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Revegetation of abandoned copper mines:

The role of seed banks and soil amendments

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia, realizada sob a orientação científica da Professora Doutora Cristina Nabais (Universidade de Coimbra) e da Doutora Susana Rodríguez Echeverría (Universidade de Coimbra).

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i. Master Thesis Layout

The present master thesis is about the evaluation of the seed bank potential and the role of soil amendments to improve revegetation processes in mining areas. The study area was the Touro's mine, an abandoned open mine of copper in Galicia, Spain, where a large scale project of revegetation is occurring with the aid of technosols, an artificial mixture of several organic residues.

The thesis is divided in three main parts. A first part entitled "Seed Bank Assessment", a second part entitled "Soil amendments" and a third part of "General Conclusions". In the first part, the seed bank potential and seedling establishment is compared between the mine soil, several types of technosols and a control soil outside the mining area. In the second part, two grasses (*Lolium perenne* and *Dactylis glomerata*) and two legumes (*Trifolium subterraneum* and *Medicago sativa*) were grown in mine soil and two types of soil amendments, addition of nutrients, by mixing the mine soil with garden soil (1:1), and by increasing the pH using $CaCO_3$. The growth in height, number of leaves and biomass was recorded and compared among the different treatments. The third part integrates the results of part one and two, summarizing the main conclusions of the thesis.

ii. Resumo

Terras contaminadas, quer com poluentes orgânicos ou inorgânicos, são um problema muito comum. As áreas mineiras são uma das principais causas de entrada de metais pesados no ambiente, além de terem um forte impacto visual. A revegetação é o método mais eficaz para prevenir a erosão e a consequente disseminação de contaminantes para áreas circundantes. No entanto, o crescimento das plantas é condicionado por factores limitantes dos solos de mina tais como o baixo pH, a baixa fertilidade, o baixo teor de nutrientes, as elevadas concentrações de metais pesados e um banco de sementes reduzido para iniciar o processo de estabelecimento de plantas. Em muitos casos, para este estabelecimento ser bem-sucedido é necessário melhorar as propriedades físicas e químicas dos parâmetros do solo.

Na mina de cobre de Touro (Galiza, Espanha), a nossa área de estudo, decorre um projecto de larga escala de alterações de solo com o uso de technosols, uma mistura de vários resíduos orgânicos, para melhorar as condições dos solos mineiros. Duas experiências foram realizadas. A primeira para avaliar o potencial de banco de sementes e o desenvolvimento destas em vários tipos de technosols comparativamente a um controlo fora da mina e ao solo de mina original. O número de plantas foi contado e as espécies identificadas. A segunda experiência foi realizada com solo de mina para avaliar o papel das alterações no solo, especificamente o aumento do conteúdo de nutrientes e pH, na performance de duas gramíneas e duas leguminosas em um estudo individual e em uma mistura de espécies. A altura e o número de folhas foram registados ao longo da experiência e a biomassa final determinada.

Os solos de mina revelaram um potencial muito baixo para a germinação e crescimento de plantas, enquanto os technosols, em geral, facilitam a germinação e

podem ter um bom impacto na revegetação. *Lolium perenne* parece ser a espécie com maior capacidade para suportar as condições adversas de solos de mina. O melhor melhoramento de solo para o crescimento das plantas nas quatro espécies testadas foi a mistura de solo de jardim com solo de mina original. Assim, não é suficiente aumentar o pH, é também necessário adicionar nutrientes para melhorar a germinação e o estabelecimento de plantas.

Em conclusão, a principal razão pela qual as plantas não germinam nem se desenvolvem em solos de minas é o baixo pH e o baixo conteúdo em nutrientes e não tanto devido à não existência de um banco de sementes, tendo em conta que ao redor das áreas mineiras existe vegetação que pode constituir uma fonte de sementes para a área. O aumento do pH reduz a solubilidade dos metais pesados, mitigando um dos problemas associados aos solos de minas. Assim, para garantir e acelerar o estabelecimento de plantas nestes solos, as propriedades destes devem ser melhoradas. É também importante ter uma base de dados das espécies locais e mais comuns dos diferentes grupos funcionais para enriquecer a qualidade e quantidade do banco de sementes. As gramíneas, com o seu sistema de raízes altamente desenvolvido são importantes para estabilizar e reduzir a erosão do solo e as leguminosas, a longo prazo, enriquecem os solos com azoto, devido ao processo de fixação de azoto, preparando a entrada de espécies mais típicas de fases de sucessão ecológica tardia.

Palavras-Chave: Solo de Mina; Technosol; Revegetação; Melhoramentos de solo.

iii. Abstract

Contaminated land, either with organic or inorganic pollutants, is a very common problem. Mining is one of the main causes of entry of heavy metals in the environment, besides having a strong visual impact. Revegetation is the most effective method to prevent erosion and the consequent spread of contaminants to surrounding areas. However, plant growth and establishment is conditioned by limiting factors of the mine soils such as low pH, low fertility, low content of nutrients, high heavy metal concentrations and a reduced seed bank to initiate plant establishment. In many cases for the successful plant establishment it is necessary to improve the physical and chemical properties of the soil parameters.

In Touro's copper mine (Galicia, Spain), our study area, there is a large-scale project of soil amendment with the use of technosols, a mixture of several organic residuals, to improve the conditions of the mine soils. Two experiments were performed. The first one to evaluate the seed bank potential and seedling development on several types of technosols compared with a control outside the mine and with the original mine soil. The number of existing plants was counted and the species were identified. The second experiment was made with mine soil to evaluate the role of amendments, namely increasing the amount of nutrients and pH, in the performance of two grasses and two legumes in an individual and in a mixture of species study. The height and number of leaves were recorded throughout the experiment and the final biomass determined.

Mine soils revealed a very low potential for plant germination and growth while technosols, in general, facilitate plant germination and can have a good impact on the revegetation. *Lolium perenne* seemed the species with the best capacity to support the

adverse conditions of mine soil. The best amendment for plant growth of the four species tested was the mixture of garden soil with the original mine soil. Thus it is not enough to increase the pH, but is also necessary to add nutrients to improve the germination and establishment of plants.

In conclusion, the main reason why plants do not germinate and develop on mine soils is the low pH and nutrients, and not so much because there is no seed bank, taking into account that around the mining areas there is vegetation that can constitute a seed source for the area. Increasing the pH reduces the solubility of heavy metals, mitigating one of the problems associated with mine soils. Thus, to guarantee and accelerate the establishment of plants in mine soils, the properties of the soils must be improved. It is also important to have a database of local and common plant species of different functional groups to enrich the quality and quantity of the seed bank. Grasses, with their highly developed root system are important to stabilize and reduce soil erosion, and legumes, on a long term, enrich the soils with nitrogen, due to the process of nitrogen fixation, preparing the entrance of species more typical of late ecological succession stages.

PART I: SEED BANK ASSESSMENT

1. INTRODUCTION

1.1 Contaminated land and remediation

Contaminated land is a worldwide spread problem and often result from the legacy of industrial activities, waste management practices and mining activity (Gay and Korre, 2006) with a potential threat to human health (Vidali, 2001).

Human assisted pathways for contamination of the environment are many and include disposal of industrial effluents and wastes, sewage sludges, the use of chemicals on agriculture areas, land-fill operations and mining (Prasad and Hagemeyer, 1999; Jabeen *et al.,* 2009).

Conventional techniques to recover the soil, as displacement, excavation or soil washing (Wu *et al.,* 2012), besides being economically expensive, can also have some side effects, like spreading even more the contamination. To prevent these associated problems, other processes to clean up contaminated sites through techniques that decrease or eliminate contamination *in situ* are preferred (Prasad and Hagemeyer, 1999). To achieve those aims other remediation technologies have been developed.

Bioremediation, also known as green technology, is an alternative to conventional techniques for pollutant clean-up (Singh *et al.,* 2008) with the use of microorganisms to reduce or even destroy contaminants in a given polluted area (Boopathy, 2000). This technology has many advantages. The more prominent one is related to the low cost compared to conventional techniques, with *in situ* destruction of the contaminants without harming the surrounding environment (Vidali, 2001). The main disadvantages are a longer time to achieve a reduction in the contamination, the high specificity demanded at the site and the contaminant, the resistance of some compounds to degradation and the question if some products of biodegradation are more noxious than the initial compound (Vidali, 2001).

1.2 Phytoremediation and mining

Phytoremediation, a sub-field of the bioremediation, uses plants to degrade, assimilate or metabolize organic and inorganic pollutants (Susarla *et al.*, 2002) present on contaminated soil, sludges, sediments, and ground water (EPA, 1999). Phytoremediation can be applied to organic and inorganic pollutants but one of the major targets are heavy metals, like copper (Cu), lead (Pb), zinc (Zn), mercury (Hg) and cadmium (Cd) (Salt *et al.,* 1998; Prasad and Hagemeyer, 1999). Within the area of phytoremediation, depending on the contaminated substrate and aim, it is possible to separate in other sub-areas: Phytoextraction, Phytodegradation, Rhizofiltration, Phytostabilization and Phytovolatilization (Salt *et al.,* 1998; EPA, 1999; Lone *et al.,* 2008; Susarla *et al.*, 2002; Jadia and Fulekar, 2009).

One of the major sources of land contamination with metals is mining (McGrath *et al.*, 1995). Mining affects landscapes and its effects are largely irreversible (Haasea and Larondellea, 2012). This activity is associated to an historical soil and groundwater pollution by heavy metals around the world (Chiang *et al.,* 2012). Heavy metal pollution, as a consequence of mining activities, is one the most serious environmental issues (Colin et al, 2012) due to the fact that heavy metals cannot be degraded like other organic contaminants (Ghosh and Singh, 2005; Jadia and Fulekar, 2009) or be broken to non-toxic forms (Jabeen *et al.,* 2009).

Open mine areas have a strong impact on the environment (Álvarez *et al.,* 2011) and to reduce that impact revegetation is the most effective method to restore and integrate these areas into the surrounding landscape (Remon *et al.,* 2005). However, revegetation is not always easy due to the fact that mine soils usually have low fertility, low content of nutrients and contain high heavy metal concentrations which slows

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down, or prevents the revegetation process and consequent stabilization of the mine tailings (Vega *et al.,* 2004). The use of vegetation to stabilize mine tailings is important to decrease the area exposed to erosion and to limit the spread of the metals to nearby communities (Vega *et al.,* 2006; Conesa *et al.*, 2006; Mendez and Maier, 2008).

1.3 Case study: Touro's copper mine, Galicia (Spain)

Touro's mine was an open-sky mine for extraction of cooper, with two opencast mines named Arinteiro and Bama. The area exploited by this mine is associated with the Precambrian basic massif near Santiago de Compostela (Álvarez *et al.,* 2010) and the geological substrate is amphibolite with amounts of Fe and Cu sulphides as pyrite, pyrrhotite, limonite and chalcopyrite (Calvo de Anta and Otero, 1994; Vega *et al.,* 2006). The main problem associated with these mines is the acidic soil and the high solubility of metals such as Al or Fe (Álvarez *et al.,* 2010).

The copper extraction stopped in 1988 but the environmental impact of this area continued not only because of the exposed area *per si* but also because the contaminated mine spoils were used for the construction of rural roads, spreading the contamination potential of the mine (Arias *et al.,* 1998).

The recovery measures started in the beginning of 2003 with the addition of residuals and/or sludge and the planting of *Eucalyptus globulus* Labill (Vega *et al.,* 2005) and continued over the years with success (Fig.1). One of the amendments used were technosols, which according to the World reference base for soil resources (IUSS, 2006) are defined by their "*technical* origin" "dominated or strongly influenced by human-made material" or "sealed by *technique hard rock* (material created by humans, having properties unlike natural rock)" and is used to cover soils "with a layer of

natural soil material in order to permit revegetation". They can be found all over the world in mines, roads, and oil spills. According to the same source many technosols contain toxic substances resulting from industrial processes so it is advisable to take some precautions on handling. It is also possible to add some additional material to technosols, like mussel shells, to aid the soil recovery (Fig.2).

Figure 1: Artificial lake in Touro's copper mine (Galicia, Spain)

with revegetation (2011).

Figure 2: Touro's mine. A: Elaborated residuals (technosol). B: Eucalyptus

with mussel shells.

