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Ecophysiological Characterization of Metallophyte Species Candidates for Phytoremediation

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Abstract

The largest metalliferous area in the Iberian Peninsula, known as Iberian Pyrite Belt, is now an area with several abandoned mines whose intense mining activity has resulted in a degraded environment with serious pollution problems, such as dust, acid mine drainage, tailings, soil heaps and others. Although these sites exhibit an inhospitable environment for most species, there are plant species that tolerate high contaminations of toxic metals and the low soil pH. These so called metallophyte species have physiological adaptations that allow them to survive, establish and reproduce in metalliferous environments. Moreover, most of these species contribute with phytoremediation processes and thus decreasing the bioavailability of toxic metals. For these reasons, ecological restoration of these sites should take in account the use of the already present vegetation. Furthermore, with an increase of the aridity in Portuguese Iberian Pyrite Belt, it's essential to increase the resilience of these sites and this can be achieved by considering the United Nation's *Sustainable Development Goal* 15 – Mining and Life on Land.

The aim of this work was to determine the ecological niche and the physiological performance of eight plant species present in *São Domingos* mine: *Erica australis*, *Erica andevalensis*, *Lavandula stoechas*, *Cistus ladanifer*, *Cistus salviifolius*, *Cistus monspeliensis*, *Agrostis castellana* and *Anarrhinum bellidifolium*. To accomplish this, two types of experiments were held: greenhouse and in-situ tests; using the pH as a proxy for contamination. Greenhouse tests focused of germination and further following of physiological parameters (height and reflectance) on four contamination level's substrates: commercial, mixed, contaminated 1 (from *São Domingos* mine) and contaminated 2 (from *Santa Bárbara de Padrões*). In-situ characterization was achieved by using line-intercept transects in four areas to register the cover, height and reflectance. In addition, the soils from the greenhouse tests and the in-situ points where characterized in pH, organic matter, electrical conductivity and granulometry. Generally, the results indicated a preference for the mixed substrate (amended with commercial substrate) and a general physiological adaptation to the toxic metals contamination and low pH. *E. andevalensis* was the species that withstand lowest values such as pH≈2.5. Furthermore, as these species are already adapted to the extreme characteristics of the mining area, this work concluded that the competition was a stronger driver than the pH, especially for the *Erica* sp. and *Cistus* sp. Therefore, this project has contributed to increase knowledge about the ecophysiological performance of the species that inhabit metalliferous areas, to support future ecological restoration projects of the polluted zones degraded by the mining industry.

Keywords: Abandoned mines; Contaminated soils; Iberian Pyrite Belt; Performance; Plant cover.

Resumo Detalhado

A mais extensa área metalífera da Península Ibérica, de nome Faixa Piritosa Ibérica (FPI), abrange parte de Portugal e Espanha. Atualmente, na zona portuguesa da FPI, existem apenas duas minas ativas: Aljustrel e Neves Corvo. Esta última encontra-se localizada na povoação de Santa Bárbara de Padrões. Até há poucas décadas, a atividade mineira desta formação vulcano-sedimentar era bastante intensa, no entanto tem vindo a diminuir. Como resultado deste decréscimo de atividade, existem hoje diversas minas abandonadas, como é o caso da mina de São Domingos. Além disso, uma outra consequência da atividade intensa diz respeito à degradação ambiental, causada acima de tudo por elevados níveis de poluição, nomeadamente poeiras, águas ácidas, erosão, entre outros. Estes locais apresentam um ambiente inóspito para a maioria das espécies. Existem, no entanto, algumas espécies - vegetais e não só - que conseguem tolerar as contaminações elevadas de metais tóxicos e pH ácido do solo. Estes organismos possuem adaptações fisiológicas que lhes garantem não apenas a sobrevivência, mas também a reprodução em ambientes metalíferos, sendo designadas, em face disso, de espécies metalófitas. Uma vantagem adicional da maior parte destas espécies passa pela contribuição para uma diminuição da biodisponibilidade de metais tóxicos através de processos de fitorremediação. Desta forma, para o sucesso de projetos de restauro ecológico que se venham a realizar, é essencial que os mesmos tenham em consideração a existência das espécies vegetais presentes nesse local. Um projeto de restauro ecológico que integre as espécies metalófitas vegetais provenientes do mesmo local terá fortes probabilidades de alcançar o Objetivo de Desenvolvimento Sustentável 15 das Nações Unidas – Mineração e Proteção à Vida Terrestre.

Este trabalho teve por base dois objetivos, ambos relacionados com oito espécies vegetais presentes na zona da mina de S. Domingos. O primeiro foi avaliar o desempenho fisiológico dessas espécies e o segundo foi avaliar o nicho ecológico das mesmas. As espécies em questão foram: *Agrostis castellana*, *Anarrhinum bellidifolium*, *Cistus ladanifer*, *Cistus salviifolius*, *Cistus monspeliensis*, *Erica andevalensis*, *Erica australis* and *Lavandula stoechas*. Como tal, foram realizados dois tipos de testes: um em estufa e outro no local. Ambos os testes foram realizados utilizando o pH como indicador de contaminação. Isto porque, os minérios descobertos, em contacto com a água e o ar, são submetidos a um processo de hidrólise. A hidrólise é um processo químico que causa a diminuição do pH, pelo que podemos inferir que o aumento da concentração de metais tóxicos está correlacionado com um pH mais acído. Os testes de estufa consistiram na germinação de sementes das oito espécies escolhidas, sementes essas que foram submetidas a dois pré-tratamentos de calor em quatro substratos de diferentes graus de contaminação, designadamente: comercial, mistura (combinação comercial:contaminado 1 50%:50%), contaminado 1 e contaminado 2. Os substratos contaminados utilizados, designados *contaminado 1* e *contaminado 2* são originários das minas de S. Domingos e Santa Bárbara de Padrões, respectivamente. A mina de São Domingos encontra-se atualmente abandonada, situação que se verifica desde 1966. Por sua vez, Santa Bárbara de Padrões é, presentemente, uma povoação afetada pelas poeiras derivadas da mina ativa Neves Corvo, que ainda se encontra em atividade. O solo proveniente desta povoação tem uma contaminação bastante inferior ao da mina de S. Domingos. Apesar de pertencerem a formações litológicas diferentes, estas duas minas encontram-se na zona da Faixa Piritosa Ibérica. Como tal, crê-se que esta razão nos permite justificar o uso de ambos os solos para avaliar se as espécies em causa poderão ser utilizadas em programas de restauro de outras minas, também localizadas na mesma zona.

Em cada tratamento, foi escolhido apenas um indivíduo sobrevivente, que foi seguido fisiologicamente através de dois parâmetros – altura e refletância das folhas. Para o efeito, foram usados três índices, tais como: *Normalized Difference Vegetation Index* (NDVI), *Photochemical Reflectance Index* (PRI) e *Chlorophyll* (CHL). Os testes levados a cabo no local consistiram em três transectos, realizados em quatro áreas de amostragem ao longo da mina de S. Domingos. As espécies de interesse que foram intercetadas nestes transectos, posicionados ao longo de um gradiente de pH, tiveram

diferentes medidas fisiológicas tais como: altura, cobertura e refletância das folhas. Para além disso, os substratos dos testes da estufa, assim como os solos recolhidos nos transectos, foram caracterizados a nível de pH, matéria orgânica, granulometria e condutividade elétrica.

No geral, observou-se que as espécies estudadas tendem a preferir solos acídicos, ainda que com um valor de pH superior ao encontrado na mina de S. Domingos (substrato mistura). As espécies não demonstraram alterações fisiológicas significativas entre diferentes pHs, o que poderá indicar que, como as espécies já estão adaptadas às pressões existentes, a variação destas não afeta a *performance* fisiológica. No entanto, o mesmo não se aplica ao desempenho ecológico. Foi constatado que, neste aspeto, a *performance* ecológica de algumas espécies foi afetada, especificamente, em relação à restrição do nicho. Em particular, a espécie *E. andevalensis* foi a que demonstrou maior adaptação ao pH mais ácido. Contudo, isto verificou-se apenas para o caso dos substratos provenientes da mina de S. Domingos, dado que as plantas não germinaram nos substratos comercial e contaminado 2, proveniente de Santa Bárbara de Padrões. Adicionalmente, esta foi a única espécie a conseguir estabelecer-se no substrato contaminado 1. Ecologicamente, foi observado que esta espécie tende a estar presente nas margens dos corpos de água com origem em Drenagem Ácida de Minas (DAM), cujo pH rondou o valor 2.5, ou junto a zonas de escorrência, sendo possível constatar que esta restrição do seu nicho poderá ser provocado devido à presença de outras espécies como, por exemplo, a *C. ladanifer*. No que diz respeito à *C. monspeliensis* e à *E. australis*, estas foram as espécies com menor número de indivíduos estabelecidos. Contudo, a espécie *C. monspeliensis* foi a que demonstrou menor viabilidade de germinação. Não foi possível encontrar uma razão específica para este resultado. Haverá várias hipóteses que o possam justificar, entre elas, a amostragem precoce das sementes que poderá ter inviabilizado a maturação destas. Localmente, *C. ladanifer* foi a espécie dominante da área da mina. No entanto apenas se conseguiu estabelecer nos substratos comercial e mistura, neste último substrato com um indivíduo com valor superior de NDVI. Em relação a *L. stoechas* e *A. bellidifolium*, estas duas espécies estabeleceram-se com sucesso nos substratos comercial e mistura e, no geral, mantiveram o desempenho fisiológico estável. Localmente, *A. bellidifolium* foi a espécie com menor cobertura. A espécie *A. castellana* foi a que demonstrou maior plasticidade de habitat, uma vez que foi a única a conseguir estabelecer-se no substrato *contaminado 2*, com valores superiores nos três índices para as plantas sobreviventes. Esta espécie da família Poaceae é, das oito espécies estudadas, a candidata com maior potencial para eventuais ações de cobertura vegetativa a efetuar noutras áreas mineiras, para além da mina de S. Domingos.

Adicionalmente, com base nestes testes, foi possível verificar que a competição entre espécies é um fator mais forte e determinante do que a contaminação, dado que as espécies encontradas localmente já se encontram adaptadas ao grau de contaminação existente. Devido a tudo o que foi exposto acima, este projeto contribuiu para o aumento do conhecimento sobre os desempenhos ecológico e fisiológico das oito espécies escolhidas, que habitam esta área metalífera e que poderão ser utilizadas em futuros projetos de restauro ecológico na mina de S. Domingos.

Palavras-chave: Cobertura vegetal; Desempenho; Faixa Piritosa Ibérica; Minas abandonadas; Solo contaminado.

Table of Contents

Acknowledgements ii
Abstract iii
Resumo Detalhado iv
Table of Contents vi
Table index vii
Figure index viii
Abbreviations list x
1. Introduction 1
2. Methods 6
2.1 Sampling sites 6
2.2 Greenhouse studies 6
2.2.1 Soil Sampling for Germination Tests 6
2.2.2 Species selection 7
2.2.3 Seed collection and preparation 7
2.2.4 Germination 8
2.2.4.1 Potential Seed Germination 8
2.2.4.2 Seed Germination in Soils 9
2.2.5 Plant development 10
2.3 Ecological performance in contaminated areas 11
2.3.1 Soil sampling 12
2.3.2 Vegetation Abundance 12
2.3.3 Physiological Measurements 12
2.4 Soil Characterization 13
2.4.1 Organic matter 13
2.4.2 pH and Conductivity 13
2.4.3 Granulometry 13
2.5 Data Analysis 14
3. Results 15
3.1 Greenhouse Tests 15
3.1.1 Substrate properties 15
3.1.2 Seed germination 15
3.1.3 Physiological performance 18
3.2 Ecophysiology of local populations 22
3.2.1 Soils Properties 22
3.2.2 Ecological performance 24
3.2.3 Physiological performance 28
4. Discussion 31
References 36
Appendix I – Supplementary Tables I
Appendix II – Supplementary Figures VII

Table index

Table 2.1 – Soil elements characterization in *Santa Bárbara de Padrões* . 7

Table 2.2 – Pre-treatment applied to the seeds 8

Table 2.3 – Seed pre-treatment protocol used for both tests of germination in soil 9

Table 3.1 – Greenhouse substrate characterization for texture, organic matter (OM), pH and bulk electrical conductivity (EC_b). 15

Table 3.2 – Final germination percentage (FGP %) in the greenhouse tests. 17

Table 3.3 – Mean T50 germination in the greenhouse tests. 17

Table 3.4 – Spearman's rank correlation between variables of the *S. Domingos* soil (n=738). 22

Table 3.5 – Spearman's rank correlation of the specific cover of the species of interest (n=38). 27

Table S1.1 – Description of the selected species. I

Table S1.2 – Potential germination percentage (FGP %). II

Table S1.3 – Potential mean T50 germination (days). II

Table S1.4 – Species presence in sampling area. III

Table S1.5 – Textural class of initial (P_i) and final (P_f) sampling points of each transect of each area (n=3). IV

Table S1.6 – Spearman's rank correlation between *Cistus ladanifer* ecological variables (Cover and Crown Area, n=108) in green, physiological variables (NDVI, CHL, PRI, SLA, n=9; Height, n=108) in orange and soil variables (pH, OM and EC_b, n=108) in red. V

Table S1.7 – Spearman's rank correlation between *Cistus monspeliensis* ecological variables (Cover and Crown Area, n=23) in green, physiological variables (NDVI, CHL, PRI, SLA, n=12; Height, n=23) in orange and soil variables (pH, OM and EC_b, n=23) in red. V

Table S1.8 – Spearman's rank correlation between *Erica andevalensis* ecological variables (Cover and Crown Area, n=33) in green, physiological variables (NDVI, CHL, PRI, SLA, n=12; Height, n=33) in orange and soil variables (pH, OM and EC_b, n=33) in red. V

Table S1.9 – Spearman's rank correlation between *Lavandula stoechas* ecological variables (Cover and Crown Area, n=28) in green, physiological variables (NDVI, CHL, PRI, SLA, n=9; Height, n=28) in orange and soil variables (pH, OM and EC_b, n=28) in red. VI

Figure index

Figure 1.1 – Location of the mining sites: *São Domingos* mine (*Mina de São Domingos* village) and *Neves Corvo* (*Santa Bárbara de Padrões* village); and other main ore deposits in Iberian Pyrite Belt. Adapted from Figueiredo et al. (2014). 1

Figure 1.2 – *São Domingos* mining area. 2

Figure 2.1 – Geological map of the South Portuguese Zone (1:1000000). 6

Figure 2.2 – Transects performed in sampling area of *São Domingos* mine. 11

Figure 2.3 – Example of a homogeneous vegetation area. 12

Figure 2.4 – Example of a heterogeneous area. 12

Figure 3.1 – Germinated seeds in different substrates. 16

Figure 3.2 – *Lavandula stoechas* reflectance measurement using a spectroradiometer. 18

Figure 3.3 – Shrub species medium reflectance indices in greenhouse substrates. 19

Figure 3.4 – Herbaceous species medium reflectance indices in greenhouse substrates. 20

Figure 3.5 – Average maximum height of shrub species in different substrates with different ages. 21

Figure 3.6 – Herbaceous species average maximum height in different substrates. 21

Figure 3.7 – pH gradient of the transects performed in the sampling area of *São Domingos* mine. 23

Figure 3.8 – Properties of *S. Domingos* soils of each transect areas. 24

Figure 3.9 – Species in flower found in *São Domingos* mine 25

Figure 3.10 – Total cover percentage of species selected for the study. 25

Figure 3.11 – Average species cover per area (n=3 transects per area). 26

Figure 3.12 – GAM analysis of species' cover and pH 27

Figure 3.13 – Overlapped model distribution of three dominant species cover (%) along the pH gradient, based on GAM analysis. 28

Figure 3.14 – Average shrub species reflectance indices. 29

Figure 3.15 – Average herbaceous species reflectance index. 30

Figure 4.1 – *Erica andevalensis* at the foothill of a mining heap and at the margin of an AMD waterbody. 35

Figure S2.1 – Particle-size distribution of the greenhouse substrates (n=1). VII

Figure S2.2 – Reflectance indices values of *Cistus monspeliensis* and *Erica australis* in greenhouse substrates. VII

Figure S2.3 – Shrub species maximum height in different substrates. VIII

Figure S2.4 – Average particle-size distribution with different fractions. VIII

Figura S2.5 – GAM analyses of species' cover and pH. IX

Figure S2.6 – Average reflectance indices in-situ of *Anarrhinum bellidifolium* (n=3) and *Cistus salviifolius* (n=3). X

Abbreviations list

Abel	*Anarrhinum bellidifolium*
Acas	*Agrostis castellana*
AMD	Acid mine drainage
APA	Agência Portuguesa do Ambiente
CHL	Chlorophyll
Clad	*Cistus ladanifer*
Cmon	*Cistus monspeliensis*
Comm	Commercial substrate
Cont1	*Contaminated 1* substrate
Cont2	*Contaminated 2* substrate
Csal	*Cistus salviifolius*
DW	Dry weight
DW600º	Dry weight after ignition at 600 ºC
Eand	*Erica andevalensis*
Eaus	*Erica australis*
EC_b	Bulk electrical conductivity
EDM	Empresa de Desenvolvimento Mineiro, SA
FGP	Final germination percentage
GAM	Generalized additive model
IPB	Iberian Pyrite Belt
IPMA	Instituto Português do Mar e do Ambiente
IUCN	International Union for Conservation of Nature and Natural Resources
LOI	Loss On Ignition
LNEC	Laboratório Nacional de Engenharia Civil
Lsto	*Lavandula stoechas*
Mix	*Mixed* substrate
NDVI	Normalized Difference Vegetation Index
OM	Organic Matter
PCA	Principal component analysis
Pf	Final sampling point of the transect
Pi	Initial sampling point of the transect
PRI	Photochemical Reflectance Index
rH	Relative humidity
SDG	Sustainable Development Goals
SLA	Specific Leaf Area
T	Transects
T1	Test 1
T2	Test 2
T50	Median germination time
UN	United Nations

1. Introduction

Located in the Iberian Peninsula, the Iberian Pyrite Belt (IPB), with a length of about 250 km and 35-60 km width, it's one of the world's major volcanogenic massive sulphide sites. According to Barriga *et al.* (1997), the IPB is located between *Beja* and *Setúbal* districts in Portugal, and within the provinces of *Seville* and *Huelva* in Spain (Figure 1.1).

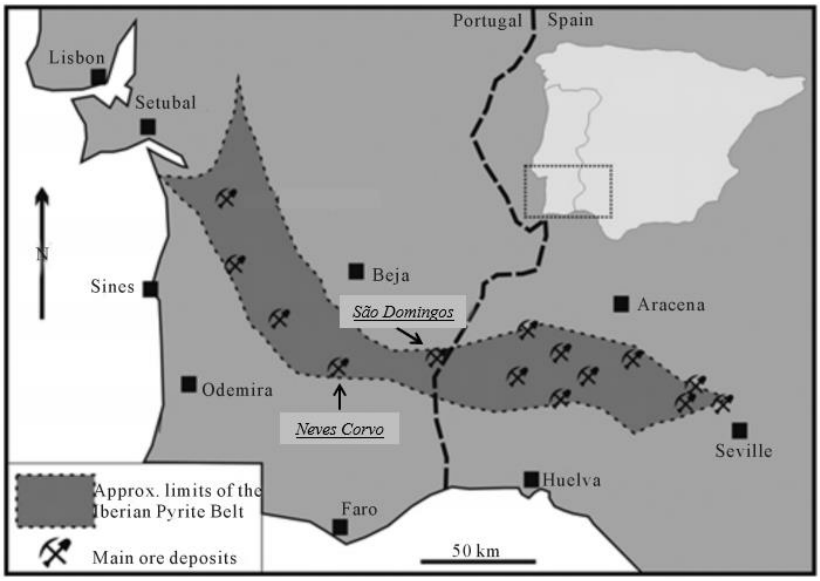

Figure 1.1 – Location of the mining sites: *São Domingos* mine (*Mina de São Domingos* village) and *Neves Corvo* (*Santa Bárbara de Padrões* village); and other main ore deposits in Iberian Pyrite Belt. Adapted from Figueiredo et al. (2014).

