect on wildling density. In its native range, mean annual precipitation in the areas where *E. globulus* occurs, ranges between 500–600 and 1250–1500 mm (Boland et al. 2006; Jacobs 1979; Kirkpatrick 1975). Our results showing a reduced wildling density in areas of lower precipitation are in accordance with the lower precipitation limit in the regions where *E. globulus* is native. In Mediterranean climates, a low annual precipitation associated with a pronounced summer drought is expected to be particularly difficult for young plant establishment. Indeed, summer drought is known to affect eucalypt regeneration by decreasing seed germination and causing high seedling mortality (e.g. Stoneman 1994).

On the other hand, our results showed a clear decrease in eucalypt wildling density in areas with precipitation above 1500 mm. Larcombe et al. (2013) found a positive relationship between eucalypt wildling establishment in Australia and mean annual precipitation up to 1400 mm, which is consistent with our results; however, in their study they did not analyse areas with precipitation above 1400 mm. Information about how *E. globulus* seed regeneration performs in areas with high precipitation is scarce. However, the species seems to be absent in areas of higher precipitation, e.g. in most western Tasmania (Williams and Potts 1996). According to Jacobs (1979) *E. globulus* does not perform well in excessively humid areas; he reports for example that it has been dropped from cultivation in the montane zone of Sri Lanka with annual rainfall above 2000 mm. Some years earlier Kirkpatrick (1975) wrote that too much water may limit the occurrence of *E. globulus*, as well as too little water. There are different possible explanations for the detrimental effect of high precipitation levels on wildling establishment. One may be the negative effect of water-saturated soil on the physiological processes of roots, as it occurs with other species (Gomes and Kozlowski 1980). Higher precipitation levels may also favour other competing vegetation, namely herbaceous plants, and reduce natural establishment as eucalypts seem to be very sensitive to competition (Kirkpatrick 1977). On the other hand, fungal diseases, which are often widespread in more humid environments (higher soil moisture and leaf wetness), are other main causes of damage and mortality of seeds and seedlings (Keane

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et al. 2000). Facelli et al. (1999) studied how leaf litter, water and fungal pathogens affect the establishment and survivorship of another eucalypt species (*E. obliqua*) in Mediterranean-type ecosystems of SE Australia, and found that litter in combination with excessive water applications reduced seedling emergence and increased seedling mortality. These authors also found that these effects were promoted by the presence of pathogenic fungi, as the addition of fungicide reverted such effects. Additionally, juvenile plants may be particularly vulnerable to fungal diseases. For example, the genus *Mycosphaerella* contains many pathogens capable of causing a severe impact on susceptible eucalypt species (Milgate et al. 2001), and nowadays, Mycosphaerella leaf disease (MLD) is one of the most important fungal diseases of eucalypts worldwide, including *E. globulus* plantations in Portugal (Silva et al. 2008) and Spain (Sánchez Márquez et al. 2011). In several eucalypt species, MLD incidence and severity is much greater in juvenile than in adult leaves (Hunter et al. 2004; Pinkard and Mohammed 2006). Indeed, a recent study in Galicia (NW Spain, near north Portugal), confirmed that juvenile leaves of *E. globulus* are not only more susceptible to MDL, but also to a wide range of fungi (Sánchez Márquez et al. 2011) which can cause much damage and seedling mortality (Keane et al. 2000).

Distance from the sea was the second most important variable, presenting a significantly negative influence on wildling abundance. This variable was used as a surrogate of continentality, i.e. thermal range. Usually, as distance from the ocean increases, summer temperatures are higher and winter temperatures are lower (coinciding in Mediterranean climates with summer drought and winter rain, respectively), thus it would be expectable that these conditions were less favourable for seedling establishment. Based on these results, we hypothesize that in Portugal, the temperature seasonality is more important than mean annual temperature, which would explain the relatively large importance of the variable distance from sea and the marginal importance of mean annual temperature. However, results from a previous study in Australia, which also found a negative relationship between wildling establishment and both mean annual temperature and temperature seasonality, suggested that the former plays a more important role (Larcombe et al. 2013).