The report "Una visita a la Mina de Touro: Procesos de Recuperación de Suelos y Aguas de Mina mediante la Valorización Biogeoquímica de Residuos" gives an overview about problematic residuals and the potential of technosols for mine soils recuperation and the possibility to create technosols with different properties (reductive properties, alkaline, adsorbents and fertilizers) to be applied has a function of the contaminant and the corrective aim. Soils on this mine are acidic, with high concentration of Al and deficiencies in mineral such as P, K, N and C delaying, or preventing, the establishment of the vegetation. Technosols improve the revegetation process, by correcting the pH and/or adding nutrients like P and N, mitigating the limiting conditions of mine soils (Calvo, 1991).

The areas of Touro's mine amended with technosols were almost totally covered by dense vegetation (ruderal plants and/or shrubs), while the mine waste heap with no technosols added showed no vegetation cover.

The reports "Humedales de la mina de Touro" (2007) and "Evaluación de Impacto Ambiental del «Proyecto de Recuperación de los Ríos Brandelos, Pucheiras, Felisa, Portapego, Rego das Rozas y Lañas en el entorno de las Minas de Touro»" give detail information about the past and current recovery. The results of the effect of covering the mine area with technosols are very positive in terms of the improvement of soil conditions and consequent growth of vegetation.

1.4 Objectives

In the context of the problematic issue of contaminated sites by mining areas and affected landscapes, this work aims to contribute for a better understanding on how to

improve the revegetation process of abandoned mines in order to reduce their environmental and visual impact.

In view of this goal it was made an evaluation of the seed bank potential, seed germination and plant growth on different soils of the Touro's mine: one control soil from outside the mining area, two mine soils (one from a slope and one from a top flat area) and six technosols with different locations inside the perimeter of the mine.

2. MATERIALS AND METHODS

2.1 Sampling points

Nine sampling points (Table I) were defined in Touro's copper mine (Galicia, Spain) (Figure 3). All tecnosols were made of urban waste and sludge from water treatment plants. Eucalypts were planted on all tecnosols, except on Tec-0, which had been applied just one week before our visit. Control was defined as a eucalypt plantation outside the mine area.

2573 tonnes of mixture of tecnosols (Tec-3 and Tec-4) were applied between 27th March and 4th April 2008 on an area of 5156 m^2, while 5.759 tonnes of Tec-1, Tec2 and Tec-2E were applied between 15th and 26th September 2008 to an area of 11.518 m^2.

Table I: Coordinates and description of the dominant vegetation and soil analyses of the different sampling points.

| Sampling point	Coordinates	Description	Vegetation	O.M.* (%)	pH	P (mg/kg)	K (mg/kg)	Total N (%)
Control	42.89213 N, 8.35004 W	Outside the mine	Eucalypt plantation	9.02	4.5	6	54	0.278
Mine-1	42.87536 N, 8.35345 W	Mine waste heap, slope	No vegetation	4.31	4	33	59	0.16
Mine-2	42.87536 N, 8.35345 W	Mine waste heap, top	Ruderal annual plants	4.31	4	33	59	0.16
Tec-0	42.87678 N, 8.35209 W	Recently applied tecnosol	No vegetation	8.34	8.1	1274	13	0.401
Tec-1	42.87704 N, 8.35434 W	Tecnosol	Eucalypt forest	8.76	7.9	1245	2975	0.471
Tec-2	42.87681 N, 8.35379 W	Tecnosol	Eucalypt forest	8.32	7.9	1306	994	0.229
Tec-2E	42.87704 N, 8.35434 W	Tecnosol	Eucalypt forest	7.44	7.9	1199	957	0.203
Tec-3	42.88546 N, 8.35301 W	Tecnosol + mussel shells	Eucalypt forest	16.38	7.5	1302	433	0.554
Tec-4	42.88535 N, 8.35288 W	Tecnosol	Eucalypt forest	12.82	7.8	1213	311	0.322

*O.M.: organic matter

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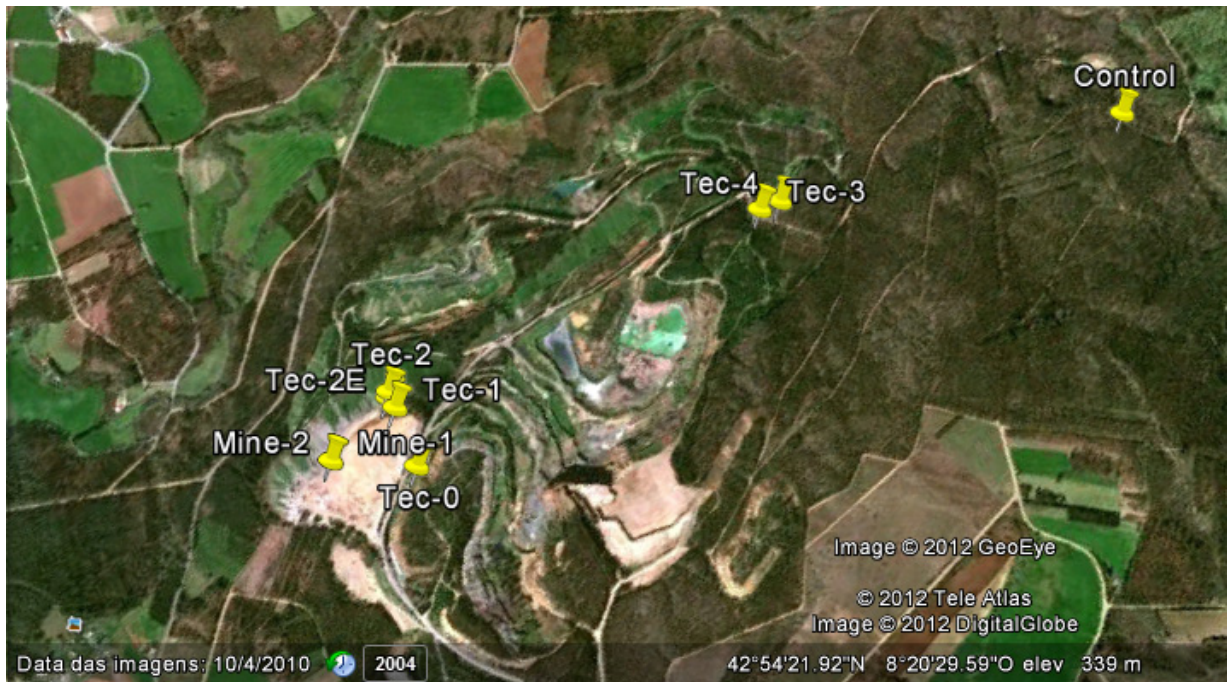
Figure 3: Image from Google Earth of Touro's mine and location of the

sampling points.

2.2 Soil

All soils used in this experiment were collected in June 2011. About 4 kg of soil from the 20-cm upper layer were collected in each sampling point and stored individually in plastic bags.

2.3 Seed bank emergence: Data collection

Each soil sample was divided in four small trays that were placed in a protected area at the Botanical Garden of Coimbra for 115 days, from 24th June 2011 until 17th October 2011. During the time of the experiment trays were kept in the shadow and watered regularly.

Trays were monitored weekly since the beginning of the experiment. At each date the number of existing plants in each tray was counted and photographs were taken to estimate plant cover. The identification of the species present in each tray was made in three different dates: day 38, 73 and 115. Plant diversity was estimated for each treatment and date using the Shannon-Wiener Diversity Index ($H = - \Sigma\ p_i\ \ln\ (p_i)$).

At the end of the experiment all plants were removed, gently shaken in order to remove particles attached to the roots, dried at 60ºC for 4 days and weighted to estimate the production of aboveground biomass in each soil.

2.4 Seed bank emergence: Statistical analysis

Kruskal Wallis and Mann-Whitney tests were applied to check for differences in the number of plants germinated at days 31, 80 and 115 (significance level of 0.05). These analyses were done using STATISTICA 7.

Correspondence analyses (CA) was applied to the log-transformed ($y'=\log(y+1)$) abundance data at days 38, 73 and 115 to analyze the plant community obtained in each soil. These analyses were carried out using Canoco and CanoDraw for Windows 4.5.

3. RESULTS

3.1 Number of individuals

Figure 4 shows the mean number of plants that germinated in the different soils during the experiment. The average number of plants that germinated ranged from values close to zero in Mine-1, Mine-2 and Tec-0 to around 100 individuals in Tec-2, with differences among the different types of soils tested.
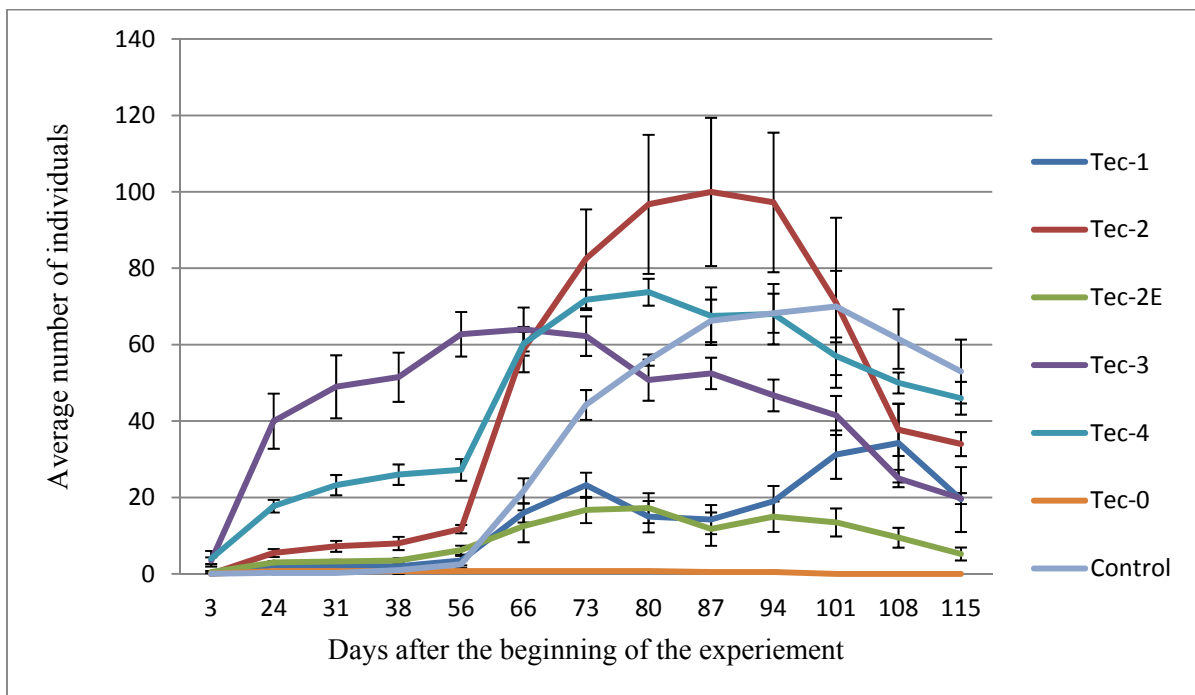

Figure 4: Average number of individuals ± SE in the studied tecnosols and the control throughout the experiment. Mine-1 and Mine-2 were not included in this graph because the average number of individuals was too small. The individual graphs of these two types of soil were included in the Appendix I.

There was a general increase on germination from day 56 onwards. The number of plants in the control soil reached values around 70 plants/tray. In Mine-1 there were no plants until day 66, being the maximum average number of plants 6.25 on day 94. In Mine-2 there were plants just in day 73 and 80, with an average number of individuals

of 0.25. These results indicate that seedling emergence and survival in the mine waste is very difficult. The absence of germination in soil Tec-0 can be explained by the absence of seeds in this soil since it was placed in the field just before our sampling. In Tec-1, Tec-2, Tec-2E, Tec-3 and Tec-4 there were plants throughout all the experiment. Tec-2 had the highest number of plants and Tec-2E had the lowest number. There was a general decrease on the number of plants found in each soil at the end of the experiment, likely due to the absence of new germinated seedlings and the mortality of young seedlings.

Significant differences ($p<0.05$) were found between the different treatments in the three sampling dates analyzed (Table II and Figure 5).

Table II: Results of Kruskal Wallis's test on days 31, 80 and 115 concerning the number of germinated plants in the different soils.