The IPB is a combination of massive sulphide deposits with volcanic and sedimentary rocks affected by hydrothermal fluids-induced processes, with origin in the Devonian-Carboniferous period (Barriga, Carvalho and Ribeiro, 1997; Tornos, Lopez Pamo and Sanchez España, 2008). Originally, this metalliferous area is believed to have around 1600Mt of massive sulphides and 250 Mt of sulphide-rich veins, known as stockwork ore. These are associated with other elements like Lead (Pb), Antimony (Sb), Sulfur (S), Arsenic (As), Zinc (Zn), Iron (Fe), Silver (Ag), Copper (Cu), Cadmium (Cd) and Mercury (Hg) (Barriga, Carvalho and Ribeiro, 1997; Tornos, 2006; Tornos, Lopez Pamo and Sanchez España, 2008). From the 90 known deposits of the IPB, only 20% have been explored (Barriga, Carvalho and Ribeiro, 1997). Currently, the mining exploration of the Portuguese IPB resulted predominantly in abandoned mines without any closing plan or environmental remediation strategy. One example, the *São Domingos* mine located in a village with the same name, is among the most emblematic copper and pyrite mines in Portugal. This mine was valuable for the Phoenicians and Carthaginians during the Calcholithic period (Barriga, Carvalho and Ribeiro, 1997) but the exploration gained more prominence in the Roman occupation from 12 to 397 AD (Fundação Serrão Martins, 2019). The Romans mined from galleries on the gossan, the exposed area of the ore deposition (Ferreira, 2012). Afterwards, the last mining period occurred between 1859 and 1966, at which point the mine was closed due to ore depletion. Along the 107 years of modern exploration, more than 20 million tons of chalcopyrite were extracted using an open-pit method (Ferreira, 2012; Oliveira, 2018; Fundação Serrão Martins, 2019). As a result of almost 5000 years of intensive mining activity, the *São Domingos* mine, as well as all other abandoned mines, are now a modified landscape with exposed tailings, mining heaps, water bodies of acid mine drainage (AMD), erosion problems and soil, air and water pollution with heavy metals (Figure 1.2).

Figure 1.2 – *São Domingos* mining area.

Nowadays, in Portugal, according to the General-Directorate for Energy and Geology, only two mines remain active: *Aljustrel* and *Neves Corvo* (DGEG, 2019). The latter is located in *Santa Bárbara de Padrões* parish. *Neves Corvo* mine is a subterranean mine whose Copper (Cu), Tin (Sn) and Zinc (Zn) exploration began after 1988 (Laboratório Nacional de Energia e Geologia, 2010; SOMINCOR SA, 2019).

Heavy metals are metallic/metalloid elements with high atomic weight, more than five times higher density than water and causing toxicity even at low exposures (Tchounwou *et al.*, 2012). Although they occur naturally, when in high concentrations become a source of toxicity and environmental contamination (Masindi and Muedi, 2018). For example, the increased concentration of Cu and Zn enhances the acidity of the waters, and Zn can also interrupt the normal activity of soil microorganisms and earthworms, consequently decreasing the organic matter breakdown (Wuana and Okieimen, 2011). Under a certain limit, some of these toxic metals like Cu, Zn, Fe and Magnesium (Mg) are also essential for biochemical functions, being called trace elements due to the importance of small concentrations for the normal development of biological organisms (Tchounwou *et al.*, 2012). However, elements like Cd, As and Pb, do not show evidence of importance to biological systems (Duruibe, Ogwuegbu and Egwurugwu, 2007; Tchounwou *et al.*, 2012).

Not only the mining exploration originates air, water and soil contamination, these can be magnified due to poor containment of the residues from the mining industry. Most toxic metals compounds can only be degraded to a certain point. Afterwards, these elements, non-degradable by microorganisms or chemical reactions, may remain bioavailable for uptake by plants, into the food chain, either in place or lixiviated away by run-offs. Residues not contained will originate contaminated water (AMD) and contaminated dust which can travel by wind, triggering skin and eyes irritation, respiratory functions' problems and others (Masindi and Muedi, 2018). Acid mine drainage is the product of chemical reactions between tailings (or other mine residues), water and oxygen. The metal's hydrolysis will result in decreasing pH of the AMD and thus promoting the toxic metal compounds disintegration enhancing their bioavailability. Thus, due to the presence of sulphur and/or iron-oxidizing

bacteria (such as *Acidithiobacillus ferrooxidans*) that use these reactions as energy source in autotrophic environments, the process will be catalyzed, hence originating more AMD in a faster amount of time. Contaminated soils, unless remediated, will act as a circular pathway increasing the contamination (Wuana and Okieimen, 2011; Simões, 2012; Masindi and Muedi, 2018).

Since most of the IPB mines are abandoned and, at the time of their exploration, there was no closure plan, there is a need to mitigate the environmental impacts created. For this reason, recently, the United Nations implemented an agenda of Sustainable Development Goals (SDGs), including an atlas with targets to achieve in the mining industry, active or not. As stated by the United Nations Development Programme *et al.* (2016), active mining companies have to minimize the toxic impacts by mitigating their emissions, recovering toxic metals from waste rock, monitoring water near the mine and downstream, building climate change resilience by modelling the impact of different climate patterns on the mining residues, especially for semi-arid and arid areas, among others (United Nations Development Programme *et al.*, 2016). This agenda must be thought-out by the active companies in their closing plan. On this atlas, the SDG 15 states the need to mitigate the impacts of mining activities and recognition of the dynamic nature of habitats to preserve ecosystem services (United Nations Development Programme *et al.*, 2016). For this reason, restoration of the inhospitable environment must be an objective to achieve. However, as mentioned before, only two mines remain active and have a chance to apply these recommendations, many others are waiting.

Currently, the environmental remediation and economic, cultural and environmental valorization of *São Domingos* mine is under public concession by EDM – Empresa de Desenvolvimento Mineiro, S.A. (EDM, 2005). Due to being an open-pit mine, the EDM plan consists mainly in a contaminated water contention and diversion of the clean water exterior to the mine (EDM and DGEG, 2011).

Although technical solutions for soil remediation already exist, they are mostly not environmentally friendly and are economically challenging, particularly for a mining site with a considerable area. Usually, different techniques are used at different stages of the remediation process (Liu *et al.*, 2018). Hence, a solution that meets the objectives established by the UN, is the use of vegetation cover as an in-situ less disruptive method: phytoremediation, which minimizes mining impact. According to Favas *et al.* (2014), phytoremediation is the use of plants and associated microorganisms to remove, degrade and/or isolate toxic metals, and can be described by the following strategies:

- Phytostabilization – direct action of root exudates resulting in metal precipitation as insoluble forms. By trapping these toxic metals in the soil matrix, bioavailability decreases, and the contaminant's diffusion is mitigated, it may or not include:
    - Rhizodegradation – roots' growth promotes rhizosphere microorganisms that degrade organic compounds by using the plants' exudates and metabolites as energy source.
- Phytoaccumulation – absorption of toxic pollutants by the roots, translocation into the plants aerial parts and further accumulation. When these species have the ability to store high concentrations, between 0.01% to more than 1% of their dry weight, they are called hyperaccumulators (Branquinho *et al.*, 2007); phytoaccumulation may or not include:
    - Phytodegradation – enzymatic degradation of organic contaminants inside plant cells;
    - Phytovolatilization – the plants' roots absorb toxic elements, converting them into non-toxic forms later released into the atmosphere;
- Phytofiltration – plants take in, accumulate and/or precipitate the toxic elements from roots, or other organs, on aqueous environments.

Plant covers also decreases the transport of metallic dust, by acting as a biomass curtain, and diminish rainwater infiltration (Favas *et al.*, 2014).

To establish a plant cover, it is necessary to determine which species are most likely to survive in a habitat characterized by arid soils, low pH, few nutrients and, especially high concentration of toxic metals, since the selective pressures provided by this type of soils are very severe. Thus, suitable species to be included in this vegetation cover would be the native flora from metalliferous areas, already adapted to those types of environments. These species survive and reproduce in environments with toxic concentrations of metals due to the existence of adaptations in their cellular mechanisms. However, these species - known as metallophytes - can only thrive if soil toxicity levels do not exceed their tolerance levels (Whiting *et al.*, 2004; Favas *et al.*, 2014).

The metallophytes rely on two different strategies to thrive in harsh environments. They can be classified as: (i) accumulators, these species have detoxicating mechanisms at cellular level, which enables accumulation of toxic metals in shoots; or they can be (ii) excluders, due to reduced uptake, translocation or selective accumulation by the roots, these species maintain low concentrations of these toxic elements in the leaves (Baker, 1981). Some generalist ubiquitous species can also thrive in these environments, as well as in others without the metal pressure, due to its capacity to adapt to a wide range of conditions, thus being called pseudometallophytes, according to Whiting *et al.* (2004).

Some studies have demonstrated the phytostabilization potential of some endemic species, such as *Erica andevalensis* (Rossini-Oliva, Abreu and Leidi, 2018), *Erica australis* (Pérez-López *et al.*, 2014), *Cistus salviifolius* (Abreu *et al.*, 2012), *Agrostis castellana* (De Koe, 1994) and others. Although those species use different mechanisms, the metals do not reach toxic levels, so the species do not display phytotoxic effects. Nevertheless, none of these studies has described these species' ecophysiological performance. To characterize species, ecologically and physiologically, it is important to understand their preferences and limitations in terms of environmental stress and relationships with other species. Thus, selecting which plants to use represents an important strategy in a sustainable vegetation cover. By rehabilitating the ecosystem using indigenous species already present in the surroundings improves the chances of viability and survival of the vegetation cover, reducing the need for maintenance, and approaching communities that existed prior the impact caused by the mining activity - ecological restoration (Vaugh *et al.*, 2014).

The extreme metalliferous environments are the habitat of particular rare species, not found elsewhere because of their special adaptations. This is the case of *E. andevalensis* that falls within the category of critically threatened species (IUCN Red List, 2016). An advantage of the use of these species as vegetation cover, is the integration of species belonging to the IUCN Red List. By studying their habitat and ecological preferences, we are also conserving these species (Whiting *et al.*, 2004) and complying with the SDG15 proposed by the UN (United Nations Development Programme *et al.*, 2016).

Objectives

The aim of this project is to contribute to improve the knowledge on ecological and physiological performance of the plant species that inhabit these metalliferous environments of the Iberian Pyrite Belt to support ecological restoration projects in areas degraded by the intense mining activity.

The following research hypotheses were addressed:

(i) We hypothesized that the ecological and physiological performance of the species varied over a pH gradient. The pH was used as a proxy for the toxic metals, as their bioavailability increases when the pH decreases; i.e. a more acid environment

corresponds to greater toxicity and less available nutrients (Wuana and Okieimen, 2011).

(ii) Another hypothesis is that, in places with higher pH (neutral), the competition between species was higher, therefore species more tolerant to environmental stress that are weaker competitors tend to disappear out of the community. On the other hand, where the pH is lower, the stress tolerant species tend to thrive due to advantage of their adapted physiological responses.

To test these hypotheses, the chosen species' ecological and physiological performances was characterized, both in situ and in greenhouse conditions.

2. Methods

2.1 Sampling sites

The Iberian Pyrite Belt was the chosen region for the collection of soils and plant material, namely the areas of *Mina de São Domingos* and *Santa Bárbara de Padrões*, two mining sites about 40 km apart located in *Beja* district, Portugal (Figure 1.1). This region was selected for being a metalliferous area (Figure 2.1), hence it is expected the existence of plant species with more tolerance to high concentrations of toxic metals. Based on climate normals between 1971-2000, both locations are characterized by a Mediterranean warm temperate climate of hot and dry summers (Kottek *et al.*, 2006), with a mean annual temperature of 16.5 ºC (IPMA, 2013) and mean annual precipitation of 402.8 mm, for *Mina de São Domingos*, and 457.4 mm, for *Santa Bárbara de Padrões* (SNIRH, 2014). These areas are described as being a lithological complex of metamorphic and sedimentary formations, namely shale, greywacke and sandstone (SNIAmb, 2015).

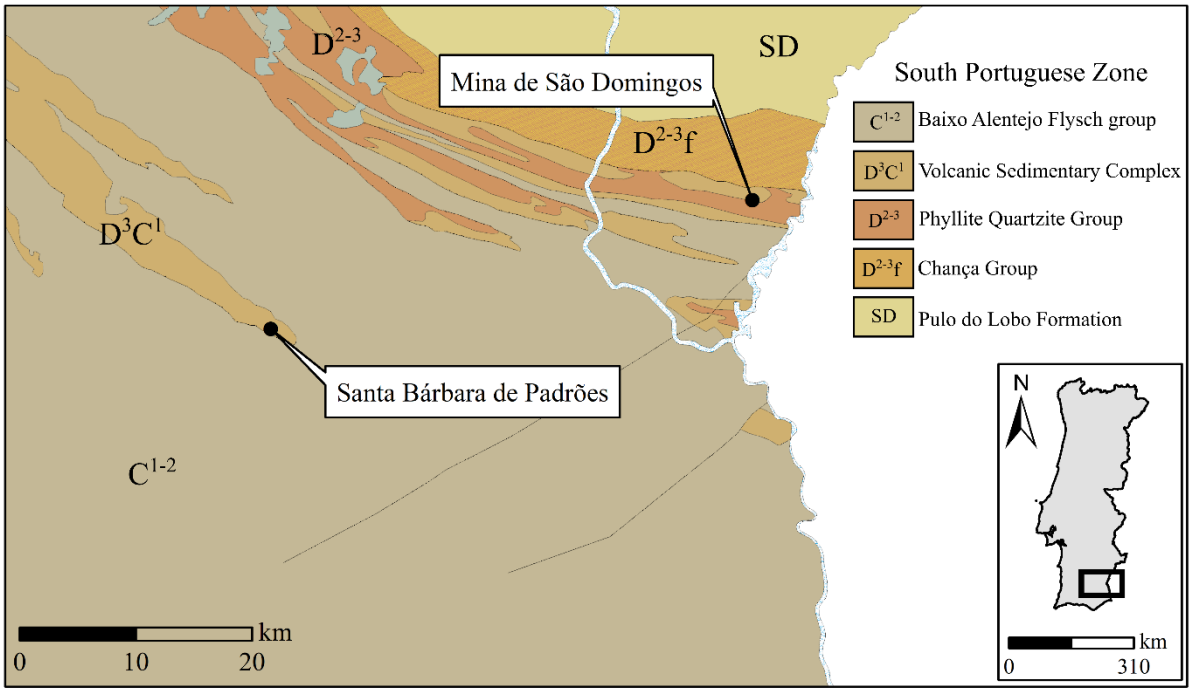

Figure 2.1 – Geological map of the South Portuguese Zone (1:1000000). Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroRID, IGN, and the GIS User Community.

2.2 Greenhouse studies

2.2.1 Soil Sampling for Germination Tests

In late November 2018, samples of soil were collected from the two different locations, *S. Domingos mine* and *Santa Bárbara de Padrões* (Figure 2.1), to use on germination tests with soils identified as *contaminated 1* and *contaminated 2*, respectively. Five topsoil subsamples, around 10 cm deep, were collected from each site and then mixed into composite samples (25 kg total). The two soils came from areas of the mines affected by acidic drainage from the tailings or lagoons, thus expected to be highly contaminated. Due to unavailable funding, in this work it was not possible to measure the toxic metals concentrations. The *São Domingos* mine soils, according to Andráš *et al.* (2018), had the

following composition: 5100 mg kg^{-1} Ca, 3100 mg kg^{-1} Mg, 101 g kg^{-1} Fe, 3050 mg kg^{-1} Pb, 1191 mg kg^{-1} As, 960 mg kg^{-1} Zn, 650 mg kg^{-1} Cu and others.

Beside the climate and edaphic similarities and the proximity, both areas differ from each other as the *S. Domingos* mine is a deactivated mine whereas *Santa Bárbara de Padrões* has an active mine (*Neves-Corvo*). Nevertheless, *Santa Bárbara de Padrões*' soil is expected to have a lower metal concentration than the deactivated and abandoned *São Domingos* mine (Table 2.1).

Table 2.1 – Soil elements characterization in *Santa Bárbara de Padrões* (H. C. Serrano, not published). Soil extraction with: *HNO_3; **EDTA; ***H_2O; N – nitrogen, OM – organic matter.

Elements	Total* (mg kg^{-1})	Bioavailable** (mg kg^{-1})	Soluble*** (mg kg^{-1})
Ca	150-370	90-210	150-210
Mg	190-3000	20-40	20-40
K	1400-2000	20-80	20-80
Cu	500-2600	400-1100	60-80
Zn	150-220	30-60	10-20
Fe	30000-35000	640-1900	3-5
Other characteristics			
N %	0.1		
OM %	3.9-9.2		
pH	2.63-4.29		

Santa Bárbara de Padrões soil was collected from an area impacted by run-off and leaching from a mine slag heap. For these reasons, *Santa Bárbara de Padrões* soil was used as a second contaminated substrate for the germination tests. The collected soils were left air-drying for two weeks and then sieved (2 mm mesh) in order to obtain homogeneous soil samples. Substrates were stored at room temperature until further use.

2.2.2 Species selection

A total of eight species were selected and are described in Table S1.1. The criteria defined for the selection of species were the local abundance, conservation status, and native distribution, moreover the existence of mature fruits at the time of collection. Were selected six shrub species: *Cistus ladanifer, C. salviifolius, C. mosnpeliensis, Erica andevalensis, E. australis, Lavandula stoechas* and two herbaceous perennials: *Agrostis castellana* and *Anarrhinum bellidifolium.*

2.2.3 Seed collection and preparation

In late summer 2018, plant material was collected, namely fruits and flowers from the species previously described. Before cleaning, the seeds were cold stored (+9º C) in paper bags. Seed cleaning consisted of removing material, such as particles of leaves, flowers, fruit capsules, and others. This procedure was carried out with the help of sieves with different mesh sizes according to the species seed size: *A. castellana, A. bellidifolium, E. australis, E. andevalensis* – 200 µm; *C. ladanifer* – 700 µm, *C. salviifolius, C. monspeliensis* – 900 µm; and *L. stoechas* – 1 mm. After proceeding to seed cleaning, the seeds were stored at +9 ºC in a plastic container until use.

2.2.4 Germination

2.2.4.1 Potential Seed Germination

In order to define the potential germination, a first test (Test 1) was performed in vitro, including seed pre-treatment methods.

The seeds were divided into two batches, each one with three replicates with 10 seeds. One of the batches was the control (without treatment) and the second batch underwent heat pre-treatment, specific for each species (Table 2.2), in a muffle oven (L3/11, Nabertherm, Germany). After the pre-treatment, the seeds were surface sterilized by soaking in a 1% sodium hypochlorite solution for 3-5 minutes, depending on the size of the seeds, to prevent pathogenic fungi contamination. Then, the seeds were washed thoroughly with deionized water (Bicksler, 2011). Seeds of each species were placed in Petri dishes with ca. 20 mL of 1% agar (m/v). These were incubated in a growth chamber (FITOCLIMA s600, Aralab, Portugal), at 20ºC and 50% relative humidity (rH), with a 16h light/8h dark photoperiod, and were followed for at least one month. Germination was recorded when the cotyledons were exposed. The germination of some *Cistus* spp. and *Erica* spp. was not observed after 20 days, thus then the germination test was repeated (Test 2) with moist filter paper instead of agar in the Petri dishes, and with modified pre-treatments applied to the seeds. To the Cistaceae seeds heat pre-treatments with different temperatures and times were applied (except for *C. ladanifer* that repeated the treatment). For the heathers (*Erica* spp.), a chemical treatment was applied, i.e. the first filter paper irrigation was with a solution of NH_4NO_3 10mM, while the following were with distilled water. Both pre-treatments are described in Table 2.2. The growth chamber conditions, and observation regime were similar to Test 1.

Table 2.2 – Pre-treatment applied to the seeds. NA – not applicable. The chosen treatments to be used in soil germination tests are in bold.