Topography, frost and soil type also played a significant role in wildling abundance, though their importance was considerably lower when compared to the previous variables. There was a greater abundance of wildlings at altitudes around 200–600 m, when compared to locations at lower or higher elevations, though the major decrease occurred at altitudes above 700 m. In its native range *E. globulus* has been reported to occur from the sea level up to 330 m (Jacobs 1979) or up to 540 m of altitude (Boland et al. 2006), though in other regions where it is planted it can be found at much higher altitudes (up to 3400 m in tropical regions; Jacobs 1979). Kirkpatrick (1975) stated that low temperatures may set the upper altitudinal limit for the species in its natural distribution range. Altitude likely acted as a surrogate of local edapho-climatic conditions. We hypothesize that in continental Portugal the lower temperatures and increasing frost (and often poorer soils) could be the main factors limiting wildling establishment at higher altitudes. Terrain slope was also related to wildling abundance, with increasing plant densities for increasing slope up to about 30°. This was contrary to our initial expectations as steeper slopes often have a thinner soil layer that could reduce wildling establishment; however, this factor could have also caused reduced vegetation competition or waterlogging, favouring eucalypt establishment. Another possible reason could be the better visibility of wildlings in sloping roadsides.

Wildling abundance also decreased consistently with increasing number of frost days. Frost occurrence is known for its negative effects on *E. globulus* and it has been reported to be particularly damaging to seedlings and juvenile foliage (Jacobs 1979; Kirkpatrick 1975). For example Kirkpatrick (1975) observed 74 % of damage in leafs and buds of unhardened *E. globulus* seedlings at −5 °C. In its native range, *E. globulus* occurs in sites where frost days are less than 50 per year (Boland et al. 2006), which is consistent with our results.

Another factor affecting eucalypt wildling abundance was soil type, with lower densities registered in Lithosols and Luvisols when compared to Cambisols, Podzols and other soil types (other soils representing only 1.3 % of the transects). Lithosols (included in Leptosols) are thin soils consisting mainly of partially weathered rock fragments, thus with limited potential for tree development (FAO 2001). On the other hand Luvisols are soils in which clay is washed down from the surface soil to an accumulation horizon at some

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depth; the wide range of parent (unconsolidated) materials and environmental conditions led to a great diversity of soils in this soil group (FAO 2001), being difficult to associate it to lower plant establishment. Nevertheless, the existing literature reports that soil is less important than climate in limiting E. globulus development (Jacobs 1979), which is in agreement with our results showing the low importance of this variable.

Survey limitations

The adopted methodology, based on car and remote sensing (GSV) surveys, has the clear advantage of enabling a quick and extensive assessment of wildling establishment in large regions when logistic resources for more detailed and rigorous assessments are scarce. However, this methodology also presents some limitations. First, the generalization of the obtained results to the whole universe of eucalypt plantations may present some drawbacks, as roadsides constitute a particular case of stand edges. In fact there are characteristics which may favour the establishment of new recruits, while others may have an opposite effect. For example roadsides are often associated with a higher availability of light, water and nutrients (Gelbard and Belnap 2003), which may favour wildling establishment (Florence 1996). The preference of invasive exotic species for disturbed sites like roadsides has been referred by several authors (Abella et al. 2009; Milton and Dean 1998; Wilson et al. 1992). On the other hand, the fact that many roadsides are managed using mowing equipment, may counteract the higher suitability of these places for wildling establishment. Also, in the case of flat roadsides, soil may be compacted and vegetation may be destroyed by vehicles, also contributing to lower wildling establishment, when compared to other plantation edges.

Another set of factors potentially responsible for the observed variability is related with the detection methodology. The results obtained with accuracy tests on car surveys suggest that there was an underestimation of wildling density (of about one-third) compared to the real density, and that there was some bias in the counts. Such underestimation of plant density was apparently higher in transects with more wildlings (areas with a higher establishment potential) and in larger transects (wider roadside). This is not surprising given the existence of visual obstacles (plants, stones, etc.) and the fact that the observer had a limited amount of time to perform the counts. We also found that an important cause of density underestimation during accuracy tests was the presence of a large number of small seedlings, which could not be detected from the road. We tried to minimise these constraints by using ordered abundance categories, rather than original values, which has the disadvantage of creating a less accurate estimate of densities.

Besides these factors, the characteristics of the surveyed stands are certainly another important aspect that would have been advantageous to take into account. Propagule pressure (Rouget and Richardson 2003) in particular, may have played an important role in the observed spatial patterns. Although all transects were located adjacent to eucalyp stands (so distance to potential invasion foci was quite similar), we did not have information on the age structure and density of adjacent and surrounding stands, a key aspect that will influence the potential seed rain (Rouget and Richardson 2003; Rejmánek et al. 2005; Larcombe et al. 2013). Other local unassessed variables, such as roadside vegetation cover and structure (other than eucalypts), may have influenced the observed variability, masking the effect of variables which were included in the present study.