Day	H	P
31	33.59178	0.0000
80	33.21133	<0.0001
115	32.49321	<0.0001
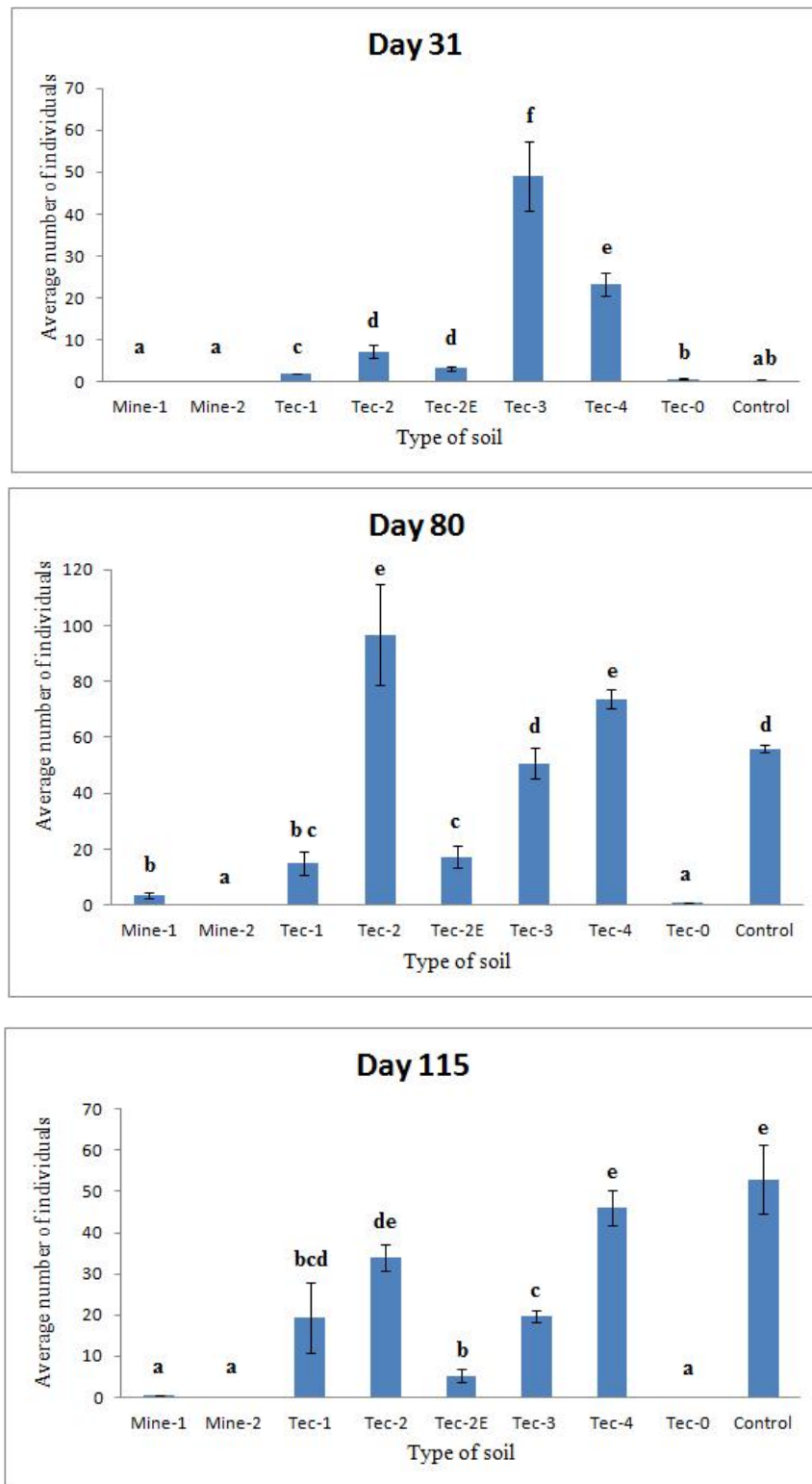
Figure 5: Average number of individuals ± SE at day 31, 80 and 115. Different letters above the bars mean significant differences between each soil type after non-parametric tests. Note that the scale used on the graph of day 80 is different from the scale used in the other two days.

On day 31, there were no statistical differences between Control, Mine-1 and Mine-2 and between Tec-2 and Tec-2E. Tec-3 was the soil with the highest number of individuals followed by Tec-4. On day 80, the highest number of individuals was found in Tec-4 and Tec-2 and these values were significantly higher than the control. There were no significant differences between Mine-2 and Tec-0. On day 115, there were no statistical differences between the soils from Mine-1, Mine-2 and Tec-0 which had very few plants. Tec-2, Tec-4 and Control had the highest values reaching 50 plants in the Control soil.

3.2 Plant cover

The percentage of ground covered by plants, showed in figure 6, was almost zero in Mine-1, Mine-2 and Tec-0 due to a very low number of individuals. The three types of soil that showed a higher plant cover were Tec-3, Tec-4 and Tec-2. These three types of soil also contained the highest number of plants.
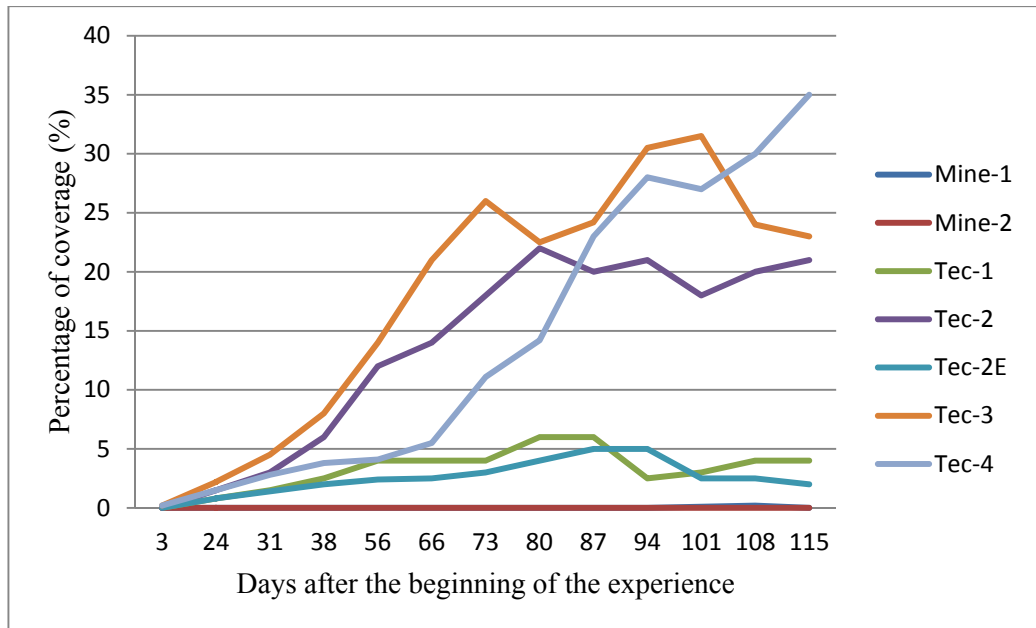
Figure 6: Percentage of plant cover for all types of soil throughout the experiment. Mine-1 and Mine-2 were not included in this graph because the % of coverage was too small.

Plant cover on day 31, 80, 115 is illustrated, for all types of soil, in figure 7. A more detailed photographic record of the evolution of the germination and plant growth in the different soil types is presented in Appendix II.

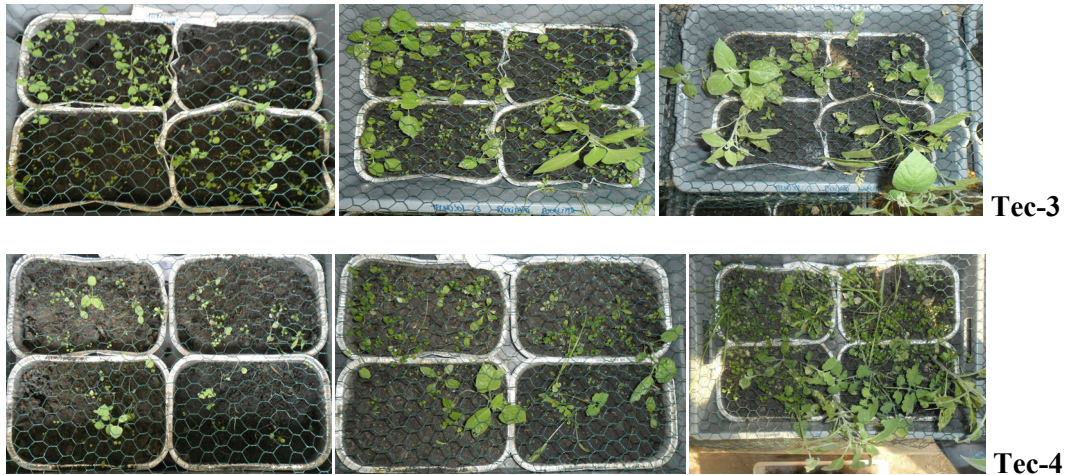Day 31   Day 80   Day 115

Mine-1

Mine-2

Control

Tec-0

Tec-1

Tec-2

Tec-2E

Tec-3

Tec-4

Figure 7: Photographs of the trays showing the evolution of the plant cover

in all types of soils on day 31, 80 and 115.

3.3 Richness and species diversity

Figure 8 and 9 shows the species richness and the Shannon-Wiener diversity index, respectively, on days 38, 73 and 115. Tec-4 showed the highest number of identified species in all three days. Mine-1 and Mine-2 had no identified species due to their small size.

According to the Shannon-Wiener index and analyzing the three days, the diversity increases over time in soils Tec-2 and Tec-3 and decreases in Tec-1. In Tec-4 increases from day 38 to day 73 but decreases in day 115. This index is directly related to the number of identified species (Figure 8) and the number of individuals of each species. However, these results have to be taken with some caution since not all plants were identified because of their small size.
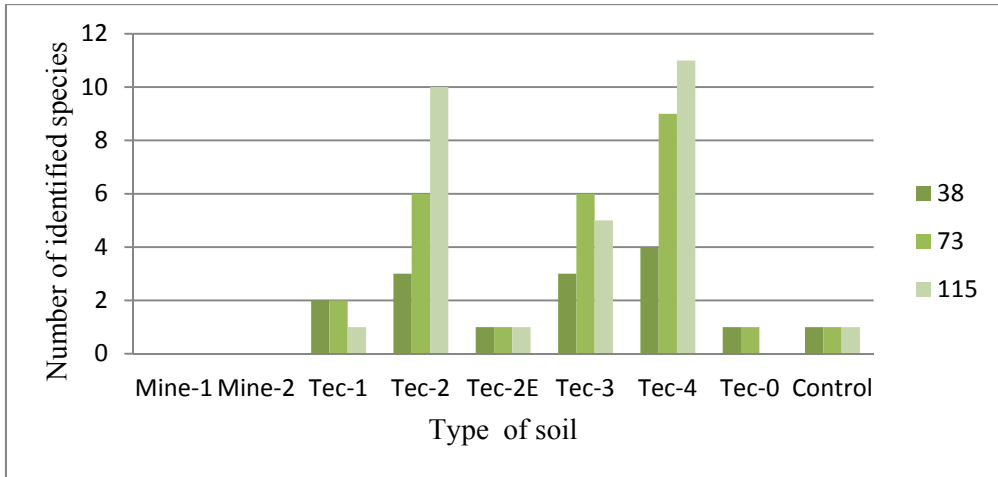
Figure 8: Species richness in all types of soil on days 38, 73 and 115.
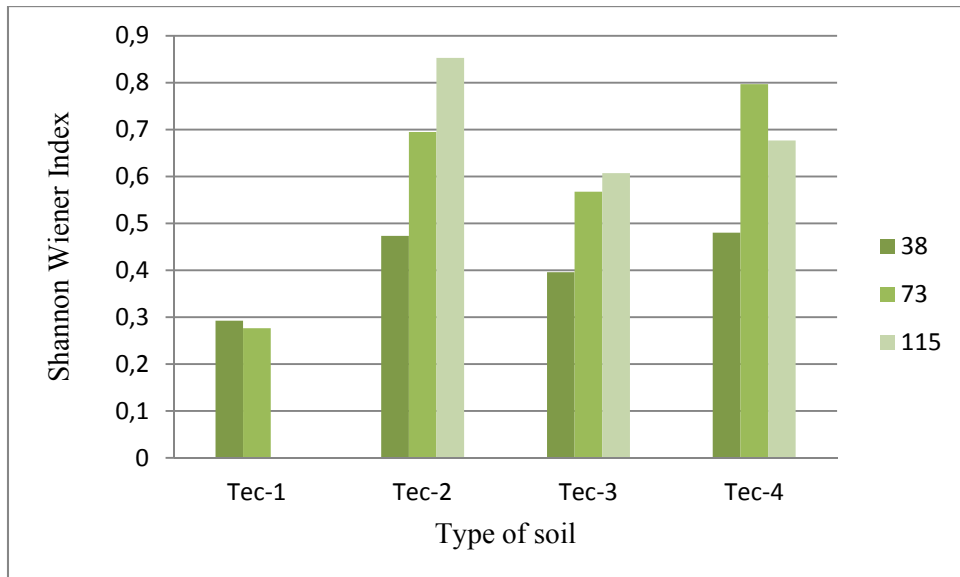

Figure 9: Shannon Wiener Index in four types of soil on days 38, 73 and

115. Mine-1, Mine-2, Tec-0, Tec-2E and Control were not included in this

graph because the Index was zero in these three days.