Species	**Test 1** Agar	**Test 2** Filter paper	**References**
Agrostis castellana	**80 ºC/ 10 mins**	NA	(Luna *et al.*, 2007)
Anarrhinum bellidifolium	**80 ºC/ 10 mins**	NA	
Cistus ladanifer	100 ºC/ 30 mins	**100 ºC/ 30 mins**	(Trabaud and Oustric, 1989; Pérez-García, 1997)
Cistus monspeliensis	**100 ºC/ 30 mins**	150 ºC/ 1 min	
Cistus salviifolius	100 ºC/ 30 mins	**100 ºC/ 10 mins**	
Erica andevalensis	**110 ºC/ 10 mins**	NH_4NO_3 10mM	(Rossini Oliva, Leidi and Valdés, 2009; Vera, Martín and Oliva, 2010)
Erica australis	**110 ºC/ 10 mins**	NH_4NO_3 10mM	
Lavandula stoechas	**80 ºC/ 10 mins**	NA	(Moreira *et al.*, 2010)

Final Germination Percentage (FGP) was calculated by dividing the number of germinated seeds by the total number of seeds placed to germinate. Time for 50% germination (T50) was calculated as the number of days until 50% of the maximum germination was reached. To calculate this value, it was necessary to establish a time limit for mathematical evaluation of the outcomes, so it was considered that 90 days would be the maximum germination time before the seeds would be considered inviable.

2.2.4.2 Seed Germination in Soils

To understand the contamination influence in the germination and physiological performance of the collected species, the seeds were germinated in four different substrates, under greenhouse conditions:

1) commercial substrate (peat),
2) mixed 50:50 (commercial:*contaminated 1*, v/v),
3) contaminated 1 (from *São Domingos* mine) and
4) contaminated 2 (from *Santa Bárbara de Padrões*).

The commercial substrate (Pindstrup, Universal) was composed of Sphagnum blonde peat, composted plant material and perlite. Only the substrate *contaminated 1* was used for the mixture because it is the original soil where the plant material was sampled, therefore it would be useful to understand if increasing the organic matter can enhance the physiological performance of the plants. The use of a second contaminated substrate increases the knowledge of how the chosen plant species would perform in a different contaminated soil, in this case from a nearby region with similar climate and edaphic origin.

Germination seed pots (4.5x4.5x4.5 cm) were used under greenhouse conditions, with randomly distributed substrates. Seed pre-treatment with the best one-month in vitro germination results was used for the germination tests in soil. The seed treatments applied for each species are specified in Table 2.3. A control test without seed treatment was also performed in order to consider the possibility of a different germination influenced by the substrate instead of the treatment. Ten seeds were seeded in each pot, with a total of three replicates for each treatment, and for each species (Test 1, Table 2.3). Germination was noted after cotyledons were exposed and recorded for one month.

Considering that seed dormancy might be influenced by temperature/light and that the greenhouse conditions were not controlled, rather environment dependent, a second test (Test 2, Table 2.3) was performed after two months. Due to seed number constraints, the experiment only had two replicates and *E. australis* could not be used. After one month, FGP and T50 in soil conditions were calculated as before.

Table 2.3 – Seed pre-treatment protocol used for both tests of germination in soil. n. – number of replicates (10 seeds per replicate except for *Lavandula stoechas* with 3 seeds per replicate); NA- seeds not available.

Species	**Treatment**	**Test 1** (n.)	**Test 2** (n.)
Agrostis castellana	80ºC/ 10 mins	3	2
Anarrhinum bellidifolium	80ºC/ 10 mins	3	2
Cistus ladanifer	100ºC 30 mins	3	2
Cistus monspeliensis	150ºC/ 1 min	3	2
Cistus salviifolius	100ºC/ 10 mins	3	2
Erica andevalensis	110ºC/ 10 mins	3	2
Erica australis	110ºC/ 10 mins	3	NA
Lavandula stoechas	80ºC/ 10 mins	3	2

Although the temperature and relative humidity of the greenhouse were not controlled, these conditions were monitored every day for the experiment period using an iButton data logger (Hygrochron DS1023, Maxim Integrated). The greenhouse had an average 22 ± 7 ºC of temperature and 57 ± 22 % of relative humidity.

2.2.5 Plant development

Germination was followed for one month, after which only the three healthiest looking plants were left in each replicate pot. After the second month, only the best individual was left for development. The plants were watered 2-3 times per week when the soil was apparently dry by touch. The commercial substrate was watered fewer times as this substrate retained water better.

To assess the plant's physiological performance in different substrates, growth and physiological parameters were used:

1. Leaf development and plant height (initially, the number of leaves was recorded but proceeded to height record when that number became too high). Plant development was followed for three months, when the saplings were five (Test 1) and three (Test 2) months old.

2. Physiological parameters were measured by leaf reflectance using a spectroradiometer (UNISPEC-SC Spectral Analysis System, PP Systems, USA) when the replicates were five (Test 1) and three (Test 2) months old (June 2019), once a month, for three months. With this device, leaf reflectance is measured between 300 to 1148 nm.

Leaf reflectance data were used to calculate physiological indices related to the production of pigments. According to Sims and Gamon (2002), in plants under stress, chlorophyll tend to decline faster, and the carotenoids are used to avoid damage to the photosynthetic system. On the other hand, hairs and waxes protect the leaves by increasing the reflectance, as the light reflected does not enter the cells and does not influence the pigments content. On the contrary, the absorbed light causes changes in the pigments content. These variations reflect the physiological state of the leaves thus providing more information about the plant (Sims and Gamon, 2002). The indices used were:

i) Normalized Difference Vegetation Index (NDVI, eq. 2.1.), is the most used index for chlorophyll and it varies between -1 and 1, with values <0.1 usually not being vegetation, and values close to 1 having the higher chlorophyll content (Sims and Gamon, 2002; Zawawi, Shiba and Jemali, 2014; IDB, 2019b):

(eq. 2.1.) $$NDVI = \frac{R800 - R670}{R800 + R670}$$

ii) Photochemical Reflectance Index (PRI, eq. 2.2.), used for photosynthetic light use efficiency based in estimation of carotenoids (Sims and Gamon, 2002; Filella *et al.*, 2009; IDB, 2019c). Lower PRI values is a standard response to the action of numerous stressors (including excess light, salinity, water stress, increased temperature, etc.) on plant leaf (Sukhova and Sukhov, 2019):

(eq. 2.2.) $$PRI = \frac{R530 - R570}{R530 + R570}$$

iii) Chlorophyll Index (CHL, eq. 2.3.), used as a simple ratio formula to provide information on the chlorophyll content (Datt, 1999; IDB, 2019a). According to Gitelson *et al.* (1997), the coefficient correlation between this index and chlorophyll is positive, thus higher values are associated with higher chlorophyll concentration, thus generally less stress.

(eq. 2.3.) $$CHL = \frac{R750}{R700}$$

2.3 Ecological performance in contaminated areas

In late May 2019, to evaluate the ecological performance of the species in the field, four areas of *São Domingos* mine were chosen: the first area (A1), located away from the acidic bodies, was characterized by crossing zones and heterogeneous vegetation composition; whereas the remaining three areas were acid mine drainage water bodies (A2, A3 and A4). Within each area we expected to have different contamination degrees. In each area, three geo-referenced transects of 30 m were oriented perpendicular to the margin of the water bodies, or the areas expected to have more contamination and/or a soil pH gradient (Figure 2.2). The edaphic characterization and ecological performance, including species abundance and physiological parameters analyzed, are described in the following sections.

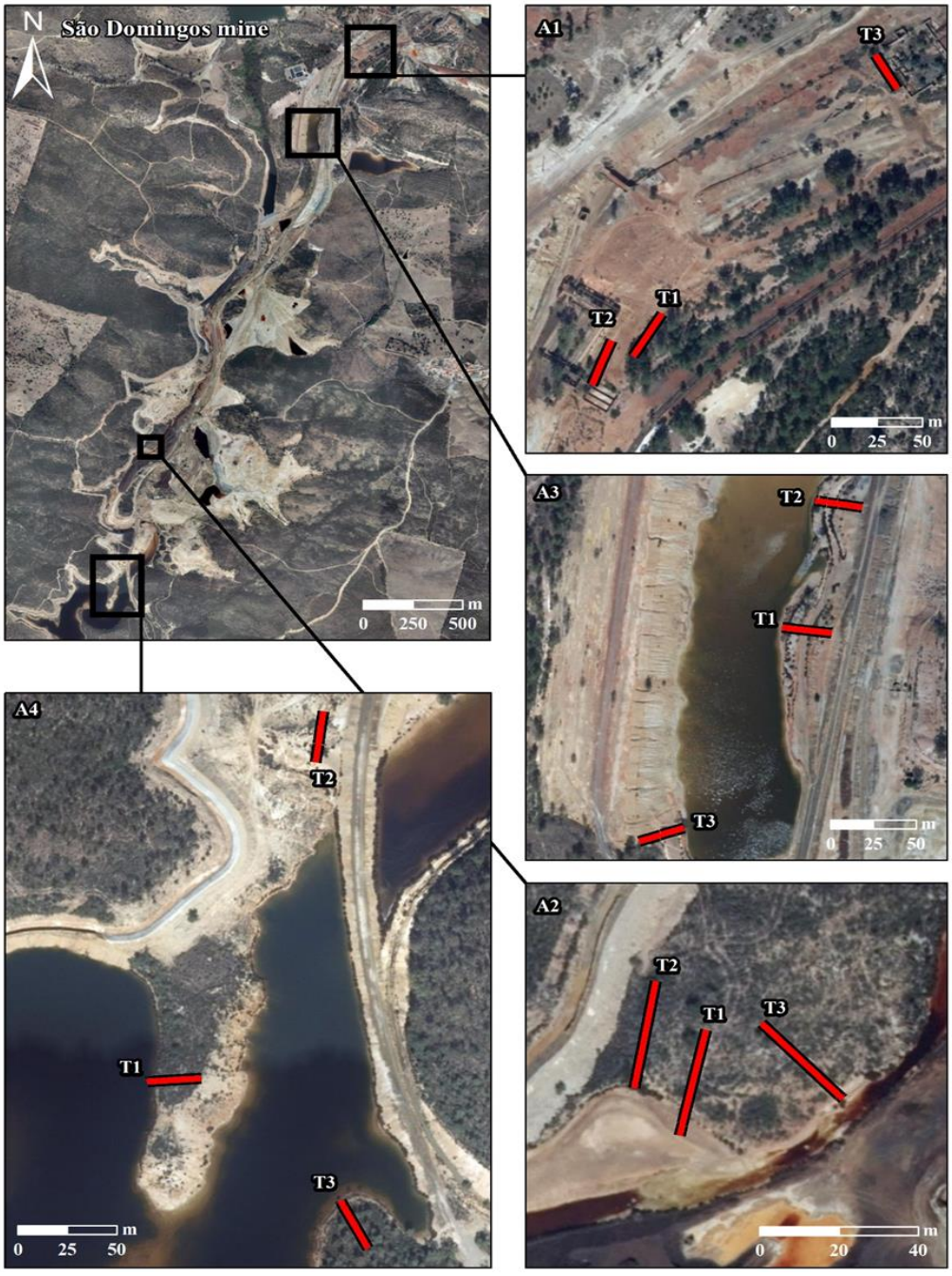

Figure 2.2. – Transects performed in sampling area of *São Domingos* mine; A – area, T - transect. Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroRID, IGN, and the GIS User Community.

2.3.1 Soil sampling

On each transect, according to the vegetation composition heterogeneity and local characteristics (e.g.: crossing area, slopes, etc.), zones for sampling points were defined. Each zone had three sampling points: one at the beginning of the zone, one in the middle and one at the end of the zone. For example: if the transect had homogeneous vegetation, then it only had one zone thus having three sampling points (Figure 2.3). If the vegetation was heterogeneous, the transect would have six or more sampling points, depending on plant zonation (Figure 2.4). On each sampling point, about 500 g of topsoil (10 cm deep) was collected for further pH, organic matter and granulometry studies. Conductivity variables namely bulk electrical conductivity (EC_b) was measured directly in soil using an HH2 Moisture Meter with WET sensor probe (Delta-T Devices, UK) on each sampling point. Between each reading, the probe was washed with deionized water to avoid cross contamination. For data analysis purposes, the soil variable's values between the points sampled were calculated from interpolation.

Figure 2.3 – Example of a homogeneous vegetation area. Transect with one zone. Yellow dots represent sampling points.

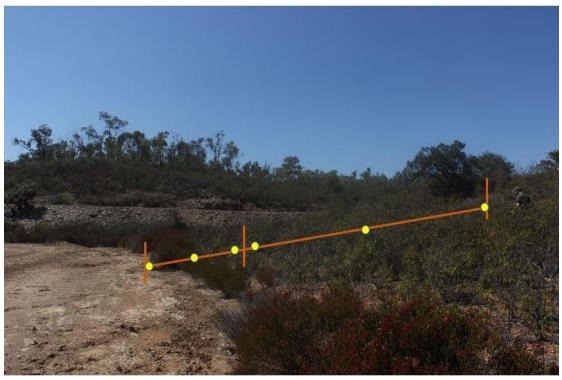

Figure 2.4 – Example of a heterogeneous area. Transect with two zones: one zone with *E. andevalensis* and a second zone with *C. ladanifer*. Yellow dots represent sampling points.

2.3.2 Vegetation Abundance

The species abundance in the sampled population, e.g. cover and frequency, are considered a proxy of their ecological performance in the local community. Therefore, vegetation abundance was recorded using the line-intercept transect method (Elzinga, Salzer and Willoughby, 1998). Within each transect, the length of interception for the crown of each species was recorded, resulting in a transect-interception length. This distance was used for plant cover estimation. Additionally, height and two diameters (wider and smaller) were also recorded. Every plant intercepted was recorded per species, except for the Poaceae and Asteraceae plants, recorded as a family and not as species.

2.3.3 Physiological Measurements

Within each area, one of the three transects was randomly selected for each of the eight studied species. Three plants were then chosen along the pH gradient in order to measure the reflectance on 15 to 20 leaves, scattered over different branches. Leaf reflectance measurements were conducted using a spectroradiometer (UNISPEC-SC Spectral Analysis System, USA). From the same three individuals, 15 leaves of each plant were collected, and the leaf area was measured using a portable leaf area meter (AM350, ADC BioScientific Ltd, UK) in order to evaluate the specific leaf area (SLA). The leaves were oven-dried at 60ºC and their dry weight registered. To assess the dry weight of the heathers' leaves, as they were very light, 15 leaves were weighted together, to calculate an average weight per leaf.

2.4 Soil Characterization

Both the substrates used for germination tests and the soils from the field transects were characterized to assess the soil effects on the performance of the studied species. The variables measured were organic matter (OM), pH, granulometry and conductivity (EC_b and ε_b). Soil samples were previously 2 mm sieved and oven-dried for, at least, 48h at 60 ºC, before processing.

2.4.1 Organic matter

The soil organic matter was estimated using the weight loss on ignition method (LOI) (Heiri, Lotter and Lemcke, 2001) in a muffle furnace (L3/11, Nabertherm, Germany). This method is based upon measuring the weight loss of a dry soil sample (DW) when exposed for 6h to high temperatures (dry weight after ignition at 600 ºC, DW600º) and calculated using the following equation 2.4.:

(eq. 2.4.) $$LOI\ (\%) = \frac{DW - DW600^{º}}{DW} \times 100\%$$

For each soil sample, three replicates were made with circa 10 g of dry soil per crucible.

2.4.2 pH and Conductivity

Soil pH was determined in a soil solution of 3.0 g sieved soil to five times its volume of deionized water. This suspension was then mixed for 1 min on a vortex (RSLAB-6PRO, Auxilab, Spain) agitated for 1h (150 rpm) in a mechanical shaker (Lab Companion IST-4075, Jeio Tech, Korea) and another hour for deposition (Cools and Vos, 2016). A pH probe (inoLab pH 730, WTW, Germany) was used to measure three times each soil sample. pH readings were taken when the value stabilized for 1 minute. Between each reading, the probe was washed with deionized water to avoid cross contamination (LNEC internal method). Bulk Electrical Conductivity (EC_b) was measured in a single replicate with an HH2 Moisture Meter and WET sensor probe (Delta-T Devices, UK).

2.4.3 Granulometry

For the measurement of the particle size distribution, circa 100 g of dry, sieved soil were used (n=1). The fractionation was carried out in a vibratory sieve shaker (Analysette 3, Fritsch, Germany) using three sieve sizes (2000 µm, 425 µm and 63 µm). The particle size classes used were (Cools and Vos, 2016):

- medium sand (2000 – 425 µm),
- fine sand (425 – 63 µm) and
- silt & clay (< 63 µm).

The greenhouse substrates test retrieved 96.24 % (±0.02) of the particles. The commercial substrate was not considered for granulometry analysis due to being an organic substrate. The total lost fraction of the in-situ soils test was 2 % (±0.04).

2.5 Data Analysis

Data analysis was conducted using Microsoft Excel 2016 for the mean, standard deviation. The remaining data analysis were carried out using the software R version 3.6.1. Unless otherwise stated, all tests were run with a significance level of $P \leq 0.05$.

To assess the assumptions of Normality and Homoscedasticity, the Shapiro-Wilk and Bartlett test were used, respectively.

The Kruskal-Wallis test was conducted to test for significant differences in germination and substrate characterization, followed by post-hoc Dunn tests.

In order to test for significant differences between substrates, the reflectance indices and height data the Dunnett's modified Tukey-Kramer pairwise multiple comparison test was used, as the data had different number of replicates.

Analyses of in-situ variables were performed based on interpolated values of pH, OM and EC_b. The interpolation was calculated using two adjacent points of the same transect zone. The values were calculated according to equation 2.5.

(eq. 2.5.) $$Y = y1 + [[(y2 - y1)/(x2 - x1)] * (X - x1)]$$

Where:

Y – interpolated value
y_1 – known soil point (initial)
y_2 – known soil point (final)
X – distance of the interpolated value
x_1 – distance of soil point (initial)
x_2 – distance of soil point (final)

Spearman's rank correlations were calculated based on the soil properties' interpolated values, intercepted plant cover, height, crown area and reflectance indices. Pedersen *et al.* (2019) described Generalized additive models (GAM) as being used for estimation of smooth functions between variables, therefore we have used this model to assess the relation of the different species with the pH gradient.

Unless otherwise stated, all photographs present were taken by the thesis' author.

3. Results

3.1 Greenhouse Tests

3.1.1 Substrate properties

The properties of the substrates used for seed germination in the greenhouse tests are shown in Table 3.1. The commercial substrate presented the highest organic matter content, in contrast to the substrate *contaminated 2* ($P \leq 0.01$). There were no significant differences (probably due to the low number of replicates) between the commercial substrate OM and the remaining contaminated substrates OM. For the pH results, the *contaminated 1* substrate was the more acidic with significant differences to the commercial substrate that was the less acidic ($P \leq 0.01$).

Table 3.1 – Greenhouse substrate characterization for texture, organic matter (OM), pH and bulk electrical conductivity (EC_b). Mean values with a standard deviation between parenthesis; Significant differences between substrates ($P \leq 0.05$) are indicated by different letters; Kruskal-Wallis test ($\alpha=0.05$).

Substrate	**Textural Class** n=1	**OM (LOI %)** n=3	**pH** n=3	**EC_b ($mS.m^{-1}$)** n=1
Commercial	Organic	90.1^{a} (± 1.5)	5.34^{a} (± 0.02)	9.3
Mixed	Sand	17.5^{ab} (± 0.8)	4.43^{ab} (± 0.02)	13.0
Contaminated 1	Sand	9.2^{ab} (± 0.3)	2.99^{b} (± 0.02)	4.3
Contaminated 2	Sandy loam	4.8^{b} (± 0.3)	4.09^{ab} (± 0.02)	12.3

The percentage of each fraction (medium sand, fine sand and silt and clay fraction) that define the textural class of each substrate used for the greenhouse germination tests (contaminated 1, contaminated 2 and mixed) is shown in Figure S2.1. The *contaminated 2* substrate was classified as being a sandy loam substrate, thus having more fine particles than mixed and *contaminated 1*, were rated as sand substrates (Table 3.1) (Cools and Vos, 2016). The commercial substrate was not considered for this classification as it is an organic substrate (more than 90% OM) (Cools and Vos, 2016).