Finally, the surveys were not all performed at the same time, which could also have some influence on the number of wildlings detected, because both the number of plants (emergence and survival) and their visibility (height and colour of surrounding annual plants) may vary among seasons. However, these potential problems should be relatively minor in our study, as in most cases we did not detect small seedlings (with much higher survival fluctuations), and because of the conspicuous nature of eucalypt wildlings (bluish colour). Ideally, monitoring should be concentrated in the same season, although this is difficult to attain when surveying very large areas.

Management considerations

E. globulus plantations in Portugal represent several benefits. These include mainly direct economic benefits for both the industry and the population (which owns much of the land), through timber production (essentially for pulp and paper companies, but also for construction, energy, etc.); additionally, they can also represent other benefits like employment and carbon

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sequestration. On the other hand, exotic plantations and the wildlings they originate may also cause several negative impacts, particularly on the environment (by altering native biodiversity, soils, hydrology, fire regimes, etc.; e.g. Calviño-Cancela et al. 2012; FAO 1985; Farley et al. 2005; van Wilgen and Richardson 2014), and on land management. However, contrarily to the economic benefits which are in general much easier to assess, most of the environmental impacts are complex and challenging to quantify monetarily (often also take more time to be noticed and are hardly perceived by most people); thus, calculating the net value of the species is a difficult task. Additionally, the knowledge about the extension of natural regeneration from seeds is still almost inexistent. The existing natural regeneration may increase conflicts of interest, and the net value of the species may change depending mainly on the "invasion risk", and whether potential future negative impacts outweigh the economic benefits (van Wilgen and Richardson 2014).

Considering the results from this study, showing that *E. globulus* wildlings are currently able to establish in a wide range of ecological conditions, it is somewhat surprising that reports about invasion by this species remain so restricted. Some of the most important factors limiting the invasive potential of alien trees include generation time, seed production, seedling survival and dispersal ability (e.g. Gordon et al. 2012; Rejmánek and Richardson 1996). The first three factors do not appear to be determinant in preventing *E. globulus* spread (Calviño-Cancela and Rubido-Bará 2013; Potts et al. 2008; current study). The onset of sexual reproduction in *E. globulus* plantations first occurs at 3–4 years of age (Potts et al. 2008). Seed production and seedling survival can be very variable, but one recent study in NW Spain estimated that despite relatively low establishment success ratio of *E. globulus* seedlings (due to high mortality during the first months), high seed production leads to considerable seedling densities (up to 20,000 plants ha^{-1} $year^{-1}$) in habitats surrounding plantations (Calviño-Cancela and Rubido-Bará 2013). Additionally, herbivory by native species (e.g. deer) seems to be non-existent in the Iberian Peninsula (Calviño-Cancela and Rubido-Bará 2013; Catry et al. 2010), though it can occur in other regions (Becerra and Bustamante 2008). Thus, it appears that the main constraint for the spread of *E. globulus* over large areas outside plantation sites is probably their limited seed dispersal. Our study did not aim to assess dispersal distances. Nevertheless, previous studies reported maximum dispersal distances from plantations of 80–175 m, though in most cases (c. 98 %) seeds or seedlings were only found in the first 10–15 m (Calviño-Cancela and Rubido-Bará 2013; Larcombe et al. 2013). Other publications also report the limited dispersal ability of eucalypts (e.g. Booth 2012; Rejmánek and Richardson 2011), suggesting that this could be the main barrier limiting the spread of *E. globulus*.