Plants belonging to 8 different families were found during the experiment (Table III). All identified species were classified as ruderal, although most of the plants could not be identified during the seedling stage. There were differences in their relative abundance in each soil and sampling date (Table IV, V and VI).

Table III: Families and species identified in all types of soils.

Family	Species
Amaranthaceae	*Chenopodium album* L.
Asteraceae	*Carduus tenuiflorus* L.
	Coleostephus myconis (L.) Reichenbach
	Conyza canadensis (L.) Cronquist
	Gnaphalium luteo-album L.
	Picris echioides L.
	Sonchus oleraceus L.
Brassicaceae	*Cardamina hirsuta* L.
Geraniaceae	*Geranium purpureum* L.
Papaveraceae	*Chelidonium majus* L.
Poaceae	*Avena sp.*
	Poa annua L.
Solanaceae	*Solanum nigrum* L.
Urticaceae	*Parietaria judaica* L.
	Urtica membranacea (Poir.)

Table IV - Species abundance, richness and diversity on day 38 after the beginning of the experiment.

	Mine-1	Mine-2	Tec-1	Tec-2	Tec-2E	Tec-3	Tec-4	Tec-0	Control
Chelidonium majus	-	-	-	-	-	5	8	-	-
Geranium purpureum	-	-	-	-	3	-	-	-	-
Parietaria judaica	-	-	2	4	-	10	3	-	3
Sonchus oleraceus	-	-	3	4	-	24	12	1	-
Urtica membranacea	-	-	-	3	-	-	1	-	-
Unidentified plants	0	0	3	21	11	167	80	2	1

Table V - Species abundance, richness and diversity on day 73 after the

beginning of the experiment.

	Mine-1	Mine-2	Tec-1	Tec-2	Tec-2E	Tec-3	Tec-4	Tec-0	Control
Cardamina hirsuta	-	-	-	-	-	-	13	-	-
Chelidonium majus	-	-	-	-	-	36	11	-	-
Chenopodium album	-	-	-	2	-	-	1	-	-
Conyza canadensis	-	-	-	-	-	-	1	-	-
Geranium purpureum	-	-	-	-	4	5	-	-	-
Parietaria judaica	-	-	1	6	-	15	3	-	4
Picris echioides	-	-	-	1	-	-	3	-	-
Poa annua	-	-	-	1	-	-	4	-	-
Solanum nigrum	-	-	-	-	-	1	-	-	-
Sonchus oleraceus	-	-	2	4	-	26	5	1	-
Urtica membranacea	-	-	-	4	-	1	1	-	-
Unidentified plants	6	1	90	312	63	165	245	2	173

Table VI - Species abundance, richness and diversity on day 115 after the beginning of the experiment.

	Mine-1	Mine-2	Tec-1	Tec-2	Tec-2E	Tec-3	Tec-4	Tec-0	Control
Avena sp.	-	-	-	1	-	-	-	-	-
Cardamina hirsuta	-	-	-	-	-	-	35	-	-
Carduus tenuiflorus	-	-	-	-	-	-	1	-	-
Chelidonium majus	-	-	-	-	-	2	12	-	-
Chenopodium album	-	-	-	1	-	-	1	-	-
Coleostephus myconis	-	-	-	-	-	-	1	-	-
Conyza canadensis	-	-	1	1	-	-	1	-	-
Geranium purpureum	-	-	-	-	4	5	-	-	-
Gnaphalium luteo-album	-	-	-	3	-	-	-	-	-
Parietaria judaica	-	-	-	9	-	9	4	-	4
Picris echioides	-	-	-	1	-	-	2	-	-
Poa annua	-	-	-	3	-	-	4	-	-
Solanum nigrum	-	-	-	1	-	-	-	-	-
Sonchus oleraceus	-	-	-	2	-	5	3	-	-
Urtica membranacea	-	-	-	2	-	1	1	-	-
Unidentified plants	1	0	77	112	17	57	119	0	208

In Tec-1 the most abundant species on day 38 and 73 was *Sonchus oleraceus*, while *Conyza canadensis* was the most abundant species on day 115. In Tec-2 the two most abundant species on day 38 were *S. oleraceus* and *Parietaria judaica*, with *P. judaica* being the most abundant identified species on day 73 and 115. In Tec-2E the most abundant species on these three days was *Geranium purpureum*. In Tec-3 the most abundant species were *S. oleraceus* on day 38, *Chelidonium majus* on day 73 and *P. judaica* on day 115. In Tec-4 the most abundant species on day 38 was *S. oleraceus* and

Cardamina hirsuta on day 73 and 115. In Tec-0, *S. oleraceus* was the most abundant species on day 38 and 73. On day 115 there were no plants in this soil. Finally, *P. judaica* was the most abundant species on the Control soil over the three days.

3.4 Correspondence analyses

The results of the CA separated Tec-2E from all other soil types along the axis X, for every date (Figure 10). This difference seems to be related to the abundance of *G. purpureum* in this soil type. The remaining soils were separated along the axis Y defined initially by the presence of *Urtica membranaceae, P. judaica, C. majus* and *S. oleraceus*. Other new plant species also contributed to the placement of the different soils along axis Y in days 73 and 115.

For day 38 the first axis explained 70.1% of the total variance and the first and second axis explained 83.7%. The CA performed in day 38 showed that the plant community in Tec-2 was the most similar to that of the control soil. Tec-0, Tec-3 and Tec-4 were very similar probably due to the relative abundance of *C. majus*. For day 73 the first axis explained 43.7% of the total variance and the first and second axis explained 67.5%. The CA performed in day 73 showed that the plant community in Tec-1 was the most similar to that of the control soil. For day 115 the first axis explained 38.5% of the total variance and the first and second axis explained 62.2%. The CA performed in day 115 showed that the plant community in Tec-2 was the most similar to that of the control soil.
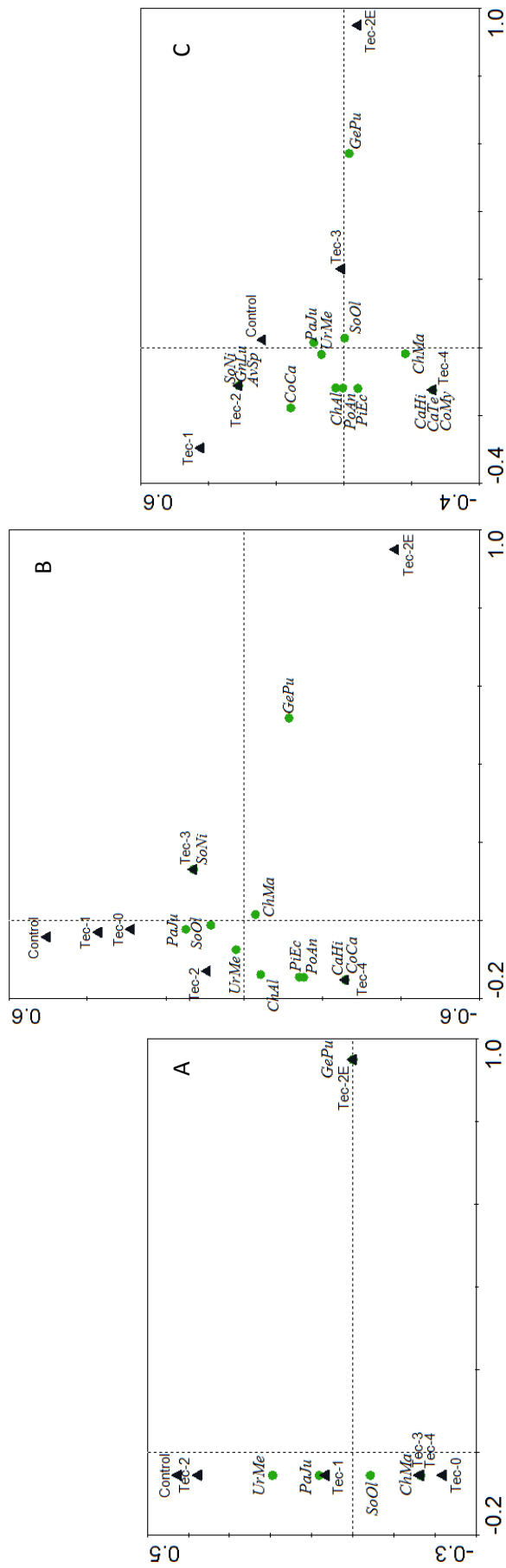
Fig. 10 - Results of CA performed on species abundance data for day 38 (A), 73 (B) and 115 (C). The species are identified by

abbreviations: AvSp: *Avena sp.*; CaHi: *Cardamina hirsuta*; CaTe: *Carduus tenuiflorus*; ChAl: *Chenopodium album*; ChMa: *Chelidonium*

majus; CoCa: *Conyza canadensis*; CoMy: *Coleostephus myconis*; GePu: *Geranium purpureum*; GnLu: *Gnaphalium luteo-album*; PaJu:

Parietaria judaica; PiEc: *Picris echioides*; PoAn: *Poa annua*; SoNi: *Solanum nigrum*; SoOl: *Sonchus oleraceus*; UrMe: *Urtica*

membranacea.

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3.5 Plant biomass

No plants were found at the end of the experiment in either of the two analyzed Mine waste soils. For Tec-1 and Control, plants were only present in one of the four trays. The mean values of plant biomass per tray for the other treatments were 0.89 for Tec-2, 0.055 for Tec-2E, 2.63 for Tec-3 and 2.16 for Tec-4.

Table VII – Total aboveground biomass in each soil type at the end of the experiment.

Type of soil	Replicate	Dry weight (g)
Tec-1	2	0.030
Tec-2	1	0.539
	2	0.510
	3	1.21
	4	1.30
	Average	0.89
Tec-2E	1	0.024
	3	0.086
	Average	0.055
Tec-3	1	0.167
	2	1.61
	3	0.330
	4	5.94
	Average	2.63
Tec-4	1	2.31
	2	1.39
	3	3.72
	4	1.20
	Average	2.16
Control	1	0.317

4. DISCUSSION

Open mine areas have a strong environmental and visual impact on the landscape. Thus efforts to improve the vegetation cover after the abandonment of the mine exploitation is important to reduce the impact of mining areas. However, there are several problems for the establishment of the vegetation, namely the lack of a proper soil, besides toxic levels of heavy metals associated with low pH which makes them even more bioavailable, and a reduced seed bank, more dependent on the vegetation from areas surrounding the mine area.

Primary plant succession, that is, the establishment of plants in a barren soil, can occur in mining areas. However, this process can take many years due to adverse conditions of these areas. To accelerate the process of plant establishment, it is often necessary to add soil amendments enriched in nutrients and with a seed bank.

In Touro's mine (Galicia, Spain) there is a large-scale project of soil amendment to the mine bare soil to improve the conditions for the establishment of vegetation. These soils, named technosols, are the result of a mixture of several organic residuals, the leftovers of mussels, etc. Thus we have collected several types of technosols within the mine, besides mine soil and a control soil outside the area of exploitation of the mine area, to evaluate their seed bank potential and seedling development.