3.1.2 Seed germination

Regarding the potential germination percentage (FGP) and the time in days to reach 50% of the maximum germination (T50) after one month in Petri dishes in a controlled environment, the best treatment type results are shown in appendix (Table S1.2 and Table S1.3, respectively).

Generally, for shrub species, the seeds collected showed good viability for germination, except for *C. monspeliensis* and *C. salviifolius*. This may have been due to low seed maturity or inadequate growth conditions, among others. The species *C. ladanifer* and *L. stoechas* had significantly higher germination rates when heat treatment was applied. Of the two, *L. stoechas* showed also higher rate of germination (lower T50) when heat-treated, taking only ±3 days to achieve T50 instead of two months besides the higher germination percentage (increased from 17 % to 77 %). The remaining species (shrubs and herbaceous) did not show any significant differences, despite also having a tendency to higher germination when heat-treated.

When analyzing the seed germination on different contaminated substrates (Figure 3.1), regarding final germination percentage (FGP, Table 3.2), the herbaceous species, *A. castellana* and *A. bellidifolium* had higher FGP values in the commercial and *mixed* substrates ($P \leq 0.05$) similar to the

results obtained in the *in vitro* tests, and also lower values of T50, taking around 5 to 8 days to germinate (Table 3.3).

Cistus ladanifer germination was higher in the commercial, *mixed* and *contaminated 1* substrates, when heat treatments are applied ($P \leq 0.01$). *C. monspeliensis*, *C. salviifolius* and *E. australis* showed very low values of FGP in all the substrates (Table 3.2). Although *C. salviifolius and E. australis* had low germination, they were positively influenced by heat treatments on *contaminated 1* substrate ($P \leq 0.05$) with faster germination rate (lower T50) and also on *mixed* substrate for *C. salviifolius* ($P \leq 0.01$; Table 3.3).

Erica andevalensis' germination was higher in the *mixed* substrate, followed by *contaminated 1* ($P \leq 0.01$; Table 3.2). Germination was much higher than tests *in vitro* (Table S1.2). *Lavandula stoechas* showed higher germination on both commercial and *contaminated 2* substrate (Table 3.2), although below their germination potential.

Figure 3.1 – Germinated seeds in different substrates: a) *Agrostis castellana* in substrate *contaminated 2*; b) *Cistus salviifolius* in substrate *contaminated 1*; c) *Lavandula stoechas* in commercial substrate.

Table 3.2 – Final germination percentage (FGP %) in the greenhouse tests. Average values of two treatments (control and heat-treated) on four substrates (commercial, mixed, contaminated 1 and contaminated 2) followed by standard deviation (mean ± SD); n. – number of replicates; Significant differences ($P \leq 0.05$) between tests for each species are indicated by different letters (Kruskal-Wallis, α=0.05).

Species	n.	Commercial		Mixed		Contaminated 1		Contaminated 2	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated
Agrostis castellana	5	**98.0^{a} (± 0.5)**	**88.0^{a} (± 1.6)**	**90.0^{a} (± 0.7)**	**94.0^{a} (± 0.6)**	28.0^{b} (± 2.4)	34.0^{b} (± 2.6)	14.0^{b} (± 1.3)	30.0^{b} (± 1.7)
Anarrhinum bellidifolium	5	**62.0^{a} (± 1.6)**	**42.0^{a} (± 2.8)**	**80.0^{a} (± 1.9)**	**70.0^{a} (± 1.0)**	6.0^{b} (± 1.3)	6.0^{b} (± 1.3)	2.0^{b} (± 0.5)	2.0^{b} (± 0.5)
Cistus ladanifer	5	24.0^{bc} (± 0.9)	**92.0^{a} (± 1.3)**	10.0^{b} (± 0.7)	**84.0^{a} (± 2.1)**	8.0^{b} (± 0.8)	**42.0^{ac} (± 1.9)**	20.0^{bc} (± 1.6)	26.0^{bc} (± 3.2)
Cistus monspeliensis	5	0.0^{a} (± 0.0)	4.0^{ab} (± 0.9)	10.0^{b} (± 1.7)	0.0^{a} (± 0.0)	0.0^{a} (± 0.0)	0.0^{a} (± 0.0)	0.0^{a} (± 0)	2.0^{ab} (± 0.5)
Cistus salviifolius	5	4.0^{bc} (± 0.6)	18.0^{a} (± 1.1)	0.0^{b} (± 0.0)	26.0^{a} (± 2.0)	2.0^{b} (± 0.5)	12.0^{ac} (± 0.8)	2.0^{b} (± 0.5)	0.0^{b} (± 0.0)
Erica andevalensis	5	2.0^{a} (± 0.5)	0.0^{a} (± 0.0)	**78.0^{b} (± 3.0)**	**78.0^{b} (± 3.4)**	**72.0^{b} (± 3.3)**	**46.0^{b} (± 5.0)**	2.0^{a} (± 0.5)	4.0^{a} (± 0.9)
Erica australis	3	3.3^{ab} (± 0.6)	13.3^{c} (± 0.6)	3.3^{ab} (± 0.6)	0.0^{a} (± 0.0)	3.3^{ab} (± 0.6)	10.0^{bc} (± 0.0)	0.0^{a} (± 0.0)	6.7^{ac} (± 0.6)
Lavandula stoechas	3	**56.7^{c} (± 3.2)**	**36.7^{bc} (± 3.1)**	20.0^{abc} (± 1.3)	6.7^{ab} (± 0.9)	0.0^{a} (± 0.0)	3.3^{ab} (± 0.5)	**40.0^{c} (± 2.5)**	**30.0^{bc} (± 2.5)**

Table 3.3 – Mean T50 germination in the greenhouse tests. Mean time (days) to reach 50% of the maximum germination for each species under two treatments (control and heat-treated) for both tests (mean ± SD) ; n. – number of replicates; Significant differences between treatment and control for each species in each substrate are in bold and indicated by: "*" $P \leq 0.05$, "**" $P \leq 0.01$ (Kruskal-Wallis test, α=0.05).

Species	n.	Commercial		Mixed		Contaminated 1		Contaminated 2	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated
Agrostis castellana	5	4.6 (± 0.5)	5.8 (± 3.5)	6.6 (± 2.3)	5.4 (± 2.7)	22.8 (± 37.6)	23.8 (± 37.1)	40.4 (± 45.3)	22.8 (± 37.6)
Anarrhinum bellidifolium	5	6.8 (± 3.3)	6.4 (± 3.4)	7.0 (± 1.2)	7.4 (± 1.7)	73.6 (± 36.7)	56.2 (± 46.3)	72.6 (± 38.9)	73.4 (± 37.1)
Cistus ladanifer	5	9.8 (± 3.9)	10.3 (± 2.0)	26.8 (± 35.4)	10.0 (± 2.1)	40.2 (± 45.6)	5.4 (± 2.2)	25.6 (± 36.6)	41.0 (± 44.8)
Cistus monspeliensis	5	90.0 (± 0.0)	73.2 (± 37.6)	57.0 (± 45.2)	90.0 (± 0.0)	90.0 (± 0.0)	90.0 (± 0.0)	90.0 (± 0.0)	72.6 (± 38.9)
Cistus salviifolius	5	56.6 (± 45.8)	12.0 (± 2.4)	**90.0** (± 0.0)**	**26.4** (± 35.6)**	**75.0* (± 33.5)**	**25.2* (± 36.4)**	73.6 (± 36.7)	90.0 (± 0.0)
Erica andevalensis	5	74.6 (± 34.4)	90.0 (± 0.0)	59.2 (± 42.2)	43.0 (± 43.0)	44.0 (± 42.0)	59.6 (± 41.6)	74.0 (± 35.8)	90.0 (± 0.0)
Erica australis	3	64.3 (± 44.5)	17.3 (± 5.9)	65.0 (± 43.3)	90.0 (± 0.0)	**65.0* (± 43.3)**	**15.0* (± 0.0)**	90.0 (± 0.0)	39.0 (± 44.2)
Lavandula stoechas	3	24.2 (± 36.9)	39.2 (± 46.4)	39.4 (± 46.2)	74.4 (± 34.9)	90.0 (± 0.0)	73.8 (± 36.2)	43.4 (± 42.6)	58.2 (± 43.6)

3.1.3 Physiological performance

The physiological performance of the seedlings growing in different substrates was evaluated through analysis of reflectance indices and growth (height), in plants with five (Test 1) and three (Test 2) months old. Measurements were made in August 2019.

Figure 3.2 – *Lavandula stoechas* reflectance measurement using a spectroradiometer.

There was high mortality among the greenhouse plants (see the legend of Figure 3.3 and 3.4 with the number of living plants). This was transversal to all treatments and explained by the greenhouse conditions that were not controlled, as such, its temperatures could reach very high values in sunny days. Additionally, the pots were very small, retaining low amounts of water. For *C. monspeliensis* and *E. australis* that presented very low survival rates, there were not enough individuals to carry out comparative studies.

Figure 3.3 summarizes the reflectance indices of shrub species. *Erica andevalensis* plants survived in *contaminated 1* and *mixed* substrates. For these, the CHL index was significantly higher in substrate *contaminated 1* plants ($P \leq 0.05$; Figure 3.3.c); for NDVI and PRI, no significant differences were detected, although there was also a tendency for higher values in the more contaminated soil. Generally, higher index values of CHL are associated with stressed plants (Oliva *et al.*, 2010), suggesting that this species performance is better in the mixed substrate than in the *contaminated 1* substrate. The other shrub species only survived in the less contaminated substrates: commercial and *mixed*. In general, there was again a tendency for lower index values in the commercial than in the more contaminated soil (*mixed* substrate, in this case). That difference was significant ($P \leq 0.05$) for CHL index in *C. salviifolius* (Figure 3.3.b and for NDVI ($P \leq 0.01$) in *C. ladanifer* (Figure 3.3.a). For *L. stoechas*, there are no differences in the reflectance indices evaluated in plants growing on commercial and mixed substrates (Figure 3.3.d).

Regarding the herbaceous species, *A. castellana* was the only species established in the substrate *contaminated 2*, where it exhibited significantly higher values for the three evaluated indices, than in the commercial and *mixed* substrates ($P \leq 0.01$; Figure 3.4.a), following the same pattern as the shrub species: higher index values in more contaminated substrates. Similarly, *A. bellidifolium* had also a higher CHL index in plants on the *mixed* substrate than on the commercial ($P \leq 0.01$; Figure 3.4.b).

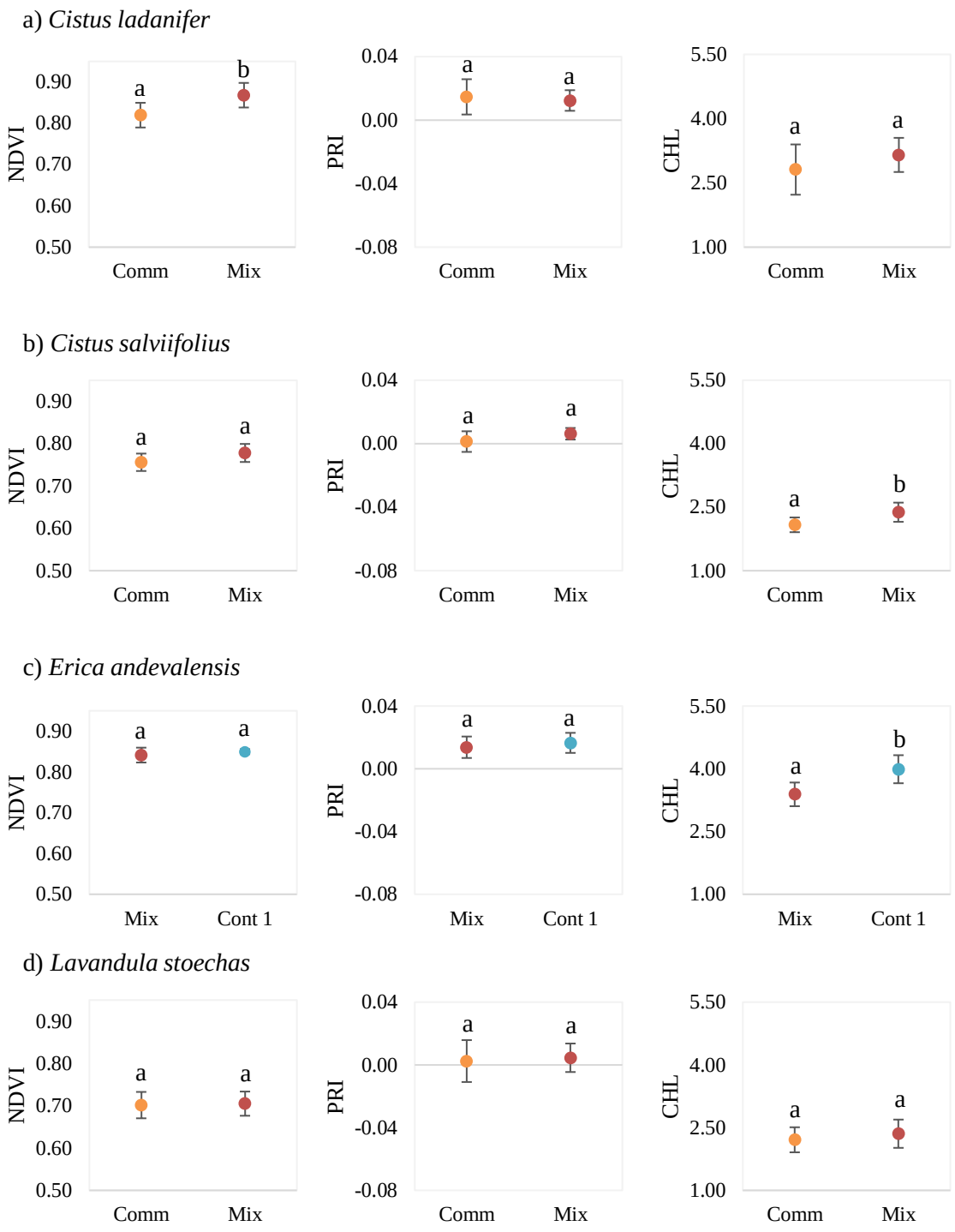

Figure 3.3 – Shrub species medium reflectance indices in greenhouse substrates: Comm – Commercial, Mix – Mixed, Cont1 – Contaminated 1. a) Comm (n=10), Mix (n=8); b) Comm (n=8), Mix (n=7); c) Mix (n=6), Cont1 (n=2); d) Comm (n=7), Mix (n=3). NDVI – Normalized difference vegetation index; PRI – Photochemical reflectance index; CHL – Chlorophyll index. Significant differences are represented by different letters (Dunnett's modified Tukey-Kramer test, α=0.05).

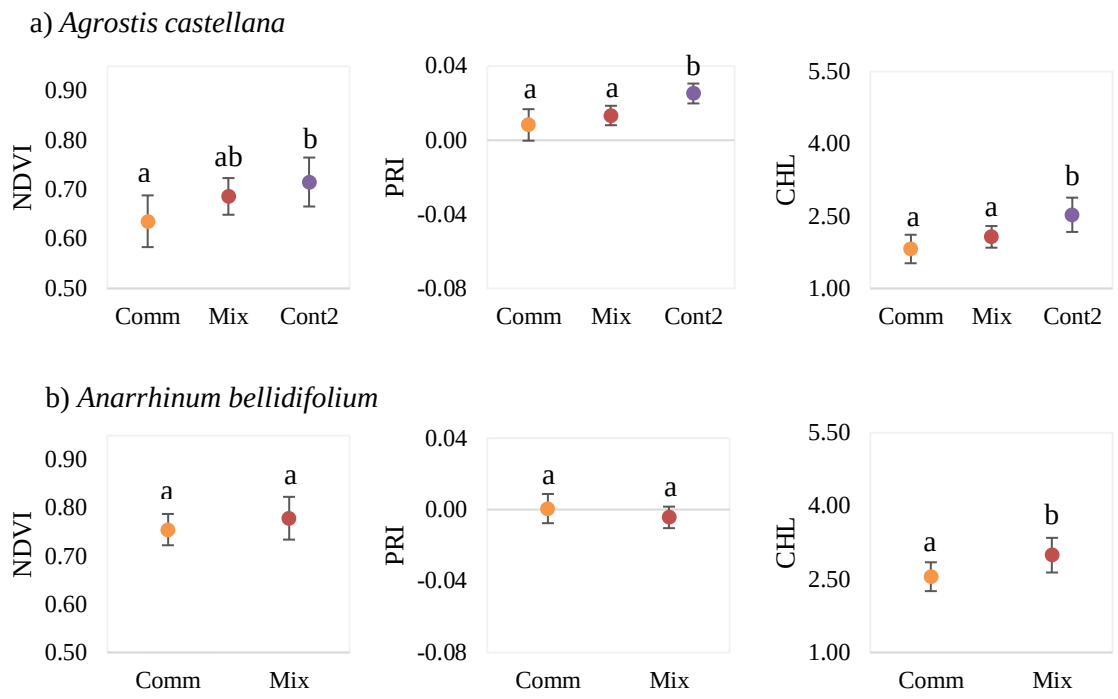

Figure 3.4 – Herbaceous species medium reflectance indices in greenhouse substrates: Comm – Commercial, Mix – Mixed, Cont2 – Contaminated 2. a) Comm (n=10), Mix (n=10), Cont2 (n=5); b) Comm (n=8); Mix (n=9). NDVI – Normalized difference vegetation index; PRI – Photochemical reflectance index; CHL – Chlorophyll index. Significant differences are represented by different letters (Dunnett's modified Tukey-Kramer test, α=0.05).

Regarding growth (final height), it's possible to observe a pattern where the *mixed* substrate plants had slightly, but not significantly, higher maximum height, thus suggesting slightly better physiological performance, than those in the other substrates (Figure 3.5 and 3.6). The only significant difference was found in *C. salviifolius* ($P \leq 0.05$; Figure 3.5.b) and in *A. castellana* ($P \leq 0.05$; Figure 3.6.a), between height of plants in *mixed* and commercial substrates. Thus, growth as an indicator of physiological performance, did not provide much information in this experiment. Nevertheless, considering the growth patterns, if the plants were evaluated for a longer period, this could be a good performance indicator.

The younger plants (T2) were usually smaller than the older plants (T1), but the growth rate in the final month was much higher for the younger plants of *C. ladanifer* and *E. andevalensis*. For these species, it might relate to their particular phenology, they grew faster when seeded late in spring.

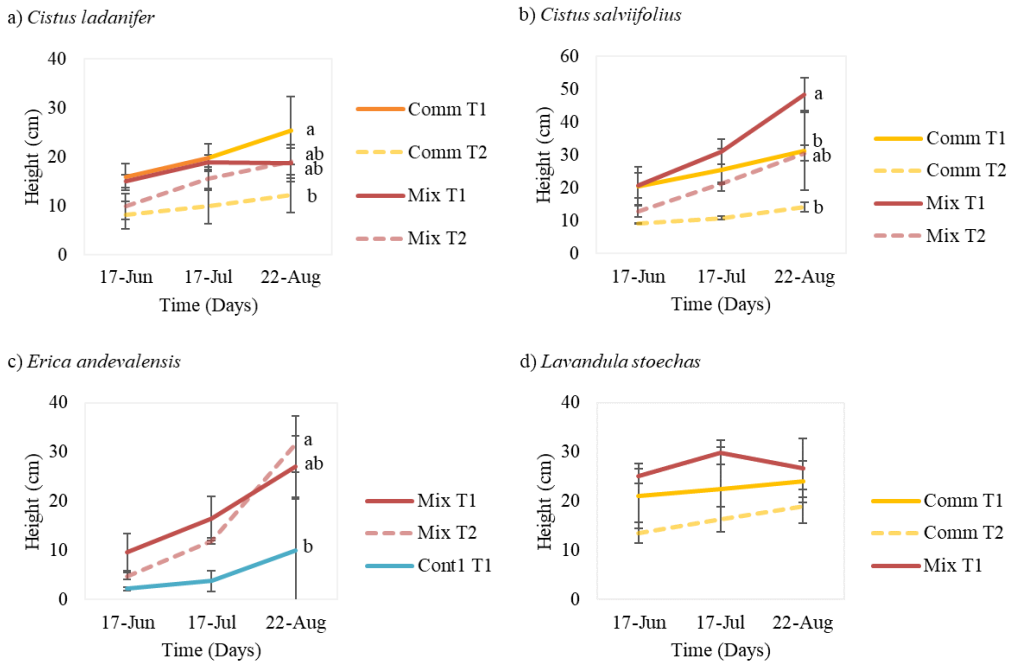

Figure 3.5 – Average maximum height of shrub species in different substrates with different ages: T1 – Test 1 (five months-old in the end of the experiment); T2 – Test 2 (three months old in the end of the experiment); Comm – Commercial, Mix – mixed, Mix – mixed, Cont1 – Contaminated 1. Measurements taken once a month, from June to August 2019. a) Comm T1 (n=6); Comm T2 (n=4); Mix T1 n=4); Mix T2 (n=4); b) Comm T1 (n=6); Comm T2 (n=2); Mix T1 (n=5); Mix T2 (n=2); c) Mix T1 (n=5); Mix T2 (n=2); Cont1 T1 (n=2); d) Comm T1(n=5); Comm T2 (n=2); Mix T1 (n=3). Significant differences in final height are represented by different letters (Dunnett's modified Tukey-Kramer test, α=0.05).