Invasion of habitats outside plantations is often the main concern for managers, and in the case of *E. globulus*, the current knowledge suggest that it will likely be relatively slow when compared to species with long-distance seed dispersal (e.g. pines). On the other hand, potential problems are not limited to invasion of habitats outside plantations. The establishment of eucalypt plants within forest plantations may also present serious management problems, mainly because of their very fast growth and resprouting ability after cutting. Eucalypt natural regeneration may increase competition with previously installed trees, hinder accessibility, and increase fire hazard. These problems may occur in pure eucalypt stands, which are usually more intensively managed, but are likely to be particularly problematic in the less managed mixed forests. In Portugal in 2006, eucalypts were present in 180,000 ha of mixed stands (usually with native pines or oaks), from which 87,000 ha were dominated by other species (AFN 2010). In these mixed forests, and in addition to the above mentioned problems, there is a real possibility of eucalypts becoming dominant or even eliminating the native species, particularly in mismanaged areas and after wildfire disturbance (e.g. Catry pers. obs., Fig. A5). Indeed, *E. globulus* is known to regenerate very well after wildfires, both vegetatively and from seed (Águas et al. 2014; Catry et al. 2013), and its faster growth constitutes a competing advantage over native species (e.g. Catry et al. 2006). Additionally, wildling establishment is not restricted to highly disturbed areas; a study in NW Spain showed that *E. globulus* is also able to spread from plantations into shrublands and pine or oak forests in the absence of fire (Calviño-Cancela and Rubido-Bará 2013). Taking these reports into account, along with the wide climatic tolerance found in the present study, we can hypothesize that *E. globulus* natural establishment is probably quite common and not limited just to roadsides.

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Because invasive alien species can have high ecological and economic costs (e.g. Pimentel et al. 2005), it is crucial to monitor (e.g. using remote sensing tools; Visser et al. 2014) and control their spread. This should definitely include trees species with limited seed dispersal capabilities, such as eucalypts. For example in Spain, Andreu et al. (2009) reported that more than half of the economic costs of alien plants management (mainly related to control and eradication) were attributed to *Eucalyptus* spp. (in Spain only *E. globulus* and *E. camaldulensis* are invasive; Sanz-Elorza et al. 2004).

Considering the apparently limited dispersal ability of *E. globulus*, the implementation of relatively simple management practices, such as maintaining clear firebreaks surrounding the stands and avoiding planting trees near drainage lines or stepper slopes, would likely greatly reduce the chances of eucalypts to invade habitats outside plantations. However, it may be very difficult to implement such practices in many situations (e.g. in highly fragmented landscapes of small and often mismanaged properties, as it is the case in central and northern Portugal). Moreover, the establishment of eucalypt seedlings within mixed forests is much more difficult to avoid. Apparently, the only way of eliminating the risk of spread both outside and inside forest stands, would be by producing and planting sterile trees, which appears to be feasible, namely in *Eucalyptus* spp. (Zhang et al. 2012). Indeed, Zhang et al. (2012) showed that it is possible to prevent pollen formation in different transgenic plants, including eucalypts, without detrimental effects on vegetative growth.

Conclusions

In the present study we showed the existence of a widespread natural establishment of *E. globulus* plants on roadsides adjacent to plantations in continental Portugal. Therefore, and despite the particular characteristics of the survey method, our results, together with those from Águas et al. (2014), suggest that *E. globulus* is able to naturally regenerate from seeds both inside and outside plantations in a wide range of environmental conditions in Portugal.

We produced a map showing the regions with higher wildling density in continental Portugal, and found evidence of significant relationships between the abundance of wildlings and several environmental variables. The abundance of wildlings was found to be higher in coastal areas of central and northern Portugal, and it was primarily affected by precipitation and distance from the sea (used as a surrogate of thermal amplitude), although topography, frost occurrence and soil type also played a significant role. Knowing the regions with higher wildling density and understanding the factors influencing plant establishment may help managers to establish and prioritize eventual control plans in regions with higher probability of recruitment. This may be useful for forest managers and for entities responsible for managing protected areas, roadsides or the space associated with other infrastructures like power lines or firebreaks (inside or near plantations).

Current results set the baseline of the current situation, but monitoring of wildling establishment should be a permanent task. The active selection of eucalypt genotypes with desirable characteristics (e.g. resistance to frost and drought), along with changing environmental conditions (e.g. climate change) also means that the establishment potential may well change over time and space (Gordon et al. 2012; Richardson and Rejmánek 2011). The high ability of *E. globulus* for dealing with fire should also be a concern in ecosystems where wildfires are a recurrent disturbance. Thus, and considering the potentially high management and ecological costs of eucalypt natural establishment, vigilant control of even apparently slow spread from cultivation sites should be assured. Future research on further assessing the seed dispersal distances and seedling survival in different habitats and ecological conditions is also a key-factor to increase current knowledge and generalization ability, and to sustain informed management recommendations.

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