Mine Soils (Mine-1 and Mine-2) revealed a very low potential for plant germination and growth. This is probably related to the occurrence of toxic levels of some heavy metals and a lower percentage of organic matter and total N, compared with the control soil. The pH (~4) and K were similar between the mine and control soils, with mine soils even showing a higher concentration of P. A low pH increases the availability of heavy metals and this can have a negative effect on the germination of seeds and becomes a growth limiting factor (Marschner, 1991). This is aggravated by the fact that mine soils probably have a low seed bank. Mineral nutrition is crucial on regulation of plant

growth and development (Gramash, 2005) and on acid soil conditions one of the most constrains to plant growth are the solubility of mineral elements and consequent deficiency (Marschner, 1991).

Control soil proved to be significantly better on plant germination and growth than mine soils. Although the content of P and K was lower and the pH was similar, the control soil showed a higher percentage of organic matter and N. Nitrogen is essential for plant growth and in soil about 95% of nitrogen is related to organic matter (Meysner *et al,* 2006), being this last one strongly related to soil fertility (Johnston *et al.,* 2009). As the soil nutrient availability increase, increase also the plant production (VanOorschot *et al.,* 1997). Additionally the control soil had a well-established vegetation cover, meaning that the probability of having a seed bank is higher.

In general, compared with the control soil, technosols showed a basic pH (~8), a high percentage of organic matter, specially Tec-3 and -4, extremely high levels of P, high levels of K, except Tec-0, and similar values of total N. Tec-0 practically showed no germination of plants. Tec-0 had been applied recently and in terms of the parameters measured in the soil, comparing with the other technosols was a lower concentration of K. The absence of germination in Tec-0 is thus probably related to an absence of a seed bank. Somehow this soil 'must wait' for seeds to disperse from surrounding areas.

Tec-2 and Tec-2E showed very similar characteristics in terms of vegetation cover and soil parameters. However, Tec-2 showed the highest number of individuals, as well as one of the highest numbers of identified species and biodiversity index, while Tec-2E was one of the technosols with lower number of germinated plants, and the only identifiable species was *Geranium purpureum*. Thus, for some reason Tec-2 had a richer seed bank.

Tec-3, the soil with the presence of mussel shells, was the soil where germination occurred faster. It had the highest percentage of organic matter and total N and a great quantity of P. Mussel shell addition increased soil pH (Paz-Ferreiro *et al,* 2012) and stabilize it over time (Álvarez *et al*., 2012). Increase also the soil fertility when combined with other soil amendments and lowered the amount of Al (Kwon *et al,* 2009).

At the end of the experiment the general decrease on the number of plants is related to the mortality of the seedlings caused maybe by the low soil depth of the trays or due to competition between the existing plants in each replicate. However, at the end of the experiment, new seeds germinated. Some of these later seeds could have been transferred by the wind from the garden surrounding the location of the trays, or can be seeds already present in the seed bank that required more time to germinate.

In terms of % of coverage, biomass and diversity of species, Tec-2, -3 and -4 showed the highest values, compared to the control and mine soils. Probably the main reason is the extreme high levels of P, a limiting nutrient in many soils. This is reinforced by the fact that all the plants that germinated were ruderals. Although the availability of P is generally lower in alkaline soils, if the soils have more than 1% of organic matter, in the pH range of 6 to 8, the phosphorus concentration in the soil solution can increase, instead of declining (Marschner, 1990).

The seeds present in the technosols can be originated from the area itself, when there is already vegetation, and/or from the surrounding environment through wind or animal dispersion. Although most of the species could not be identified, in general, all soils showed a similar community of plants. Somehow this is expectable because they come from the same area, with similar seed sources. However, in some soils there was the predominance of some species, like *Geranium purpureum* in Tec-2E and *Parietaria*

judaica in the control soil. Nonetheless the percentage of unidentified species was very high.

Our results showed that the technosols, in general, facilitate plant germination and can have a good impact on the revegetation of bare mine soils. The application of a layer of these soils on top of the mine soil can thus stimulate the initial germination, growth and establishment of plants, accelerating the process of plant succession. The initial growth of the plants in the technosol is also important because when the roots of plants reach the layer of the mine soil, with a lower pH and high toxic levels of heavy metals, the impact can be mitigated by the nutrient rich layer of the technosol.

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PART II: SOIL AMENDMENTS

1. INTRODUCTION

1.1 Soils properties

Human activities can interfere and change some soil properties through, for example, the disposal of chemicals from industries (Entry *et al.,* 2002). Drought, salinity, pH, low content of nutrients, among others, are environmental factors which can disturb plant growth (Flexas *et al.,* 2006). The soil structure and texture also influences plant growth (Passioura, 1991).

A soil is considered fertile when it can provide the proper quantity of nutrients (Janssen and Willigen, 2006) but the actual values of soil quality are not consensual (Reynolds *et al.,* 2002) due to differences between soils and their function (Karlen *et al.,* 1997). Thus the characteristics of a specific area affect the plant growth in a specific way as the soil properties influence all the reactions, transformations and mobility (Ross, 1994).

Mine soils are usually acidic, with low fertility, low content of nutrients (as P, Ca, K deficiencies), high concentrations of heavy metal, high solubility of toxic metals (as Al, Fe, Mn), constituting harsh environments that impair plant growth (Tang *et al.,* 2003; Kochian *et al.*, 2004; Goransson *et al.,* 2008). Nonetheless there are plants able to grow under these conditions, namely low pH and high Al toxicity (Osaki *et al.*, 1997), but most of them do not have mechanisms which allow them to survive with these soil conditions.

The low availability of some nutrients and the toxicity of heavy metals (Adams, 1981; Yan *et al.*, 1992; Marschner 1990) usually do not allow the establishment of new plants. The absence of vegetation makes these soils more vulnerable to erosion that can cause the spread of the contaminants to other nearby communities. Revegetation on abandoned mining areas, without modifications or amendments is thus a difficult task.

1.2 Amendments

Surface stabilization by the establishment of a vegetation cover on adverse soils minimize the soil erosion and prevent the spread of contaminants to environment around the area (Wong, 2003; Mendez and Maier, 2008; Carrasco *et al.,* 2009).

To improve the vegetation and allow a successfully revegetation it is necessary to improve the physical and chemical properties of the soil (Caravaca *et al.,* 2003). The amendments have the capacity to transform metals into less soluble or insoluble forms making them less available and can enhance ion exchange, sorption and redox reactions (Mench *et al.,* 2003). To accomplish a revegetation project, regardless of the characteristics of the disturbed land, it is imperative to plan the actions for revegetation, namely the selection of the species to introduce, the type of soil amendments, and the economical costs of all the processes associated. After the implementation, it is necessary to monitor the project with regular inspections to determine the success of the revegetation process and if it there is any need to modify the previous conditions (Anderson and Ostler, 2002).

Various amendments can be used to ameliorate the general conditions of soils to ameliorate seed germination, survival and plant growth, important for the primary plant succession. The appropriate vegetation associated to the amendments is important to maximize the revegetation process (Wong, 2003). In general, the application of lime agents and organic matter improves the revegetation potential in acid contaminated by with heavy metals (Córdova *et al.,* 2011).

1.2.1 Organic amendments

The addition of organic matter improves some soil characteristics (Khaliq *et al.,* 2006) as water retention and infiltration, aeration, and have a beneficial effect on the surface stability (Tisdall and Oades, 1982; Caravaca *et al.,* 2003) although the soil stability not only depends on soil organic matter but also on Fe and Al oxides and CaCO$_3$ soil contents (Caravaca *et al.,* 2004). Caravaca *et al.* (2003) showed that the addition of organic matter had a good effect on revegetation of semiarid areas increasing the quantity of nutrients and the soil stability.

When nutrients are added to the soil, besides an improvement of plant growth (Roldán *et al.,* 2006) the physical soil conditions are also ameliorated (Clemente *et al.,* 2012). For example, an organic amendment very used is sewage sludge, a bio-organic product of wastewater treatment, which is a source of plant macro and micronutrients and improves the soil quality in physical, chemical, and biological properties (Sajwan *et al.,* 2007; Sivapatham *et al.,* 2012) and increases the pH (Little *et al.,* 1991).

1.2.2 Liming

Soils with low or high pH, are adverse for plant growth (Kobayashi *et al.,* 2010) due to its effect on nutrient availability. Lime is an old and common amendment used to improve soil properties (Haynes and Naidu, 1998). Liming agents, such as calcite or calcium carbonate, are compounds capable of increasing the pH of acidic soils (Levonmäki and Hartikainen, 2007). Liming besides increasing the soil pH, reduces the bioavailability of heavy metals of mine soils (Little *et al.,* 1991; Lee *et al.,* 2004), and compensates calcium and magnesium losses (Persson *et al.,* 1990).

The amount of lime required to neutralize the soil acidity is not equal for all soils (Shoemaker *et al.,* 1961). Soils with low cation exchange capacity (CEC) suffer a higher pH increase after liming (Matula and Pechová, 2002). Mine soils usually present a low CEC (Vega *et al.,* 2005), thus liming will be effective in correcting the pH of those soils.

The effects of lime agents on the topsoil are relatively fast, occurring in a short time, however in the subsoil it is more difficult and takes longer (Tang *et al.,* 2003).

1.3 Objectives

The aim of the present work is to test if the change of some soil properties of mine soils, like the amount of nutrients and pH, can improve germination of seeds and growth of plants.

To achieve this goal we have tested the effect of two amendments on mine soil, addition of nutrients and increase of the pH, on the seed germination and plant growth of two grasses and two legumes, and compared with the original mine soil and a positive control, a garden soil. The height, number of leaves and biomass were the parameters analyzed to check the performance of the four plant species used.

The results will increase the knowledge about the role of soil amendments in view of their application to improve the conditions for plant growth in mining areas.

2. MATERIALS AND METHODS

2.1 Experimental set up and data collection

Four treatments were used to test the effect of pH and nutrient amendments on plant growth in the mine soil. Garden soil was used as a positive control with the best plant growth conditions. The other three treatments included the mine soil, mine soil amended with calcium carbonate ($CaCO_3$) to increase soil pH, and a mixture 1:1 (vol:vol) of mine soil and garden soil to improve texture and nutrient content. The mine soil used in this experiment was collected in February 2012 in Touro's mine (Galicia, Spain). About 20 kg of soil from the 20-cm upper layer were collected in the top of the Mine waste and stored in plastic bags in a cool place until use.

To determine soil pH, a fraction of the soil used in the four treatments was air dried and sieved through a 2-mm mesh. Subsequently, 5 g of soil were mixed with 50 mL of ultrapure water, stirred for 30 minutes and allowed to settle for 10 minutes and the pH was measured using an OAKTON pHmeter (Page *et al*., 1982). Three replicates were used to estimate soil pH.

The pH of the garden soil was 7.74, the mixture of mine soil and garden soil was 7.07, and the mine soil was 3.7. A pre-test was performed to calculate the necessary amount of calcium carbonate ($CaCO_3$) to increase the pH of the mine soil up to 6.14-6.77. Between 2.5 to 5g of calcium carbonate ($CaCO_3$) were added to 140g of mine soil and the pH was determined afterwards. This pre-test was used to calculate the proportion of $CaCO_3$ necessary to add to the volume of soil used in the pots in order to increase the pH of the soil mine. Two types of pots were used, with an approximate volume of 1790g the larger and 140g the smaller, the small ones to study the species response to the four treatments, and the larger ones to study the response of the mixture

of species to the same treatments (see below). Thus, 2g of $CaCO_3$ were added to the small pots and 25g were added to the larger pots.

Two legumes (*Medicago sativa* L. and *Trifolium subterrraneum* L.) and two grasses (*Lolium perenne* L. and *Dactylis glomerata* L.) commonly used in mine revegetation projects were selected for this study. Both grasses are perennial plants native to Europe, some regions of Asia and North Africa (FAO, 2012a; FAO, 2012b). *Medicago sativa* is an erect and perennial plant, widely distributed in temperate zones of the world (FAO, 2012c) and *T. subterraneum* is an annual plant native to southern Europe, North Africa and Southern England (FAO, 2012d).