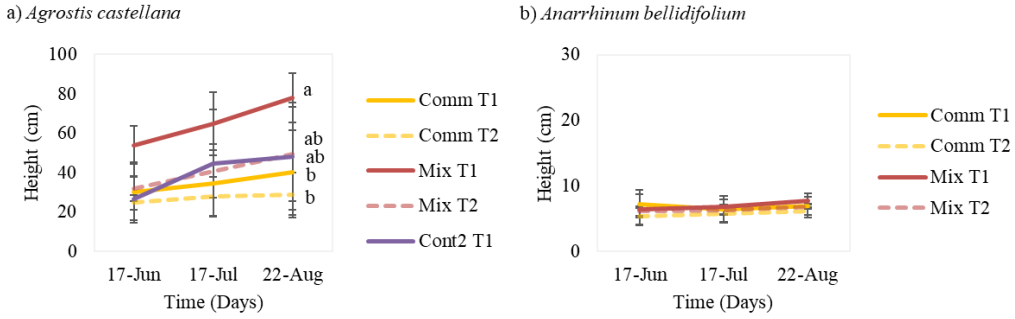

Figure 3.6 – Herbaceous species average maximum height in different substrates: T1 – Test 1 (five months-old in the end of the experiment); T2 – Test 2 (three months old in the end of the experiment); Comm – Commercial, Cont2 – Contaminated 2, Mix – mixed. Measurements taken once a month, from June to August 2019. a) Comm T1 (n=6), Comm T2 (n=4), Mix T1 (n=6), Mix T2 (n=4), Cont2 T1 (n=5); b) Comm T1 (n=4), Comm T2 (n=4), Mix T1 (n=5), Mix T2 (n=4). Significant differences are represented by different letters (Dunnett's modified Tukey-Kramer test, α=0. 05).

There was no statistical treatment of *E. australis* and *C. monspeliensis* results due to lack of data since just one plant survived. Nonetheless, Figure S2.3 shows the height of these two species along the two months of measurements.

3.2 Ecophysiology of local populations

3.2.1 Soils Properties

By examining the relationship between the soil properties, we can estimate which variables can be used to better understand relations between soil and plants. In the soils where the plants were collected we could observe the following patterns (Table 3.4). Despite not being significantly correlated, OM and pH are soil properties of high importance to plants, since OM provides nutrients and improves water retention, whereas pH regulates metal speciation, bioavailability and mineral mobility (Bot and Benites, 2005; Zeng *et al.*, 2011).

According to the user manual of Delta-T Device (2007), EC_b is the measurement of the soil ability to transmit electrical current, and is a function of pore water conductivity, soil particle conductivity, soil moisture content and soil composition. Therefore, higher EC_b values were interpreted as an indicator of increased presence of soluble ions (salts) in both greenhouse and in-situ tests. The abundance of fine sand was related to lower pH values or less organic matter, but the fraction "silt & clay" abundance was, on the contrary, related to higher soil pH values, although no significant relation to OM.

Table 3.4 – Spearman's rank correlation between variables of the *S. Domingos* soil (n=738). The soil properties represented are: pH, OM – organic matter, EC_b – bulk electrical conductivity, granulometry fractions – Medium sand fraction, Fine sand fraction and Silt & clay fraction. Significant levels are represented as: "***" $P \leq 0.001$.

	pH	OM	EC_b	Medium	Fine
OM	-0.06				
EC_b	-0.27***	0.01			
Medium	0.16***	0.34***	0.39***		
Fine	**-0.55*** **	-0.31***	0.01	**-0.58*** **	
Silt & Clay	0.32***	0.01	-0.32***	-0.35***	-0.49***

The pH gradient is represented in Figure 3.7 whereas Figure 3.8 shows the properties of the soil measured for each sampling point: organic matter, pH and bulk electrical conductivity (EC_b). The textural classes of each transect are described in Table S1.5. The percentages of each fraction are represented in Figure S2.4.

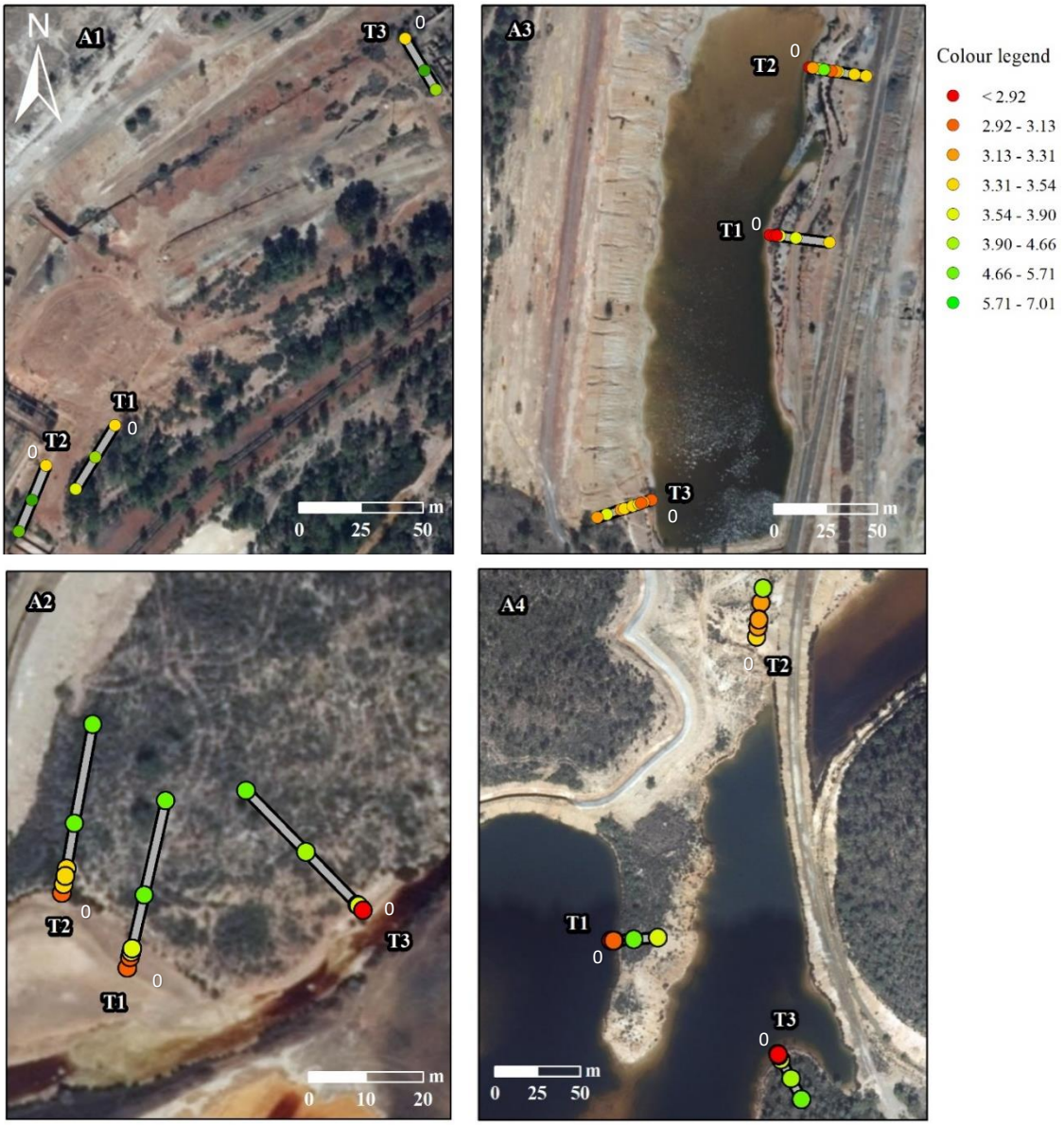

Figure 3.7 – pH gradient of the transects performed in the sampling area of *São Domingos* mine; A – area, T – transect; 0 – Transect initial point. Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroRID, IGN, and the GIS User Community.

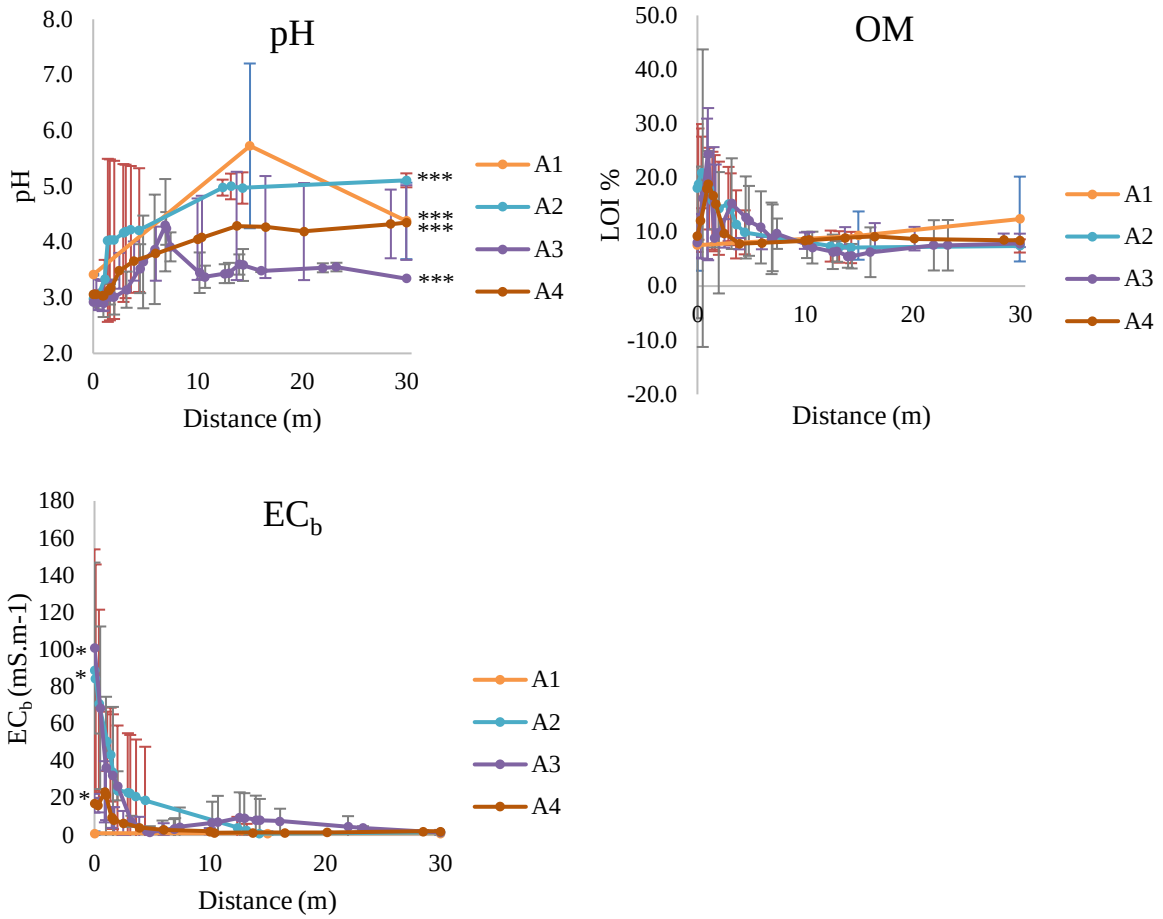

Figure 3.8 – Properties of *S. Domingos* soils of each transect areas. Significant differences between initial (0 m) and final (30 m) sampling points are represented by: "*" $P \leq 0.05$, "***" $P \leq 0.001$. Mean values (± SD) are shown for pH (n=3), organic matter (LOI%, n=3) and EC_b (n=3).

As expected, by choosing transects perpendicular to the putative more contaminated areas (lagoons or other sampling sites), the pH increased from the initial point (average pH_{0m}=3.09 ± 0.22) to final point (average pH_{30m}=4.31 ± 0.73) of transects, suggesting that bioavailable contaminating metals presence might decrease in the same direction (Figure 3.7). Accordingly, bulk electric conductivity generally decreased along the transects. For organic matter calculated by LOI, the results are more variable and site dependent, without significant differences from one end to the other of the transect areas.

3.2.2 Ecological performance

We tend to think of mining sites as inhospitable habitats but there were more species richness, beside the eight studied. Most intercepted species were identified at the species level (except for those belonging to the families of Asteraceae and Poaceae other than *A. castellana*) and are depicted on Table S1.4. From these species, four were trees, nine were shrubs and three were herbaceous.

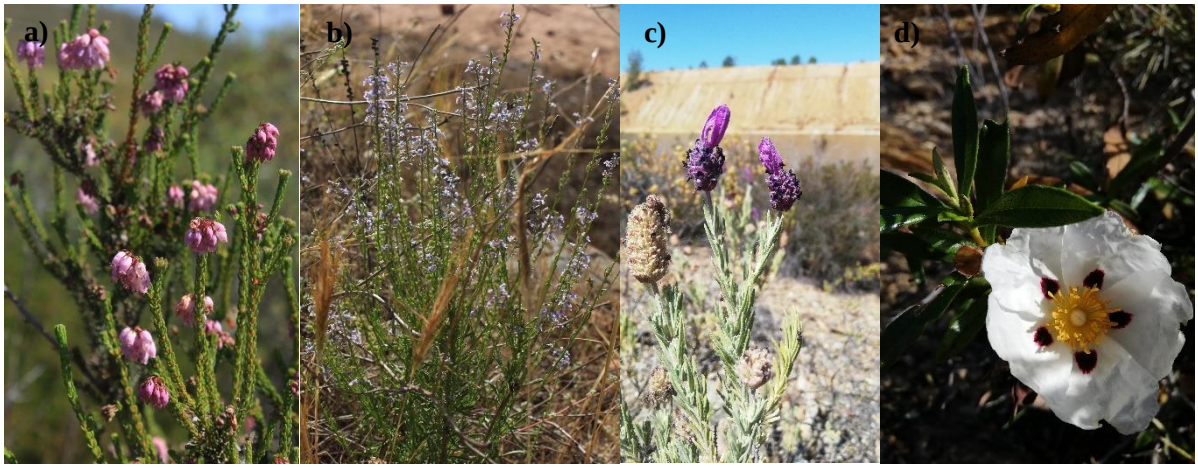

Figure 3.9 – Species in flower found in *São Domingos* mine: a) *Erica andevalensis*; b) *Anarrhinum bellidifolium*; c) *Lavandula stoechas*; d) *Cistus ladanifer*.

In order to assess the ecological performance of each species, that is the abundance in the natural conditions, the plant cover along the 30 m transects was considered in four areas, with 3 transects in each.

The four areas sampled showed a dissimilar abundance of plants (an average of 79 % ± 40% of the transects are covered by plants), but not significantly different among them (Kruskal-Wallis test, $P \geq 0.05$, Figure 3.10). The values of total plant cover exceed 100% due to overlap between the crown of some of the plants.

The eight studied species comprised a large portion of the plant cover in the study areas but were not exclusive. The cover by other species is indicated on Figure 3.10 and the species present are identified in the Figure 3.11. *Anarrhinum bellidifolium* was only found in one zone of one transect, thus it was not possible to analyze its performance. Likewise, *C. salviifolius* was also only found in one area.

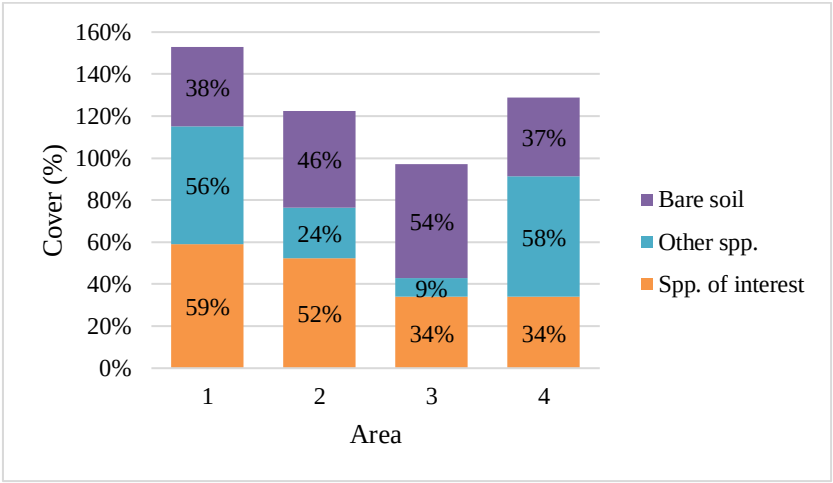

Figure 3.10 – Total cover percentage of species selected for the study (Spp. of interest), other species and bare soil in each study area (mean of three transects per area).

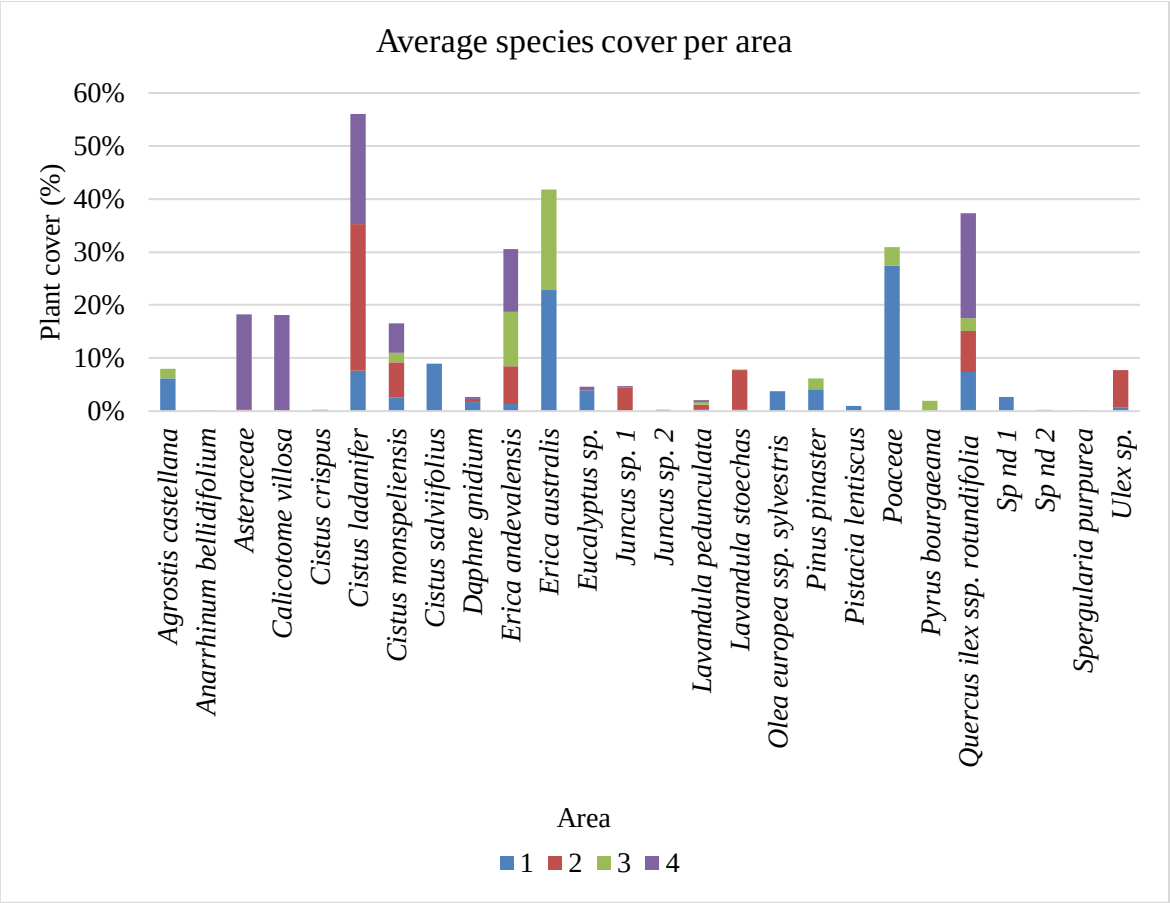

Figure 3.11 – Average species cover per area (n=3 transects per area). Sp nd - Species not identified.