Two different experiments were prepared using this soil and plant species. The first experiment investigated the growth of each individual plant species in each type of soil. The second experiment tested the growth of the mixture of these four species in the same soils. All treatments had 10 replicates and two individuals per plant species on the individual experiment and four individuals on the mixture experiment. In February, all pots were placed in a greenhouse at the Botanical Garden of Coimbra and were watered regularly during 12 weeks. Pots were seeded and 5 weeks after germination and seedling development, data on height and number of leaves were collected weekly during 7 weeks. At the end of the experiment plants growing individually (first experiment) were removed from the pots and gently washed and shaken in order to remove particles attached to the roots. Only aboveground biomass was harvested in the mixture of species due to the impossibility of separating the roots of each species. All plants were dried at 60ºC for 3 days and weighted.

2.2. Statistical analyses

Repeated Measures ANOVA and Tukey test were applied to check for differences on height and number of leaves for each plant species between the four types of soil along the 7 weeks (significance level of 0.05). One-way ANOVA and Tukey test were applied to check for differences on plant biomass for each plant species between the four types of soil (significance level of 0.05). All data were normal, except for the biomass data of *M. sativa* on the experiment of the mixture of species that had to be transformed using log (n+1). These analyses were done using STATISTICA 7.

3. RESULTS

3.1 Individual plant species performance

For all four studied species, plant height was higher in the Garden Soil, followed by Mine Soil + Garden Soil, Mine Soil + $CaCO_3$ and finally Mine Soil (Figure 11). The same result was observed for the number of leaves produced by each species in each treatment during the experiment (Figure 12).

Significant differences ($p<0.05$) were found between the different treatments in the four plants analyzed in terms of height (Table VIII) and number of leaves (Table IX). None of the four species survived until the end of the experiment in the mine soil, where growth was severely impaired.

Lolium perenne was the highest species in all treatments and *Trifolium subterraneum* was the plant with the lowest height registered. The highest value for number of leaves was obtained for *T. subterraneum*.

Statistical differences in height and number of leaves for each species were consistent over time. There were significant differences in height for *L. perenne* between all soils on the seven weeks except between Garden Soil and Mine Soil + Garden Soil on week 1. The number of leaves showed no statistical differences between Mine Soil + Garden Soil and Mine Soil + $CaCO_3$ on week 1, Mine Soil + $CaCO_3$ and Mine Soil on week 3 and 4 and between Garden Soil and Mine Soil + Garden Soil on week 4. In the remaining weeks there were significant differences among all soils.

The height of *D. glomerata* showed no differences between soils on week 1. On week 2 – 7 Mine Soil + $CaCO_3$ had significant differences compared to the Garden Soil and Mine Soil + Garden Soil. The number of leaves showed no statistical differences between Garden Soil and Mine Soil + Garden Soil and between Mine Soil + Garden

Soil and Mine Soil + CaCO$_3$ on week 1. For the other weeks statistical differences were found between Mine Soil + CaCO$_3$ and the two other soils tested.

The height of *M. sativa* on week 1 and 2 showed no significant differences between Garden Soil and Mine Soil + Garden Soil and between Mine Soil + CaCO$_3$ and Mine Soil. On weeks 3 to 7 there were statistical differences between all soils tested. For the number of leaves there were no differences between Garden Soil and Garden Soil + Mine Soil and between Mine Soil + CaCO$_3$ and Mine Soil on week 1 and 2. On week 3 no significant differences were found between the treatments. On week 4 and 7 there were significant differences between all soils and on week 5 and 6 Garden Soil and Garden Soil + Mine Soil were statistically different from the Mine Soil + CaCO$_3$.

No significant differences were found in height of *T. subterraneum* between Garden Soil and Mine Soil + Garden Soil on week 1, and Mine Soil + CaCO$_3$ and Mine Soil on week 2. On the other weeks there were differences between all soils. The number of leaves for this species showed no statistical differences between Garden Soil and Mine Soil + Garden Soil on week 1, 2 and 3. On week 4 − 7 statistical differences between all soils were found.

Grasses and legumes had a different curve shape of growth. While the grasses tended to reach a plateau in height and number of leaves, both legumes showed an initial delay in growth and an exponential increase from week 2 in height and number of leaves.
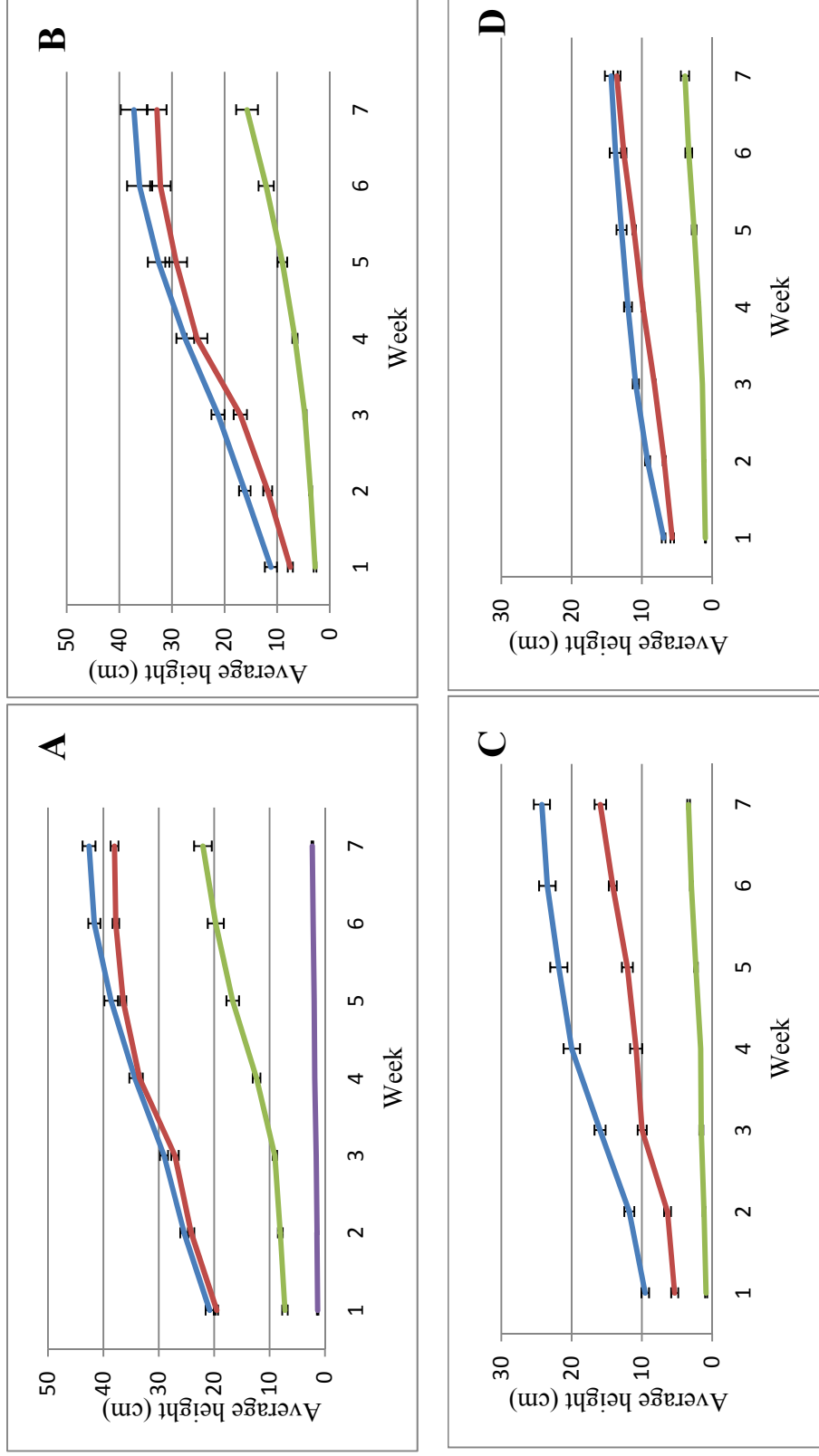
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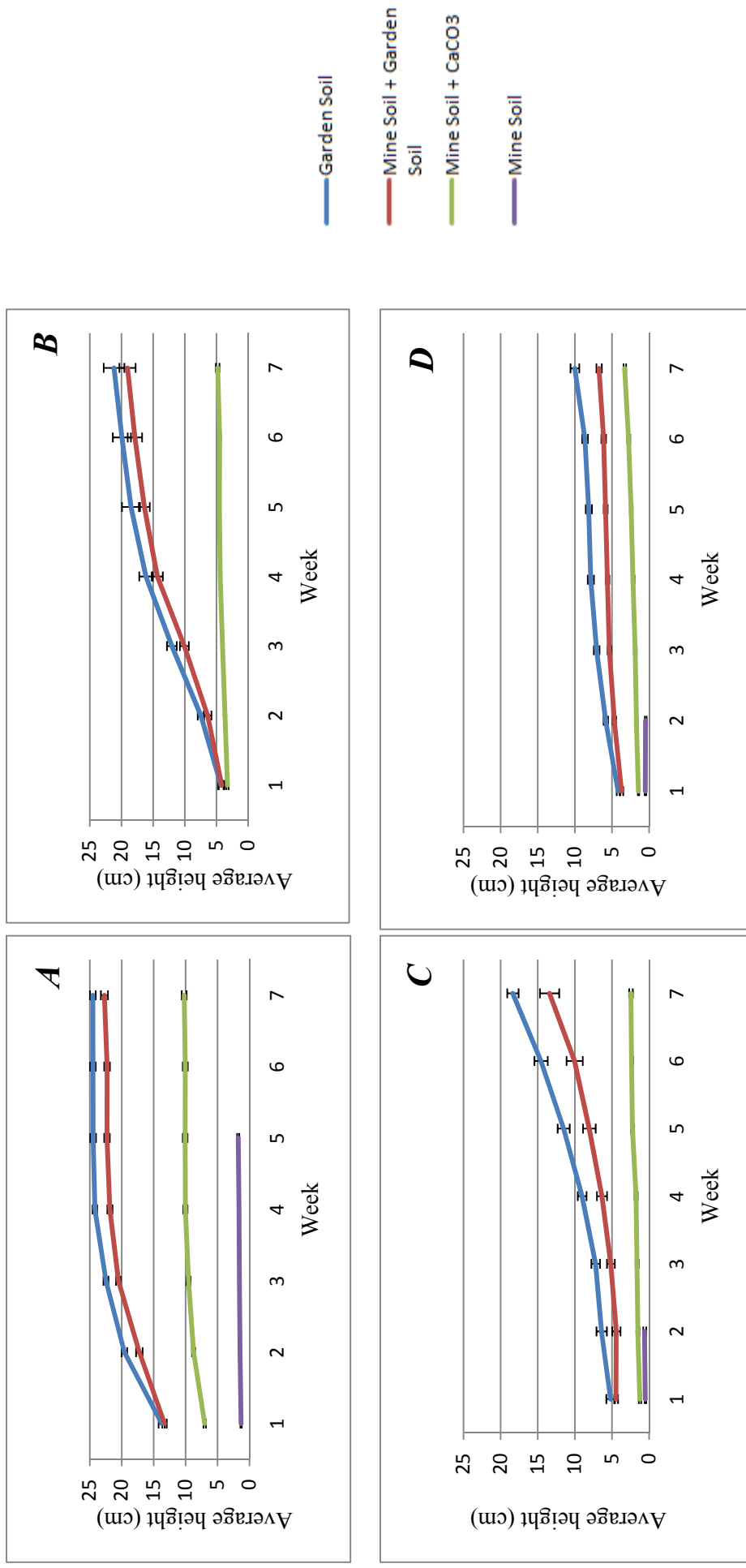
Figure 11: *Average height ± SE of the four species in the four treatments throughout the experiment. Lolium perenne (A), Dactylis glomerata (B), Medicago sativa (C) and Trifolium subterraneum (D).*
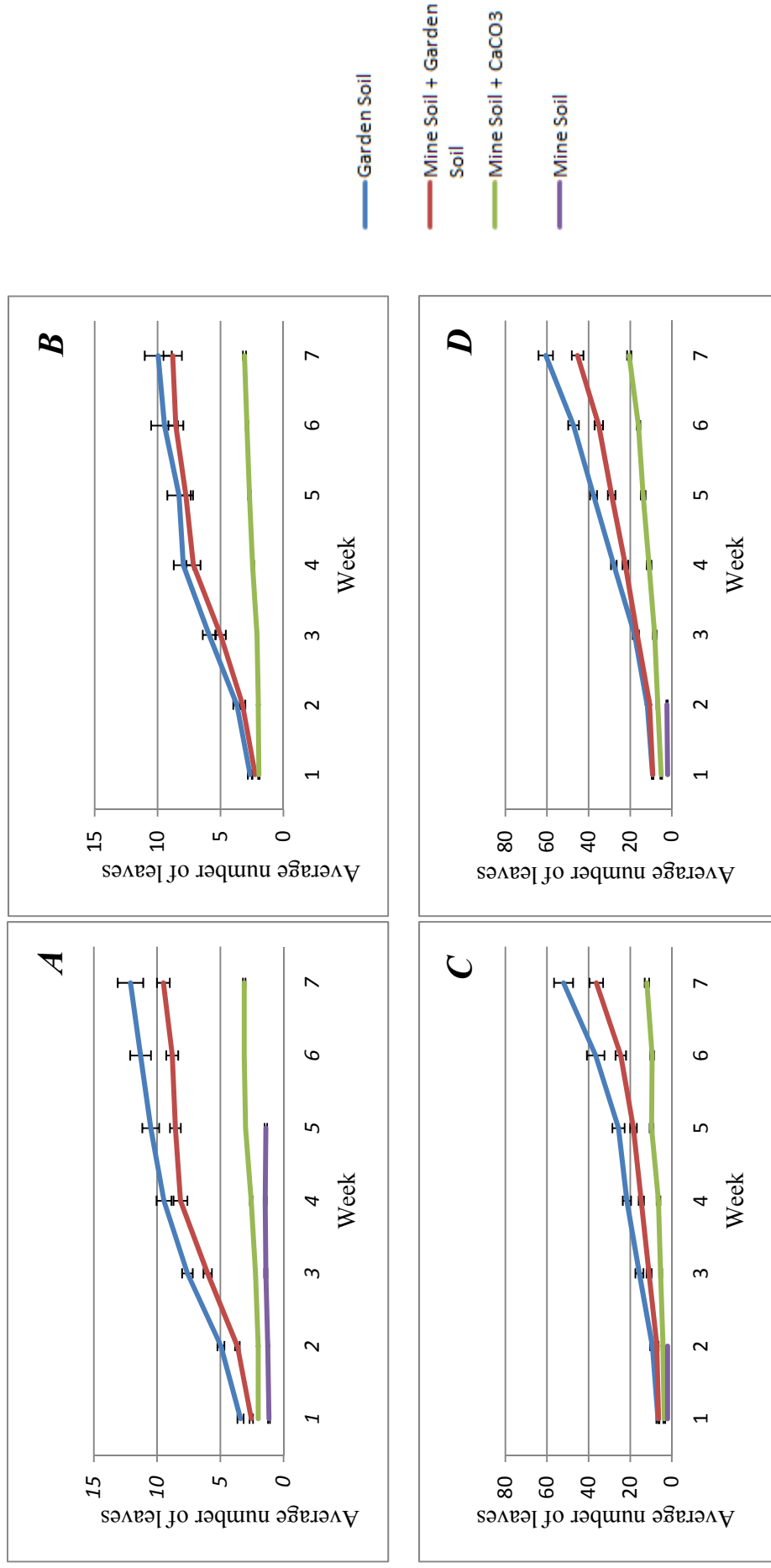
Figure 12: Average number of leaves ± SE of the four species in the four treatments throughout the experiment. *Lolium perenne* (A),

Dactylis glomerata (B), *Medicago sativa* (C) and *Trifolium subterraneum* (D). Note that the scale used on the graphs is different.
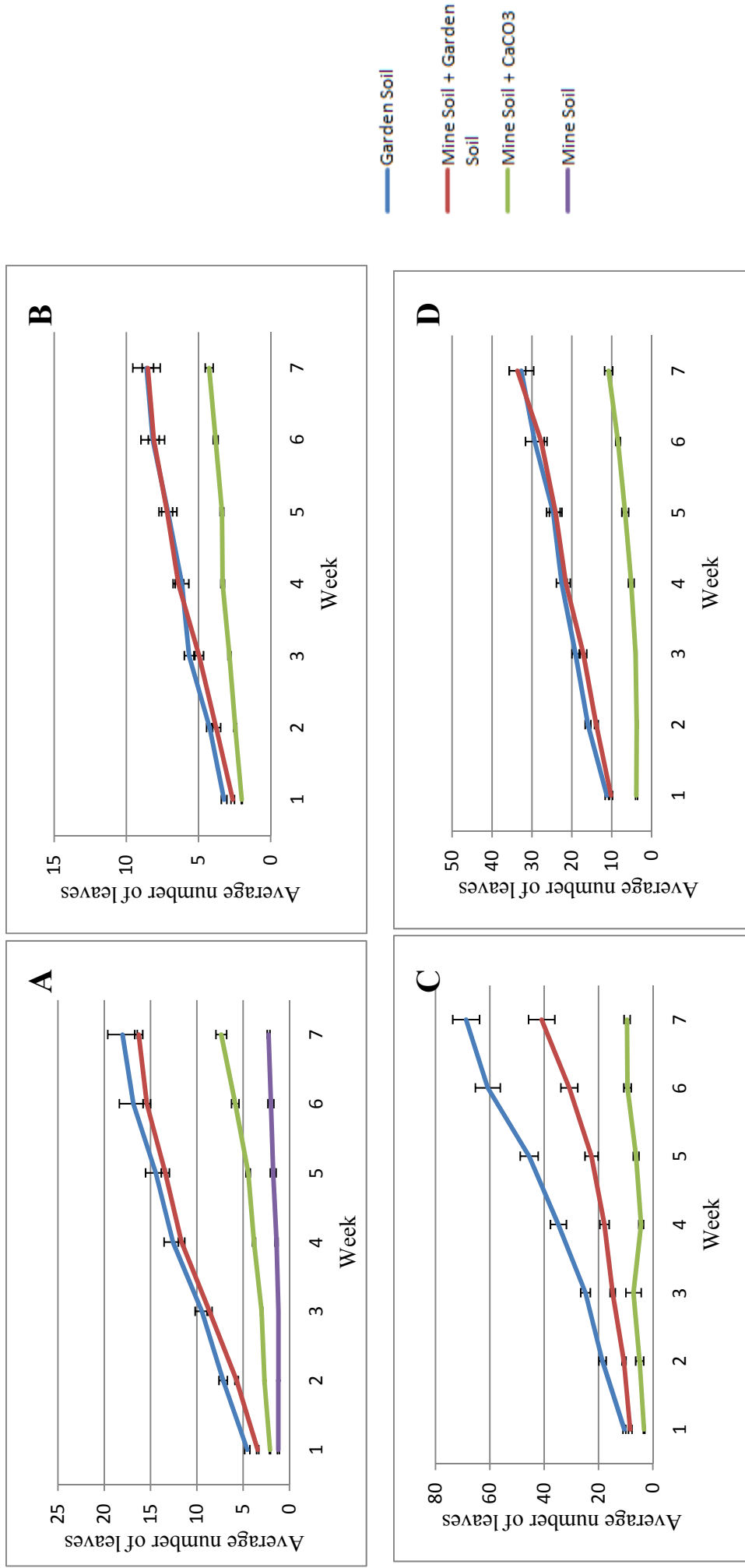
Table VIII: Results of Repeated Measures ANOVA for the height of the four
species.

Species	F	p
Lolium perenne	27.205	<0.0001
Dactylis glomerata	8.5715	0.0000
Medicago sativa	5.18397	0.0000
Trifolium subterraneum	7.5651	0.0000

Table IX: Results of Repeated Measures ANOVA for the number of leaves
of the four species.

Species	F	p
Lolium perenne	14.2358	0.0000
Dactylis glomerata	3.42129	<0.05
Medicago sativa	7.80174	0.0000
Trifolium subterraneum	8.1175	0.0000

Differences in final plant biomass between treatments were found for the four species. Final plant biomass was higher in the Garden Soil, followed by Mine Soil + Garden Soil and Mine Soil + $CaCO_3$ (Table X). No plants were found at the end of the experiment in Mine Soil.

Significant differences were found among the three treatments for the *L. perenne* and *T. subterraneum* (Figure 13). Plant growth was significantly lower in the Mine Soil + $CaCO_3$ for *D. glomerata* and *M. sativa,* but there were no differences on the biomass of these species grown on Garden Soil and Mine Soil + Garden Soil.

Table X: Results of One-way ANOVA for biomass of the four species.

Species	F	p
Lolium perenne	48.2763	0.0000
Dactylis glomerata	27.3733	0.0000
Medicago sativa	14.06937	<0.05
Trifolium subterraneum	20.7649	<0.0001
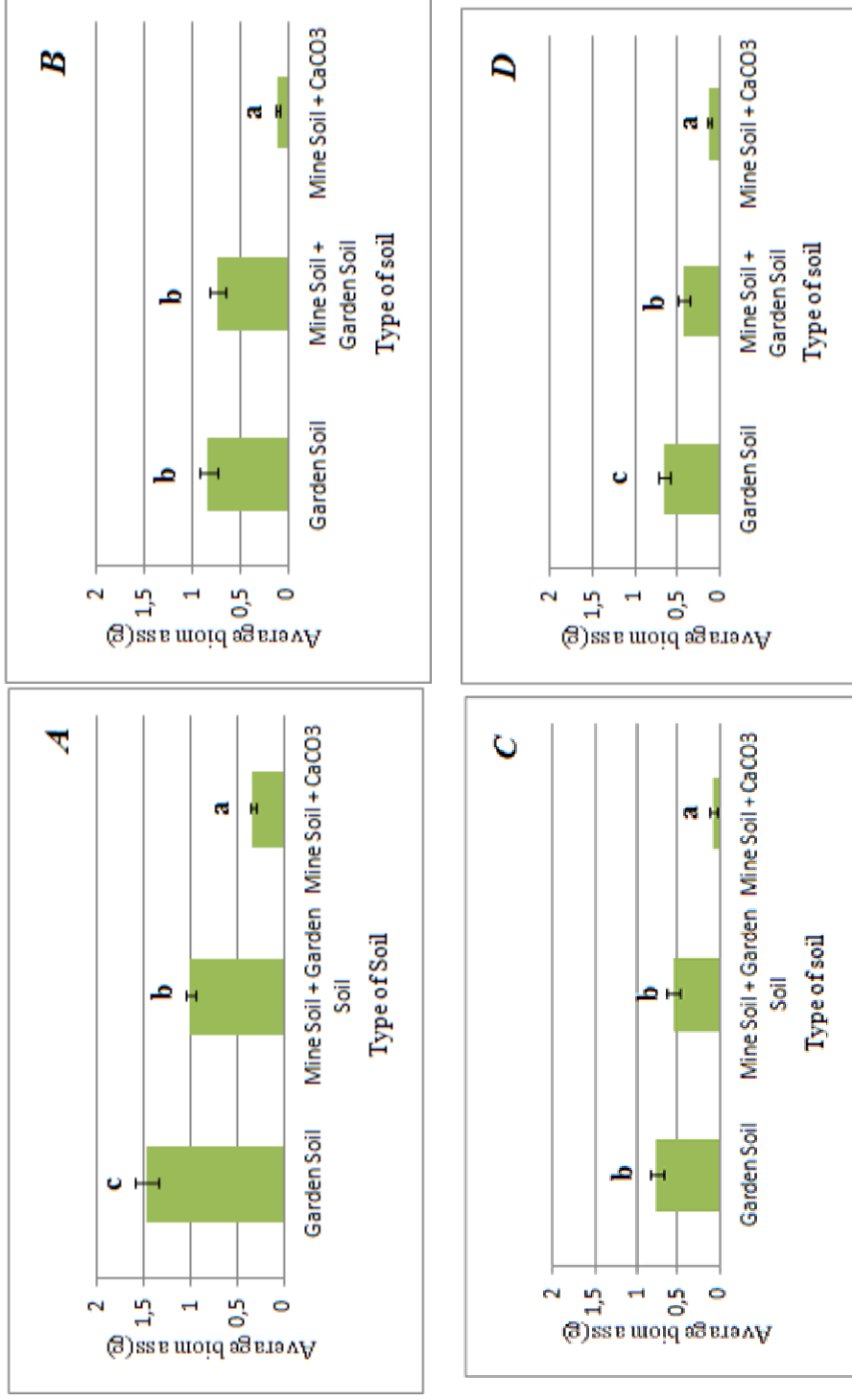
Figure 13: Average biomass ± SE. *Lolium perenne* (A), *Dactylis glomerata* (B), *Medicago sativa* (C) and *Trifolium subterraneum* (D).