Considering the possible interaction between species (e.g. competition, facilitation), their correlation was analyzed (Table 3.5). The covers of *L. stoechas*, *C. monspeliensis* and *C. ladanifer* were all positively correlated ($P \leq 0.05$; Table 3.5), confirmed by GAM analysis (Figures: S2.5.e, S2.5.c and 3.12.c, respectively). Nevertheless, *C. ladanifer* is the dominant species of the three, with higher cover in the study areas. Both *Erica* spp. are positively correlated ($P \leq 0.05$; Table 3.5).

Regarding other soil variables measured, the results were less interesting as the data was limited, therefore the significance was amplified. Apart from *L. stoechas*, the main result was a negative correlation between pH and OM with significant differences specific for each species (Table S1.6-S1.9).

From the GAM analysis we can evidence a clear pattern (Figure 3.12.a, 3.12.b and 3.13) between *E. andevalensis* and *C. ladanifer*'s cover, despite not having a significant negative correlation between these two species. This is also apparent in field observations (Figure 2.4). In contrary, *E. australis*' cover tends to increase with *C. ladanifer*'s cover. This result is confirmed by Figure 3.13, where *E. australis*' cover peak coincided with the first peak of *C. ladanifer* cover.

Table 3.5 – Spearman's rank correlation of the specific cover of the species of interest (n=38). The species represented are: Acas – *Agrostis castellana,* Abel – *Anarrhinum bellidifolium,* Clad – *Cistus ladanifer*, Cmon – *Cistus monspeliensis*, Csal – *Cistus salviifolius,* Eand – *Erica andevalensis*, Eaus – *Erica australis*, Lsto – *Lavandula stoechas.* Significant differences are represented as: "*" P≤0.05, "**" P≤0.01, "***" P≤0.001.

	Acas	Abel	Clad	Cmon	Csal	Eand	Eaus
Acas							
Abel	0.36*						
Clad	0.31	0.01					
Cmon	-0.05	0.23	**0.33***				
Csal	0	-0.13	0.13	0.09			
Eand	-0.20	-0.09	-0.08	-0.21	**-0.34***		
Eaus	0.09	0.29	0.21	-0.02	**-0.41***	**0.39***	
Lsto	**0.48****	0.3	**0.48****	**0.34***	0.25	-0.28	-0.17

However, *E. australis*, when in the presence of both *E. andevalensis* and *C. ladanifer*, tends to have higher cover between those two species (Figure 3.13). The remaining species' GAM analysis are represented in Figure S2.5 in the appendix. They represent species of low cover percentage in the study areas, thus less important in evaluation of species interaction. The species *A. bellidifolium* was chosen due to its visible representative population, however only one individual was intercepted by the transects. This may have happened as a result of a scattered population.

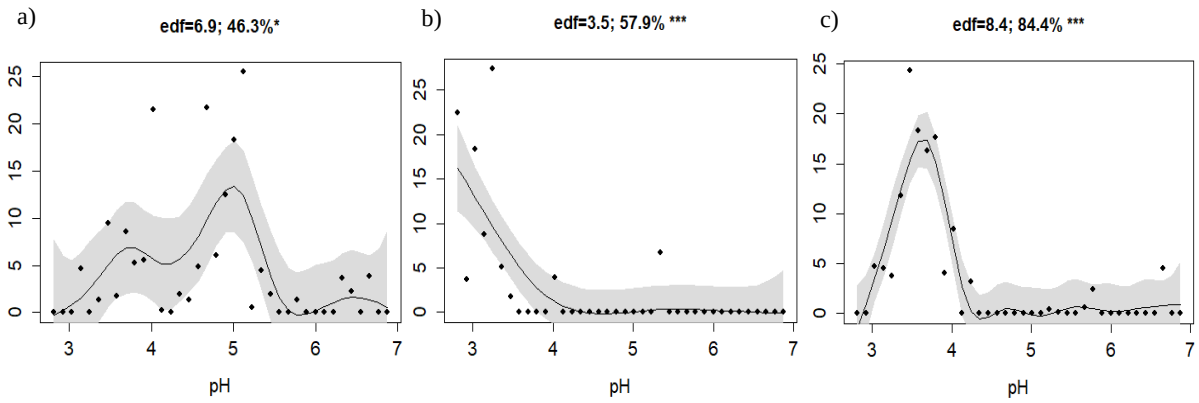

Figure 3.12 – GAM analysis of species' cover and pH. a) *Cistus ladanifer*; b) *Erica andevalensis* and c) *Erica australis*. edf – estimated degree of freedom; % – proportion of null deviance explained; "*" *P*≤0.05, "**" *P*≤0.01, "***" *P*≤0.001.

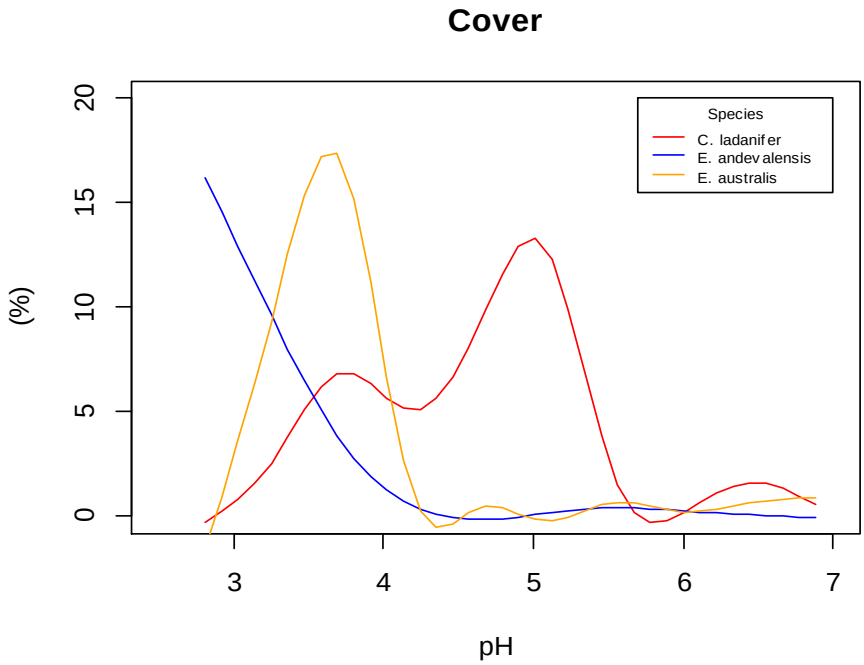

Figure 3.13 – Overlapped model distribution of three dominant species cover (%) along the pH gradient, based on GAM analysis.

3.2.3 Physiological performance

Physiological measurements were performed on the transect intercepted species of interest. Specifically, the values of NDVI, PRI and CHL indices, from each species, tended to remain constant (without significant differences) across the study areas, the same for the SLA (specific leaf area). The only exception was NDVI values of *L. stoechas* that were lower on the more acidic samples ($P \leq 0.05$; Figure 3.14.f). Reflectance indices of *A. bellidifolium* and *C. salviifolius* are represented in Figure S2.6 in the appendix, due to small number of samples.

Yet, by analyzing correlations of the more frequent species (presence in ≥3 areas, namely: *C. ladanifer*, *C. monspeliensis*, *E. andevalensis* and *L. stoechas*), it's possible to get relations between the ecological, physiological and soil variables (Tables S1.6-S1.9).

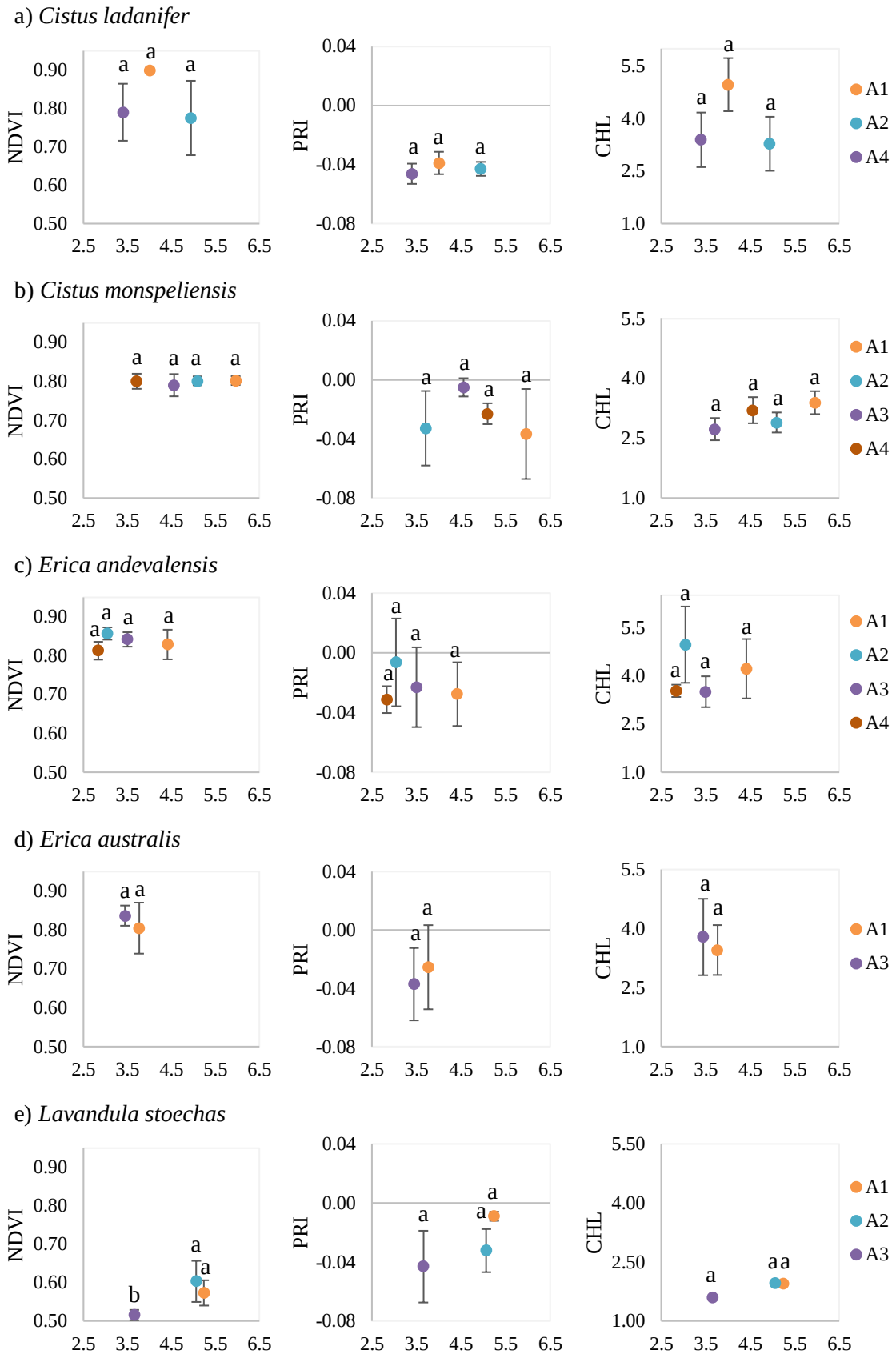

Figure 3.14 – Average shrub species reflectance indices. X axis indicates the interpolated pH of the measured individuals covers. Point colors represent the species presence areas (A = area). Significant differences are represented by different letters (Kruskal-Wallis test, α=0.05).

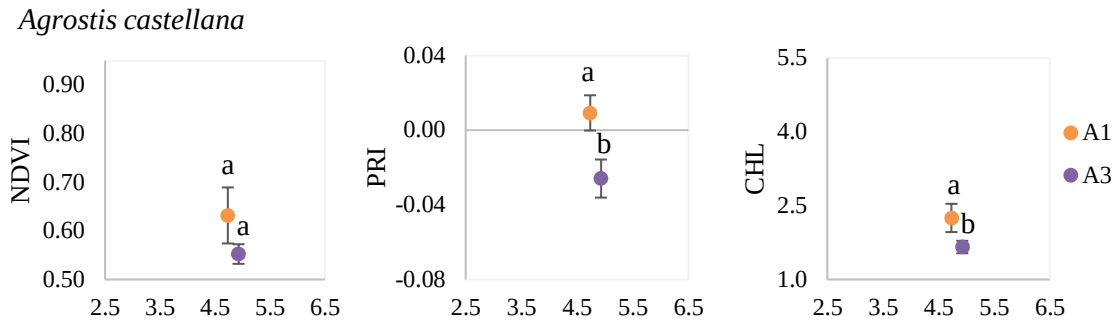

Figure 3.15 – Average herbaceous species reflectance index. X axis indicates the interpolated pH of the measured individuals covers. Point colors represent the species presence areas (A = area). Significant differences are represented by different letters (Kruskal-Wallis test, α=0.05).

Specifically, *C. ladanifer* has an increase of NDVI and CHL values, SLA and height, with the increase of crown area and cover ($P \leq 0.05$; Table S1.6), suggesting that physiological performance and ecological performance were directly related. For *C. ladanifer*, this better performance was positively correlated with the concentration of OM or negatively with soil EC_b ($P \leq 0.001$), but not strongly with pH. *Cistus monspeliensis* reveals the same pattern of positive correlation between cover and crown area, and height ($P \leq 0.01$; Table S1.7), however it has negative correlation between cover and pH, and positive with OM. Likewise, *L. stoechas* has an identical positive response between cover and the reflectance indices ($P \leq 0.05$; Table S1.9), but negative with SLA ($P \leq 0.05$), suggesting smaller or thicker leaves in larger plants or denser areas. Yet, for this species physiological performance (NDVI, CHL, PRI) and ecological performance (cover) were positively associated with both OM and pH, but negatively with EC_b.

In contrast, when *E. andevalensis*'s cover increased, the physiological reflectance indices decreased (namely CHL, $P \leq 0.05$; Table S1.8), suggesting that the species physiological performance is not at its best in the areas where the cover is higher. Furthermore, the pH and OM do not seem to have any significant relation to the species physiological performance because of its wide fundamental niche, however there is a (non-significant) moderate positive correlation between EC_b and NDVI, suggesting a better performance in areas with more salts and possibly more contaminated, but not necessarily more acidic. The same was found for the ecological performance (cover) where the higher correlations were determined with ECb ($P \leq 0.05$). On the other hand, the leaf SLA has weak positive correlation (non-significant) with pH but negative with OM, and positive with crown cover (older plants have larger leaves).

4. Discussion

The knowledge of the ecophysiological performance of spontaneous species in contaminated sites, hence adapted to toxic metals, allow us to successfully establish vegetation covers for ecological restoration projects with phytoremediation potential and low maintenance costs. This work focused on the ecological requirements and physiological performance of eight species present in *São Domingos* mine area. Considering the presence of acid mine drainage (AMD), tailings and other contamination sources, it is expected to find contamination gradients. Hence, different species are likely adapted to different contamination degrees, and this adaptation is reflected in their ecophysiological performance. Additionally, the study of the germination and physiological performance of the seedlings, in different substrates, allowed us to understand the different mechanisms of regeneration and establishment in the contaminated environments. The capacity for colonization of these contaminated areas is dependent not only on the adult plant's performance, but also on the capacity of establishment and adaptive characteristics of the species in these initial phases.

In this work, the pH was used as a proxy for toxic metals contamination, as the toxic metals' bioavailability increases with the decrease of pH (Rieuwerts *et al.*, 1998).

Plant performance varies along the pH gradient, but not always

On the field, *E. andevalensis* colonized every sampled area, mostly at the margin of AMD water bodies, usually with the soil flooded, or run-off areas where pH reaches the lowest values (pH ~ 2.8). Ecologically, the individuals' cover increased as the pH decreased, but more significantly, as the EC_b increased (Table S1.8; Figure 3.12 and 3.13). Thus, EC_b seems to be also a strong driver for the ecological performance of *E. andevalensis*, as well as for its physiological performance, namely for NDVI. This may have happened due to the low capacity of competition, as there were individuals established on higher pH (e.g. pH = 5.34) and will be discussed further. In the greenhouse, *E. andevalensis* was the only species to physiologically demonstrate stable functions in the lowest pH of this work. This species was the only to have survived and established in the *contaminated 1* substrate (the original substrate where the seeds were collected). Higher CHL reflectance index was an indicator of successful establishment in the substrate *contaminated 1* and exhibiting similar values of the remaining reflectance indices (NDVI and PRI – Figure 3.3.a) between substrates. These results are in concordance with Oliva *et al.* (2010) where it was observed that a significant increase of Cu contamination, increased the photosynthetic pigments in *E. andevalensis*. Moreover, *E. andevalensis'* growth in substrate *contaminated 1* was inferior to that of the *mixed* substrate (Figure 3.3.a). Slower growth in more contaminated soils may indicate that plants tend to divert energy/ or resources to increase mechanisms aimed at reducing translocation of toxic metals uptake (Oliva *et al.*, 2010). Although some individuals germinated on the commercial and *contaminated 2* substrates, with a pH of 5 and 4 respectively, they were not able to establish and thrive in these substrates. Acosta *et al.* (2011) showed that increased salinity enhanced the toxic metals mobility, the mobility extension being dependent of the different metals, quantity of the available metals, which soluble salts exist in the soil, pH, and others. With the enhanced mobile fraction of the toxic elements, which is the soluble fraction in the liquid phase according to Rieuwerts *et al.* (1998), together with the acidic pH and higher EC_b, the toxic metals adsorption is increased. Therefore, all these soil characteristics enhanced the toxic metals bioavailability

Nonetheless, similar physiological performance results (Figure 3.14.b) were compliant with other studies that characterize this species (*E. andevalensis*) as an excluder metallophyte. In order to survive these harsh conditions, *E. andevalensis* may rely on metal-exclusion mechanisms to prevent intracellular phytotoxicity (Rossini-Oliva, Abreu and Leidi, 2018). According to these authors, toxic

elements are mostly present in the *E. andevalensis*' roots, associated with *ericoid mycorrhizas* that reduce the uptake of toxic ions (e.g. Fe, As, Si, Mn, Pb, Cu, Zn) by chelation processes and help the micro- and macronutrients acquisition such as phosphorous (Turnau *et al.*, 2007). In shoots, the epidermis has the higher metal content to restrict accumulation in the mesophyll cells, and multicellular glandular hairs can secrete droplets of high toxic metals content (Turnau *et al.*, 2007; Oliva *et al.*, 2010). These mechanisms of metal exclusion can be combined with other metal detoxification processes such as histidine increase in leaves, to form chelators complexes with the toxic ions; peroxidase production and increased photosynthetic pigments content, such as carotenoids and chlorophyll, to reduce ROS damage (Oliva *et al.*, 2010). The association of *E. andevalensis* from early stages of seedlings with soil microorganisms (mycorrhiza or others) is suggested from the greenhouse results of germination that were almost three times more effective than the in-vitro germination where these organisms were not present. Also, substrate *contaminated 2*, being from a different origin than the plants, may lack the microbial species that would be specific for Erica.

Erica australis, another of the studied species, also appears to have thrived in low pH, between pH of 3 and 4 (Abreu, Tavares and Batista, 2008). Like *E. andevalensis*, the cover of *E. australis* was negatively correlated with the pH ($P \leq 0.01$). Although the only seedling survival was on the *mixed* substrate, the final germination percentage (FGP) was very low and similar in all substrates (Table 3.2). These results were compliant with the field observations and other authors, as *E. australis* was only found in soils with pH > 3 (Abreu, Tavares and Batista, 2008). Furthermore, despite being present in only two of the sampled areas, the reflectance indices values did not show any significant differences (Figure 3.14.d) which reflects tolerance to contaminants. Abreu *et al*., (2008) and Márquez-García *et al.*, (2009) verified the high tolerance of *E. australis*, as *E. andevalensis*, to most of the toxic elements present in *São Domingos* mine though in conditions with higher pH. For these reasons, *E. australis* would probably thrive better in slightly less contaminated soils (Figure 3.12.c). Contrary to *E. andevalensis*, this species had lower germination in soil than in-vitro, thus the microbial requirement should not be so strict.

The species, *L. stoechas, A. bellidifolium* and *A. castellana* tend to be present in soils with higher pH which points to a tolerance limit. These results were based on both germination and survival of individuals in the greenhouse tests in the less acidic (pH > 4) substrates: commercial, *mixed* and *contaminated 2* (this latter substrate only for *A. castellana*); and based on the presence of these species in situ (4 > pH > 6). Although *A. bellidifolium* was observed only in Area-1 with highest pH. Also, the greenhouse tests showed higher germination rate in less acidic and with more OM substrates (commercial and *mixed*). The seedlings showed no difference in height as well as in reflectance indices, except for a higher chlorophyll content (CHL) in the *mixed* substrate, which could contribute to lower the ROS damage (MacFarlane and Burchett, 2001). So, this species revealed a low tolerance limit towards toxic metals contamination. However, some studies have recorded tolerance for high contamination with Pb and Zn which may point to a specific toxic elements tolerance (Pratas *et al.*, 2014; Fernández *et al.*, 2017).

The species *A. castellana* has showed decreased physiological performance in extreme acidic soils as the chlorophyll reflectance and the photochemical reflectance index decreased in situ, yet this species was the only to thrive in the *contaminated 2* substrate (with pH of 4.09) with a better physiological performance (higher reflectance indices), but slower growth (lower height). A few authors have mentioned this species capacity of accumulating toxic elements such as Fe (Favas *et al.*, 2014) or As (De Koe, 1994; Schat, 2002; Pastor, Gutiérrez-Ginés and Hernández, 2015) and its capacity to colonize different disturbed environments, thus exhibiting adaptability to different stress situations (Bech *et al.*, 2002; Bleeker *et al.*, 2002; Pastor, Gutiérrez-Ginés and Hernández, 2015). According to Global Biodiversity Information Facility, this species is found around the globe (South America, North America, Europe, Africa and Australia) thus revealing an enhanced habitat plasticity.

As for *L. stoechas*, the decrease of pH may have caused slightly lower physiological values, but only significantly different for the NDVI. These results are compliant with the greenhouse studies: although the germination was higher in the commercial and *contaminated 2* substrate, the seedlings survived and thrived in the *mixed* and commercial substrate with similar reflectance indices and height. As already stated by Pistelli *et al.*, (2017), *L. stoechas* also demonstrated a strong association with the presence of *C. ladanifer* ($P \leq 0.01$; Table 3.5).

Cistus ladanifer demonstrated increased germination rate not only in the commercial substrate but also in the *contaminated 1* and *mixed* substrates. Additionally, showed similar values of physiological performance either in-situ and/or in greenhouse, though NDVI was higher in *mixed* substrate. These results may indicate that this species can adapt to different contamination levels but still prefers slightly disturbed sites (Santos *et al.*, 2009; Santos, Ferreira and Abreu, 2011). *Cistus salviifolius* showed low germination rates in all substrates and the establishment only occurred in the commercial and *mixed* substrate, with similar physiological indices and higher CHL index for *mixed* substrate, which indicates a preference for slightly higher pH soils (Santos, Ferreira and Abreu, 2011; Abreu *et al.*, 2012; Carvalho *et al.*, 2019). Just as happened with *A. bellidifolium*, the in-situ study was insufficient as this species was only intercepted in the area 1. Accordingly, this species was only observed in the least contaminated area of our study.

On the other hand, although we cannot make assumptions about the performance of *C. monspeliensis* in greenhouse, this species was present on every sampled area revealing a good physiological performance on the different transects. These results are compliant with other authors that have identified different tolerance mechanisms that allow this species to establish in low-to-high contaminated places (Arenas-Lago *et al.*, 2016, 2018).

We can conclude that there is a preference for the restrictive and disturbed environments as the species studied generally demonstrated good physiological performance in-situ and preference for the amended substrate (*mixed* substrate) in the greenhouse tests. Therefore, the native species have the highest potential for phytoremediation. Furthermore, by taking action by using the species already present, the metallophyte conservation is ensured (Baker *et al.*, 2010).

Competition vs tolerance in the pH gradient

In this work, pH did not represent a strong pressure, as these species are already adapted to acidic soils.

According to the results and field observations, *C. ladanifer* is the dominant species, beginning to establish when the pH > 3, and where OM and EC_b is also high. This species' cover is positively correlated with other species such as *L. stoechas* and *C. monspeliensis* (Table 3.5), thus stablishing a community. Despite not being negatively correlated with significance with *E. andevalensis* cover, this interaction can be observed in Figure 3.13: as the *E. andevalensis*' cover decreases along the pH gradient, the *C. ladanifer*'s cover increases. Furthermore, in the only sampled area where *C. ladanifer* wasn't present, *E. andevalensis* occurred in higher pH (pH = 4.51, Area 3). These observations may imply that *E. andevalensis* is a weak competitor and may use the low pH as an advantage/strategy for establishment (Abreu, Tavares and Batista, 2008). Abreu *et al*., (2008) cited that Buján *et al.* (2006) encountered *E. andevalensis*' individuals in soils whose pH ascend up to a value of 6.2 in the Spanish IPB, which meets our hypothesis of an wider potential niche where there are not any other competing species (Buján García, 2010). Nonetheless, Abreu *et al.*, 2008) stated that the individuals of *São Domingos* mine may present a different phenotype than those of the Spanish IPB due to the existence of more acidic soils, thus only establishing in soils with a pH up to values of 4. These statements may be valid because in the Spanish IPB, *E. andevalensis* is mostly found in pH≈3, where in *São Domingos* the individuals are found in AMD margins of pH≈2.5, thus representing an adaptation to more acidic

pH than the Spanish ones. Oliva *et al.*, (2010) also highlighted the monospecific and restricted niche and, according to Rufo *et al.*, (2010), it's a subhumid climate and acidic soil shrub community. However, in some transects this species formed mixed communities with *E. australis*: association *Ericetum australis-andevalensis,* next to *C. ladanifer* community (Abreu, Tavares and Batista, 2008; Oliva *et al.*, 2010; Rufo and de la Fuente, 2010). *Erica australis*, a subhumid climate shrub (Rufo and de la Fuente, 2010), only occurred between *E. andevalensis* and *C. ladanifer* in the sampled transects, between pH of 3 and 4, which was confirmed by the GAM analysis (Figure 3.13). This may imply that this species, although only having physiological capacity to stand higher pHs than *E. andevalensis* (according to the previous discussed results), had higher capacity than the latter species to compete against *C. ladanifer* for resources. Some authors have stated that this species may use the lignotuber structure, which is a source of carbohydrates and nutrients, allowing *E. australis* to resprout after disturbances, thus increasing survival (Cruz and Moreno, 2001). According to Cruz *et al.*, (2001), the soil pH was positively correlated with the lignotuber size which may be the reason why *E. australis* still has the capacity to compete for resources between *E. andevalensis* and *C. ladanifer*. The *Cistus* spp. have other strategies to improve competition over the few available resources, as it is an obligate seeder (Calvo *et al.*, 2005). Those strategies might be: high seedbank, leaf litter with allelopathic properties, increasing germination after dry season (Arenas-Lago *et al.*, 2018). Specifically, drought break these species seeds' hard coat (Valbuena, Tárrega and Luis, 1992) and eliminate plants that are possible resources' competitors, favoring species adapted to quickly germinate after intensive temperatures to establish without competition. The germination results are compliant with these hypotheses as heat treatment significantly increased germination for both *C. ladanifer* and *C. salviifolius*, except in *contaminated 2* substrate for the latter (Table 3.2). However, germination rate only decreased for *C. salviifolius*' treated seeds in *contaminated 1* and *mixed* substrate ($P \leq 0.05$; Table 3.3). Moreover, higher correlation between *C. ladanifer* performance and OM (Table S1.6) may be related to leaf litter productivity (Arenas-Lago *et al.*, 2018).

Overall, the results suggest that these species are well adapted to acidic pH, even though in different ranges. Furthermore, they displayed preferences for the mixed substrate which indicates preference for moderately poor soils and with acidic pH. Aside from the mechanisms used to support the toxic contamination, as these species are somewhat adapted to stress conditions, it's necessary to have into account the competition interactions between species in order to have successful establishment and further soil remediation. The soil phytoremediation not only will decrease contaminant migration by water and wind erosion (Abreu, Tavares and Batista, 2008), but has the potential to, rehabilitating former mining areas, increase local recreation activities (already ongoing as seen in field observations) and enhance tourism (Ferreira, 2012).

Final remarks

This study aimed to contribute with knowledge of the ecophysiological performance of eight different species present in *São Domingos* mine and how this knowledge can contribute to future projects on contaminated soils' rehabilitation.

From the eight species evaluated in this study, *E. andevalensis* revealed to be the best species to be used as vegetation cover in higher contaminated soils nearby the AMD water bodies. Furthermore, by providing habitat for this species, we ensure its conservation. Although *E. andevalensis* can form associations with *E. australis*, may thrive better as an alone species. *Cistus ladanifer* and *C. monspeliensis* may be used in low to high contamination soils whereas *L. stoechas*, *C. salviifolius*, *A. bellidifolium* and *A. castellana* may be used in lower contamination areas in association with *C. ladanifer* and *C. monspeliensis*. Due to its successful germination and establishment, *A. castellana* is the potential species to be used in other mining areas, such as *Neves Corvo* mine.

Nevertheless, tests more specific are needed before determining the vegetation species for a plant cover for rehabilitation projects. However, these species may represent potential success in *São Domingos* mine.

Figure 4.1 – *Erica andevalensis* at the foothill of a mining heap and at the margin of an AMD waterbody.

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Appendix I – Supplementary Tables

Table S1.1 – Description of the selected species.

Species	Description	Distribution	Notes	References
Agrostis castellana Boiss. & Reut (Poaceae)	Proto-hemicryptophyte. Perennial. Maximum height of 90 cm. Short rhizome.	Wide distribution across continents: South America, North America, Europe, Africa and Australia	Can inhabit a wide range of habitats – great environmental plasticity.	(Global Biodiversity Information Facilty, 2018; Flora-On, 2019a; Jardim Botânico UTAD, 2019; Naturdata - Biodiversidade em Portugal, 2019)
Anarrhinum bellidifolium (L.) Willd. (Scrophulariaceae)	Hemicryptophyte. Height around 100 cm. Dense or branched inflorescences. Blue or violet-colored flowers.	Present in western Europe.	Establishment on different habitats with acidic or decarbonated substrates.	(Benedí i Gonzalez *et al.*, 2009; Flora-On, 2019b)
Cistus ladanifer L. (Cistaceae)	Phanerophyte. Woody bush with sticky leaves that grows up to 200cm.	Wide Mediterranean distribution: from the occidental Mediterranean region to Morocco and Algeria.	Colonizes disturbed or burned areas forming dense populations.	(Aedo Pérez *et al.*, 2005; Flora-On, 2019c)
Cistus monspeliensis L. (Cistaceae)	Phanerophyte. Shrub of 60-180 cm of height.	Mediterranean region, Madeira and Tenerife islands.	Establishes on low xerophytic or evergreen-sclerophyllous shrublands. Usually, on dry and hot Mediterranean climate.	(Aedo Pérez *et al.*, 2005; Flora-On, 2019d)
Cistus salviifolius L. (Cistaceae)	Phanerophyte or chamaephyte. Height 20-90 cm.	Mediterranean region.	Inhabits low xerophytic scrublands and other forest stands, preferably with acidic substrates.	(Aedo Pérez *et al.*, 2005; Flora-On, 2019e)
Erica andevalensis Cabezudo & J. Rivera (Ericaceae)	Phanerophyte or chamaephyte. Maximum height of 120 cm.	Iberian Peninsula endemism. Found on two main mining areas: São Domingos (Portugal) and Rio Tinto (Spain).	Tolerance to a high concentration of toxic metals. Occupies lagoons borders with origin in mining activity. Under conservation status due to decreasing population size: IUCN threat level as "Vulnerable" for Portugal.	(Castroviejo *et al.*, 1993; Carrascosa and Castrillón, 2008; IUCN Red List, 2016; Flora-On, 2019f)
Erica australis L. (Ericaceae)	Phanerophyte or chamaephyte. Maximum height of 250 cm.	Autochthonous. Found on the Iberian Peninsula and NW Africa.	Frequently found on siliceous or ultrabasic regions, xerophilic and fire disturbed environments, usually sun-exposed.	(Castroviejo *et al.*, 1993; Flora-On, 2019g)
Lavandula stoechas L. (Lamiaceae)	Phanerophyte or chamaephyte. Perennial plant. Maximum height of 150 cm.	Autochthonous species of the Mediterranean and Macaronesia regions.	Colonizes xerophytic shrublands, dominating or under oak forests. Found on dry and sun-exposed environments, with a poor, siliceous or acidic substrate.	(Morales Valverde *et al.*, 2010; Flora-On, 2019h)

Table S1.2 – Potential germination percentage (FGP %). Average values observed under control and heat treatment followed by the standard deviation (mean ± SD); n. - number of replicates with 10 seeds; Significant differences between treatment and control for each species are in bold and indicated by: "*" $P \leq 0.05$. (Kruskal-Wallis test, $\alpha=0.05$).

Species	**n.**	**FGP Control (%)**	**n.**	**FGP Treatment (%)**	**Treatment type**
Agrostis castellana	3	83.3 (± 0.6)	3	90.0 (± 0.0)	80°C/10 min, in agar
Anarrhinum bellidifolium	3	90.0 (± 1.0)	3	93.3 (± 0.6)	80°C/10 min, in agar
Cistus ladanifer	3	**16.7 (± 0.6)**	3	**96.7* (± 0.6)**	100°C/30 min, in filter paper
Cistus monspeliensis	3	6.7 (± 0.6)	3	0.0 (± 0.0)	100°C/30 min, in agar
Cistus salviifolius	3	3.3 (± 0.6)	3	16.7 (± 1.5)	100°C/10 min, in filter paper
Erica andevalensis	3	20.0 (± 1.0)	3	23.3 (± 1.5)	110°C/10 min, in agar
Erica australis	3	20.0 (± 1.0)	3	43.3 (± 3.1)	110°C/10 min, in agar
Lavandula stoechas	3	**16.7 (± 2.1)**	3	**76.7* (± 1.5)**	80°C/10 min, in agar

Table S1.3 – Potential mean T50 germination (days). Average values observed under different treatment types followed by the standard error of mean (mean ± SD); n. - number of replicates; Significant differences for each species are indicated by: "*" $P \leq 0.05$. (Kruskal-Wallis test, $\alpha=0.05$).

Species	**Substrate type**	**n.**	**T50 Control (days)**	**n.**	**T50 Treatment (days)**
Agrostis castellana	Agar	3	3.7 (± 2.9)	3	3.3 (± 0.6)
Anarrhinum bellidifolium	Agar	3	2.3 (± 0.6)	3	3.7 (± 1.5)
Cistus ladanifer	Filter paper	3	5.3 (± 1.2)	3	5.3 (± 2.3)
Cistus monspeliensis	Agar	3	30.7 (± 51.4)	3	90.0 (± 0.0)
Cistus salviifolius	Filter paper	3	90.0 (± 0.0)	3	37.0 (± 45.9)
Erica andevalensis	Agar	3	12.3 (± 4.9)	3	13.7 (± 5.9)
Erica australis	Agar	3	10.0 (± 2.6)	3	11.0 (± 4.6)
Lavandula stoechas	Agar	3	**61.3* (± 49.7)**	3	**2.3* (± 0.6)**

Table S1.4 – Species presence in sampling area. In bold are the species studied. T – transect.

Species	**Type**	**Area 1**			**Area 2**			**Area 3**			**Area 4**		
		T1	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
Agrostis castellana	Herbaceous			x				x					
Anarrhinum bellidifolium	Herbaceous			x									
Asteraceae	Herbaceous					x		x			x		
Cistus crispus	Shrub		x										
Cistus ladanifer	Shrub	x	x		x	x	x				x	x	x
Cistus monspeliensis	Shrub		x			x		x			x	x	
Cistus salviifolius	Shrub		x	x									
Daphne gnidium	Shrub		x	x		x							x
Erica andevalensis	Shrub		x		x	x	x	x	x	x	x	x	x
Erica australis	Shrub	x	x					x	x	x			
Eucalyptus sp.	Tree	x											x
Calicotome villosa	Shrub										x		x
Poaceae	Herbaceous			x						x			
Juncus sp.	-					x	x					x	
Lavandula pedunculata	Shrub			x	x	x			x				x
Lavandula stoechas	Shrub			x	x	x	x	x					
Olea europea ssp. *sylvestris*	Shrub			x									
Pinus pinaster	Tree	x	x							x			
Pistacia lentiscus	Shrub			x									
Pyrus bourgaeana	Tree							x					
Quercus ilex ssp. *rotundifolia*	Tree			x	x		x	x			x		x
Spergularia purpurea	Herbaceous							x	x				
Ulex sp.	Shrub			x	x	x	x						

Table S1.5 – Textural class of initial (P_i) and final (P_f) sampling points of each transect of each area (n=3).

Sampling points			**Textural class**
Area 1	Transect 1	P_i	Loamy sand
		P_f	Sand
	Transect 2	P_i	Sandy loam
		P_f	Loamy sand
	Transect 3	P_i	Sandy loam
		P_f	Sandy loam
Area 2	Transect 1	P_i	Loamy sand
		P_f	Loamy sand
	Transect 2	P_i	Loamy sand
		P_f	Sandy loam
	Transect 3	P_i	Sand
		P_f	Sand
Area 3	Transect 1	P_i	Sand
		P_f	Loamy sand
	Transect 2	P_i	Sand
		P_f	Loamy sand
	Transect 3	P_i	Sand
		P_f	Loamy sand
Area 4	Transect 1	P_i	Sand
		P_f	Loamy sand
	Transect 2	P_i	Sandy loam
		P_f	Sand
	Transect 3	P_i	Sand
		P_f	Loamy sand

Table S1.6 – Spearman's rank correlation between *Cistus ladanifer* ecological variables (Cover and Crown Area, n=108) in green, physiological variables (NDVI, CHL, PRI, SLA, n=9; Height, n=108) in orange and soil variables (pH, OM and EC_b, n=108) in red. Significant levels are represented as: "*" $P \leq 0.05$, "**" $P \leq 0.01$, "***" $P \leq 0.001$.

	Cover	Crown Area	NDVI	CHL	PRI	SLA	Height	pH	OM
Crown Area	**0.72*** **								
NDVI	**0.74***	**0.69***							
CHL	**0.69***	**0.74***	**0.98*** **						
PRI	0.18	0.18	0.42	**0.52**					
SLA	**0.69***	-0.05	0.29	0.2	0				
Height	**0.69*** **	**0.80*** **	**0.69***	**0.74***	0.18	-0.05			
pH	-0.16	**-0.22***	-0.05	0.05	0	**-0.74***	-0.07		
OM	**0.37*** **	**0.20***	**0.74***	**0.69***	0.18	**0.69***	0.15	-0.02	
EC_b	-0.15	**-0.26****	**0.74***	**0.69***	0.18	**0.69***	-0.15	**0.62*** **	**0.19***

Table S1.7 – Spearman's rank correlation between *Cistus monspeliensis* ecological variables (Cover and Crown Area, n=23) in green, physiological variables (NDVI, CHL, PRI, SLA, n=12; Height, n=23) in orange and soil variables (pH, OM and EC_b, n=23) in red. Significant levels are represented as: "*" $P \leq 0.05$, "**" $P \leq 0.01$, "***" $P \leq 0.001$.

	Cover	Crown Area	NDVI	CHL	PRI	SLA	Height	pH	OM
Crown Area	**0.64****								
NDVI	-0.08	0.02							
CHL	0.31	0.06	0.34						
PRI	**0.5**	0.24	0.16	**0.79****					
SLA	0.47	0.22	-0.4	-0.25	-0.11				
Height	**0.57****	**0.75*** **	-0.08	0.31	**0.5**	0.47			
pH	-0.39	-0.33	-0.07	0.15	-0.2	-0.17	-0.35		
OM	**0.54****	**0.52***	0.17	-0.4	-0.07	-0.08	**0.45***	**-0.58****	
$EC_{b\backslash}$	0.36	**0.49***	0.07	-0.15	0.2	0.17	**0.56****	**-0.68*** **	**0.67*** **

Table S1.8 – Spearman's rank correlation between *Erica andevalensis* ecological variables (Cover and Crown Area, n=33) in green, physiological variables (NDVI, CHL, PRI, SLA, n=12; Height, n=33) in orange and soil variables (pH, OM and EC_b, n=33) in red. Significant levels are represented as: "*" $P \leq 0.05$, "**" $P \leq 0.01$, "***" $P \leq 0.001$.

	Cover	Crown Area	NDVI	CHL	PRI	SLA	Height	pH	OM
Crown Area	**0.84*** **								
NDVI	-0.27	0.11							
CHL	**-0.63***	0.15	0.38						
PRI	-0.28	-0.02	**0.54**	**0.75****					
SLA	-0.19	0.48	0.32	0.17	-0.01				
Height	**0.84*** **	**0.85*** **	**-0.53**	0.02	-0.15	-0.32			
pH	-0.04	0.03	0.11	0.15	-0.02	0.48	-0.06		
OM	0.34	0.3	-0.11	-0.15	0.02	-0.48	0.32	-0.29	
EC_b	**0.36***	**0.35***	**0.55**	0.11	0.28	0.02	**0.36***	**-0.45****	**0.59*** **

Table S1.9 – Spearman's rank correlation between *Lavandula stoechas* ecological variables (Cover and Crown Area, n=28) in green, physiological variables (NDVI, CHL, PRI, SLA, n=9; Height, n=28) in orange and soil variables (pH, OM and EC_b, n=28) in red. Significant levels are represented as: "*" $P \leq 0.05$, "**" $P \leq 0.01$, "***" $P \leq 0.001$.

	Cover	Crown Area	NDVI	CHL	PRI	SLA	Height	pH	OM
Crown Area	**0.74*** **								
NDVI	**0.84** **	0.26							
CHL	**0.74***	0.05	**0.92*** **						
PRI	0.05	**-0.69***	0.27	**0.58**					
SLA	**-0.69***	**-0.74***	-0.48	-0.4	0.18				
Height	**0.65*** **	**0.76*** **	**-0.58**	**-0.69***	**-0.74***	-0.05			
pH	0.24	0.1	**0.58**	**0.69***	**0.74***	0.05	-0.02		
OM	0.25	0.31	**0.58**	**0.69***	**0.74***	0.05	0.23	0.24	
EC_b	0.16	0.19	**-0.58**	**-0.69***	**-0.74***	-0.05	0.31	0.31	-0.3

Appendix II – Supplementary Figures

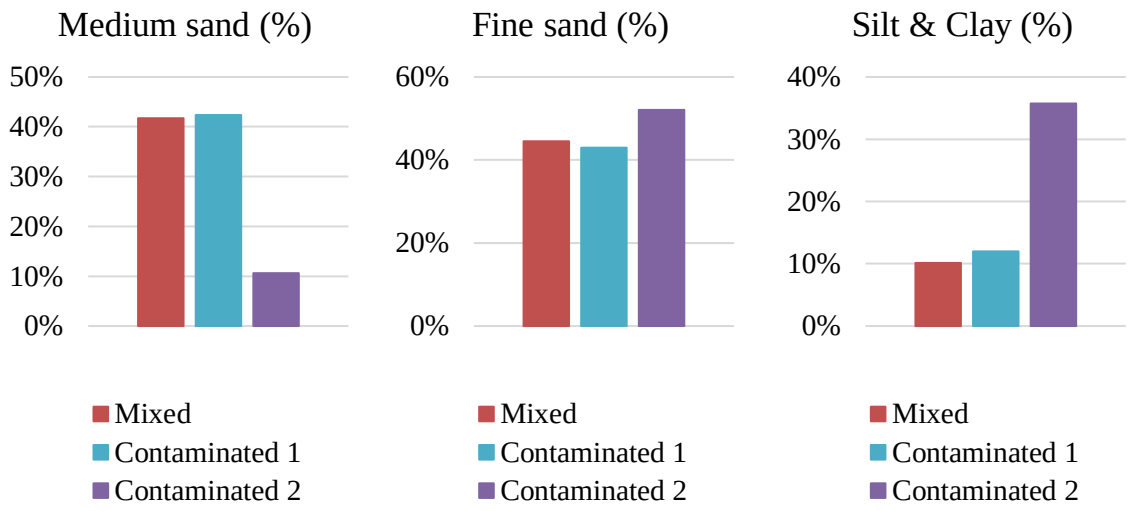

Figure S2.1 – Particle-size distribution of the greenhouse substrates (n=1)

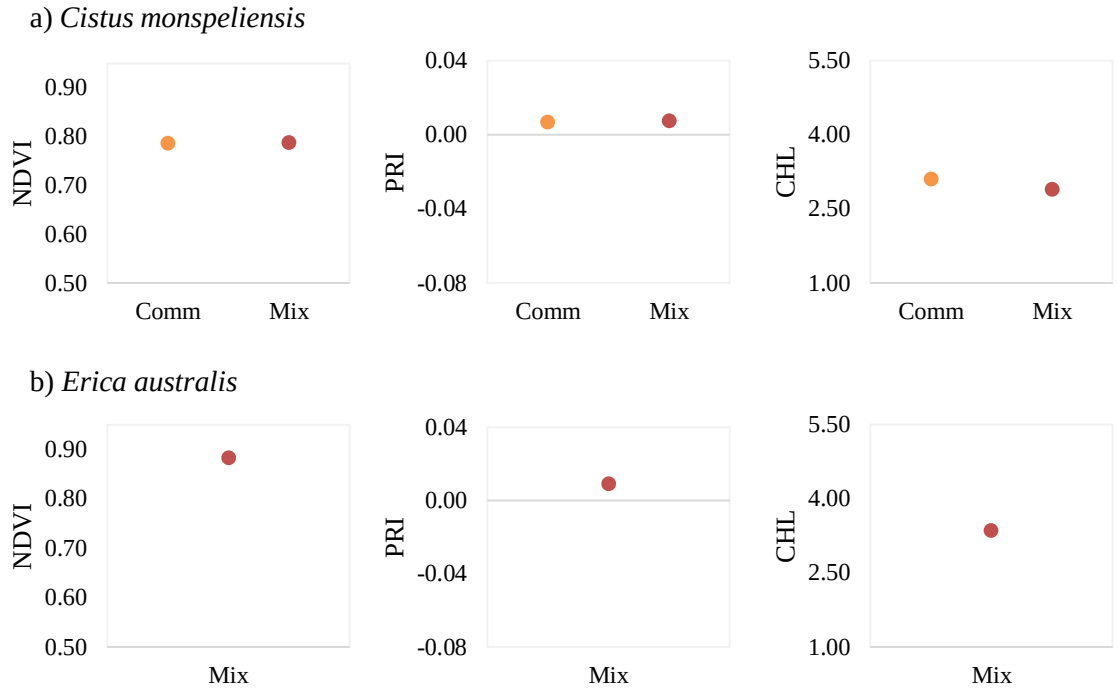

Figure S2.2 – Reflectance indices values of *Cistus monspeliensis* and *Erica australis* in greenhouse substrates: Comm – Commercial, Mix – Mixed. a) Comm (n=1), Mix (n=1); b) Mix (n=1). NDVI – Normalized difference vegetation index; PRI – Photochemical reflectance index; CHL – Chlorophyll index.

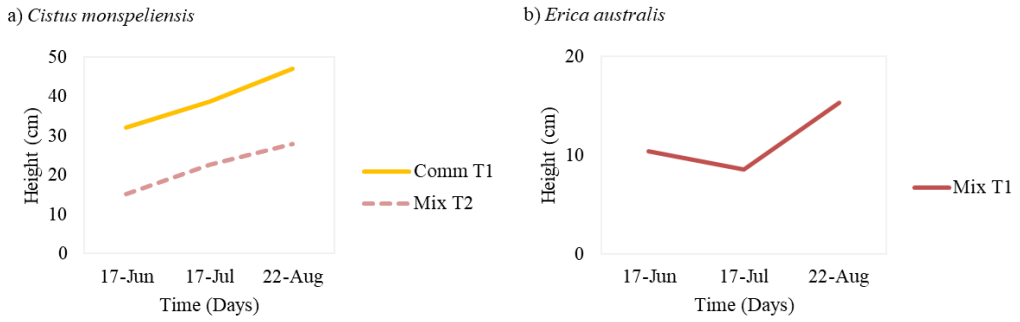

Figure S2.3 – Shrub species maximum height in different substrates: T1 – Test 1 (five months-old); T2 – Test 2 (three months old); Comm T1 – test 1 commercial, Mix T1 – test 1 mixed, Mix T2 – test 2 mixed. a) Comm T1 (n=1), Mix T2 (n=1); b) Mix T1 (n=1).

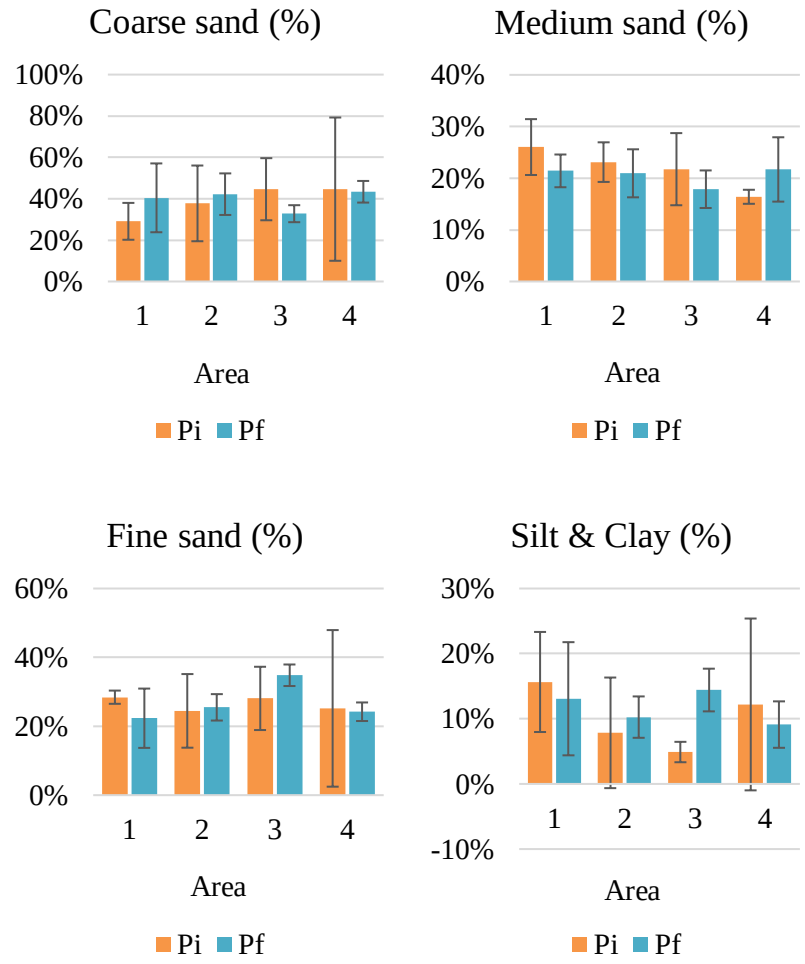

Figure S2.4 – Average particle-size distribution with different fractions: Coarse sand (n=1), Medium sand (n=1), Fine sand (n=1) and Silt & Clay (n=1) fractions; of the initial (Pi) and final (Pf) sampling points of each transect of each area.

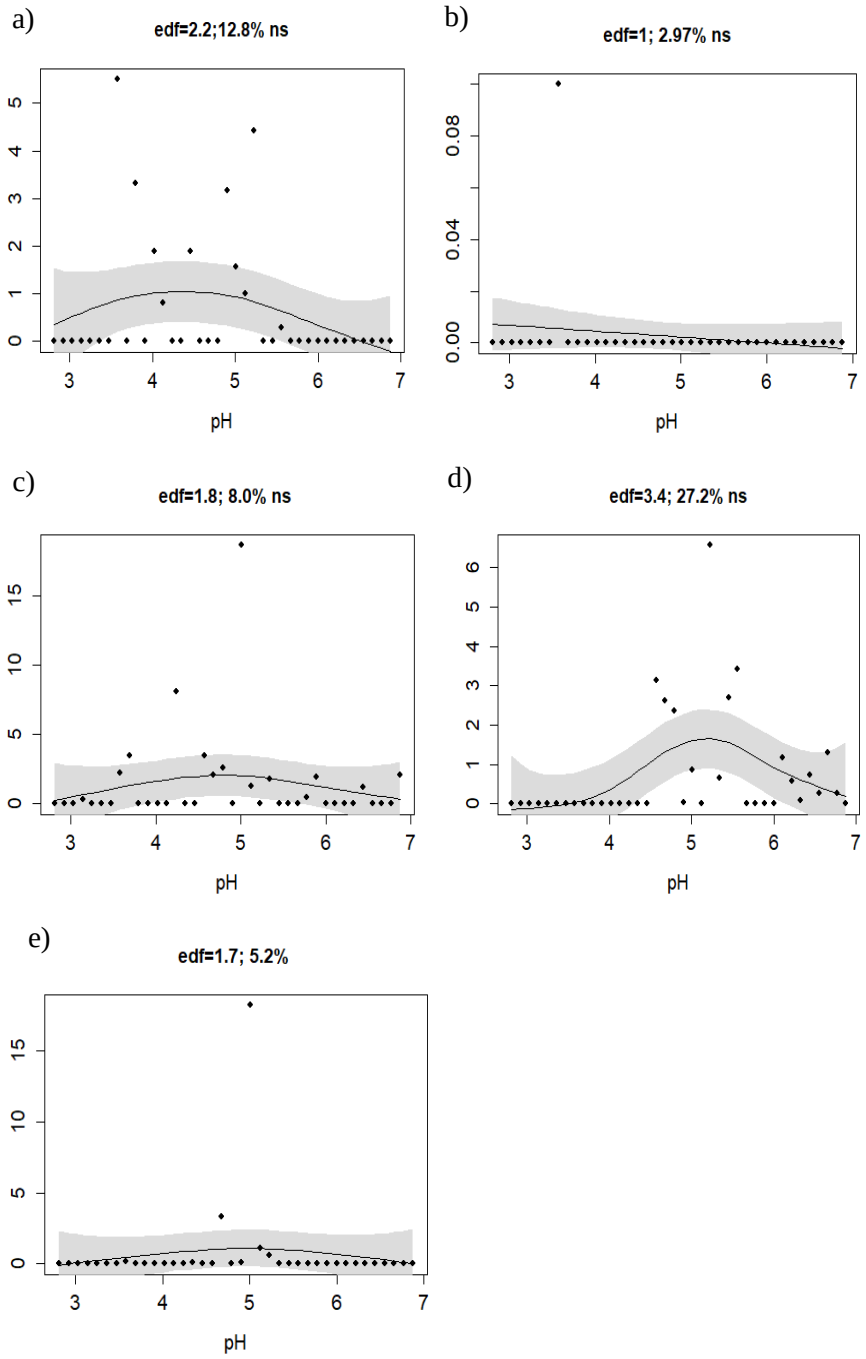

Figura S2.5 – GAM analyses of species' cover and pH. a) *Agrostis castellana*; b) *Anarrhinum bellidifolium*; c) *Cistus monspeliensis*; d) *Cistus salviifolius* and e) *Lavandula stoechas*. edf – estimated degree of freedom; % – proportion of null deviance explained; "*" $P \leq 0.05$, "**" $P \leq 0.01$, "***" $P \leq 0.001$.

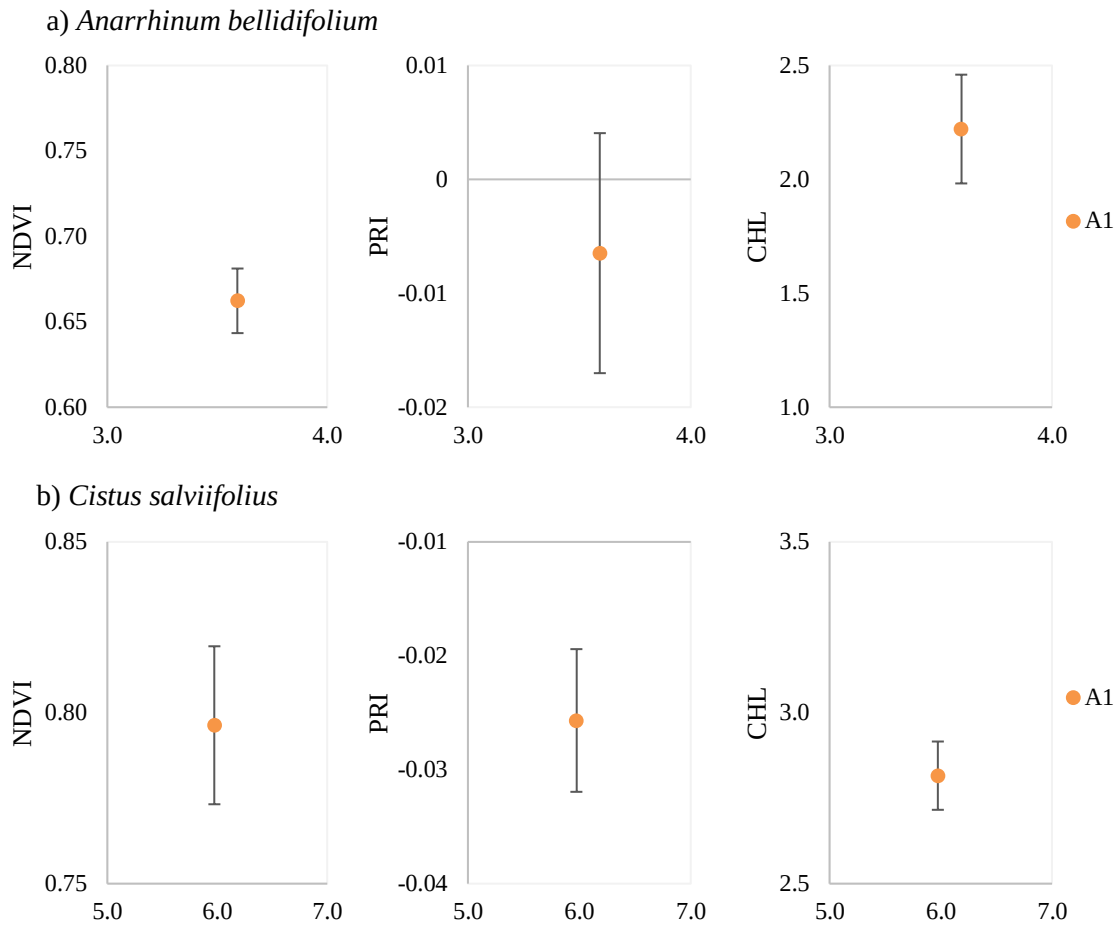

Figure S2.6 – Average reflectance indices in-situ of *Anarrhinum bellidifolium* (n=3) and *Cistus salviifolius* (n=3). X axis indicates the interpolated pH of the measured individuals covers. Point colors represent the species presence areas (A = area).