Different letters above the bars mean significant differences between each soil type after ANOVA analysis.

3.2 Plant species performance in a mixture of species

For all four studied soils, *L. perenne* was the highest species and *T. subterraneum* was the plant with the lowest height registered (Figure 14 and 15). For the number of leaves produced the highest value was obtained for *M. sativa* on Garden Soil. On Mine Soil + Garden Soil *T. subterraneum* had more leaves until week 5, but afterwards, *M. sativa* showed more leaves. On Mine Soil + $CaCO_3$ there were some oscillations in the number of leaves of the two legumes, and all species showed the lowest height and number of leaves, compared to the Garden Soil and Mine Soil + Garden Soil (Figure 16 and 17). On the Mine soil *L. perenne* was the only species which survived until the end of the experiment.

Significant differences ($p < 0.05$) were found between the different treatments in the four plants analyzed in terms of height (Table XI) and number of leaves (Table XII). Statistical differences were consistent over time. There were statistical differences in height for *L. perenne* between Mine Soil and Mine Soil + $CaCO_3$ and the other two soils in all weeks, and between Garden Soil and Mine Soil + Garden Soil only on week 6 there were significant differences. For the number of leaves there were statistical differences between all soils in the first two weeks. On week 3 – 7 there were no differences between Garden Soil and Mine Soil + Garden Soil.

The height of *D. glomerata* showed significant differences between all types of soils until week 3. On the following weeks there were only significant differences between the Mine Soil + $CaCO_3$ and the other two types of soil (Garden Soil and Mine Soil + Garden Soil). The number of leaves on Mine Soil + $CaCO_3$ was statistically different from the other two treatments in all weeks.

The height and number of leaves of *M. sativa* showed significant differences between all treatments, and for all the 7 weeks. In *T. subterraneum* statistical differences were found in height between all treatments until week 4. For the other three weeks statistical differences were only found between Mine Soil + $CaCO_3$ and the other two soils (Garden Soil and Mine Soil + Garden Soil). For the number of leaves there were statistical differences between all soils on week 2 and between Mine Soil + $CaCO_3$ and the other two soils on the other weeks.
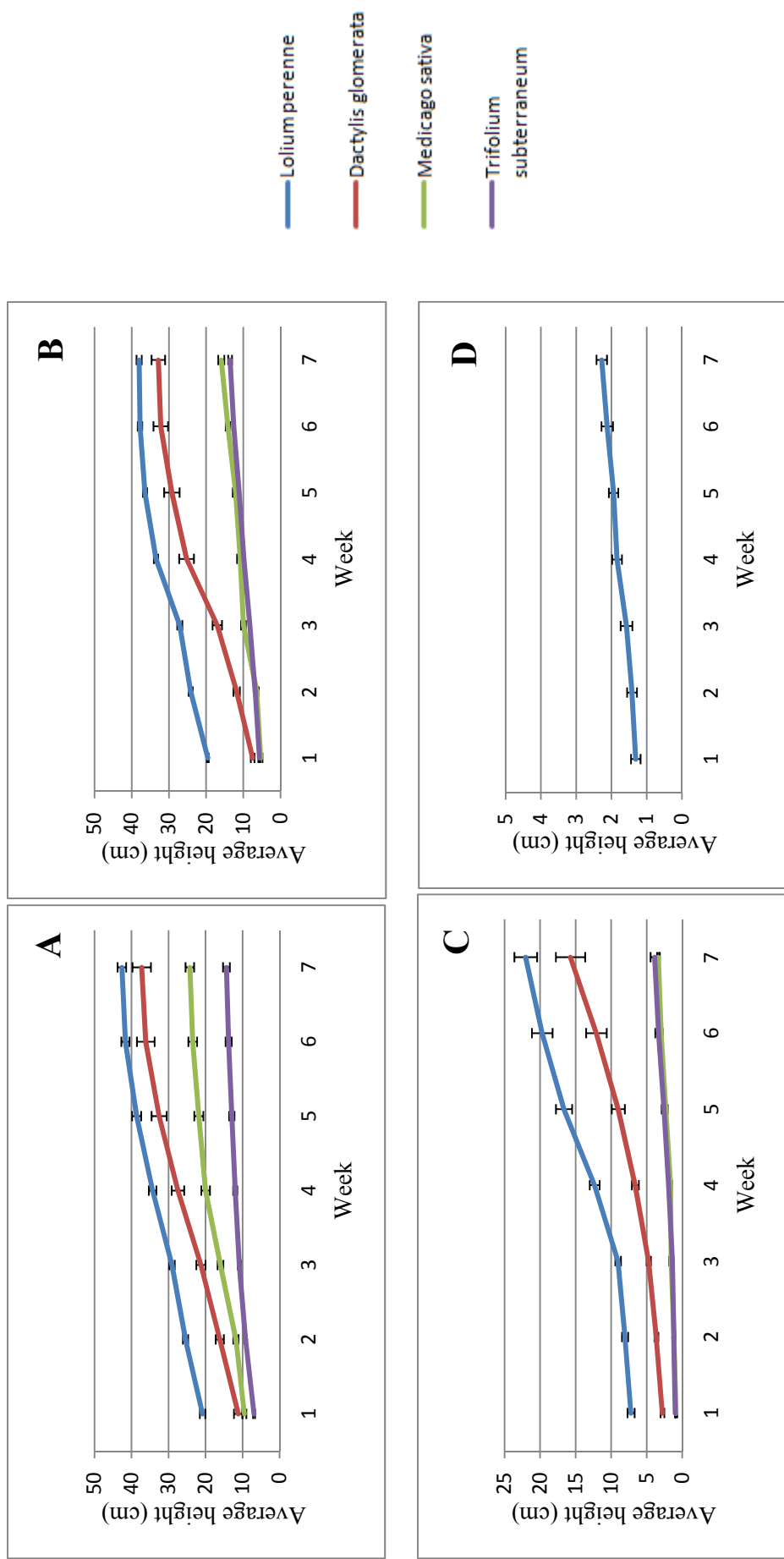
Figure 14: Average height ± SE of the four species in the four treatments throughout the experiment. Garden Soil (A), Mine Soil + Garden Soil (B), Mine Soil + CaCO$_3$ (C) and Mine Soil (D). Note that the scale used on the graphs is different.

Figure 15: *Average height ± SE of the four species in the four treatments throughout the experiment. Lolium perenne (A), Dactylis glomerata (B), Medicago sativa (C) and Trifolium subterraneum (D). Note that the scale used on the graphs is different.*
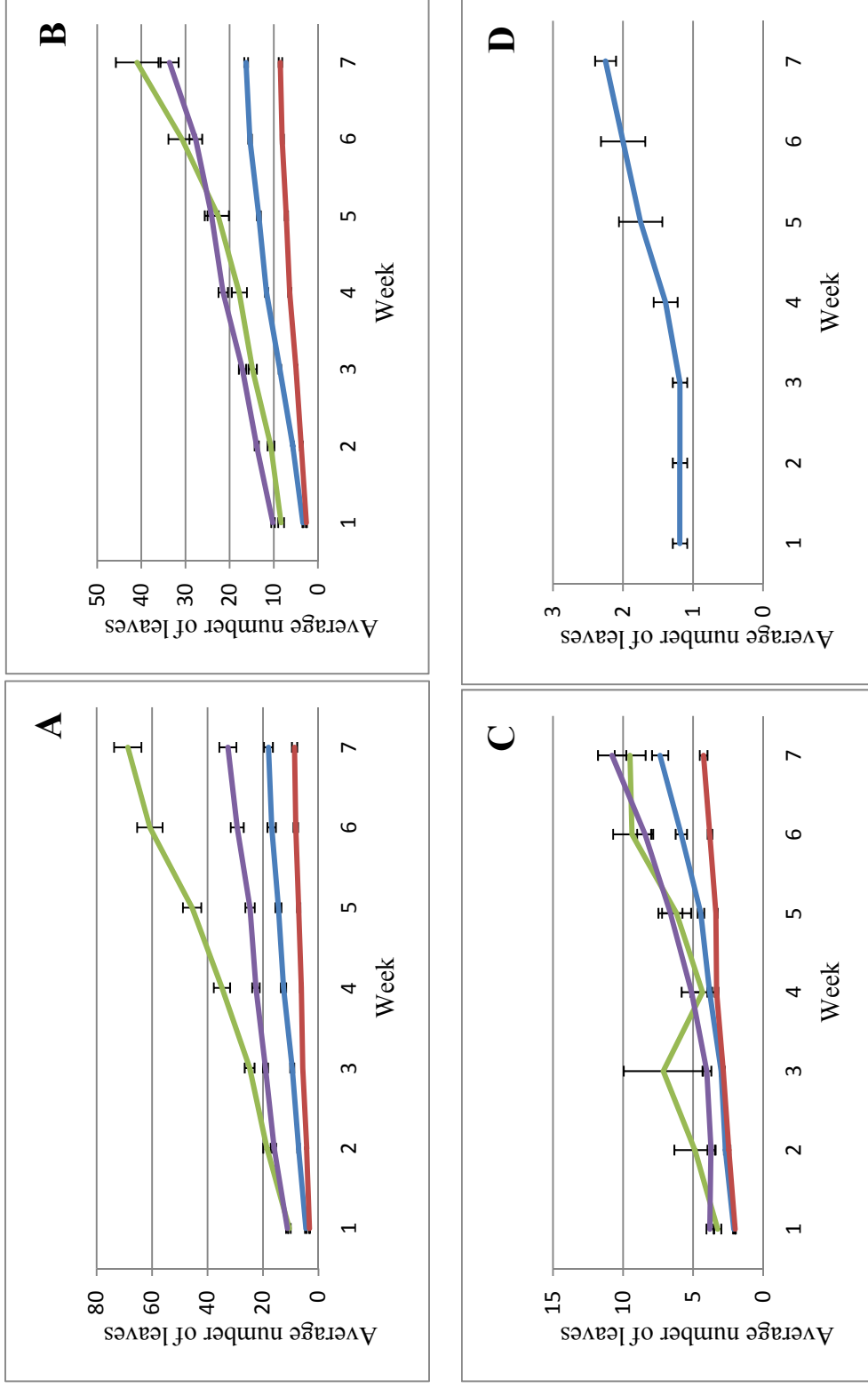
Figure 16: Average number of leaves ± SE of the four species in the four treatments throughout the experiment. Garden Soil (A), Mine Soil + Garden Soil (B), Mine Soil + CaCO₃ (C) and Mine Soil (D). Note that the scale used on the graphs is different.

Figure 17: Average number of leaves ± SE of the four species throughout the experiment. *Lolium perenne* (A), *Dactylis glomerata* (B), *Medicago sativa* (C) and *Trifolium subterraneum* (D). Note that the scale used on the graphs is different.

Table XI: Results of Repeated Measures ANOVA for height of the four species.

Species	F	p
Lolium perenne	24.3612	0.0000
Dactylis glomerata	8.67738	0.0000
Medicago sativa	12.6099	0.0000
Trifolium subterraneum	19.6725	0.0000

Table XII: Results of Repeated Measures ANOVA for the number of leaves of the four species.

Species	F	p
Lolium perenne	16.6867	0.0000
Dactylis glomerata	5.0933	<0.0001
Medicago sativa	9.93071	0.0000
Trifolium subterraneum	12.1968	0.0000

Differences in the final plant biomass between treatments were found for the four species (Table XIII). Final plant biomass was higher in the Mine Soil + Garden Soil and Garden Soil, followed by Mine Soil + $CaCO_3$ for all the species, although *M. sativa* showed significantly higher biomass in the Garden Soil, compared to the Mine Soil + Garden Soil (Figure 18).

Table XIII: Results of One-way ANOVA for biomass of the four species.

Species	F	p
Lolium perenne	94.1991	0.0000
Dactylis glomerata	19.7236	<0.0001
Medicago sativa	73.7213	0.0000
Trifolium subterraneum	16.71829	<0.0001
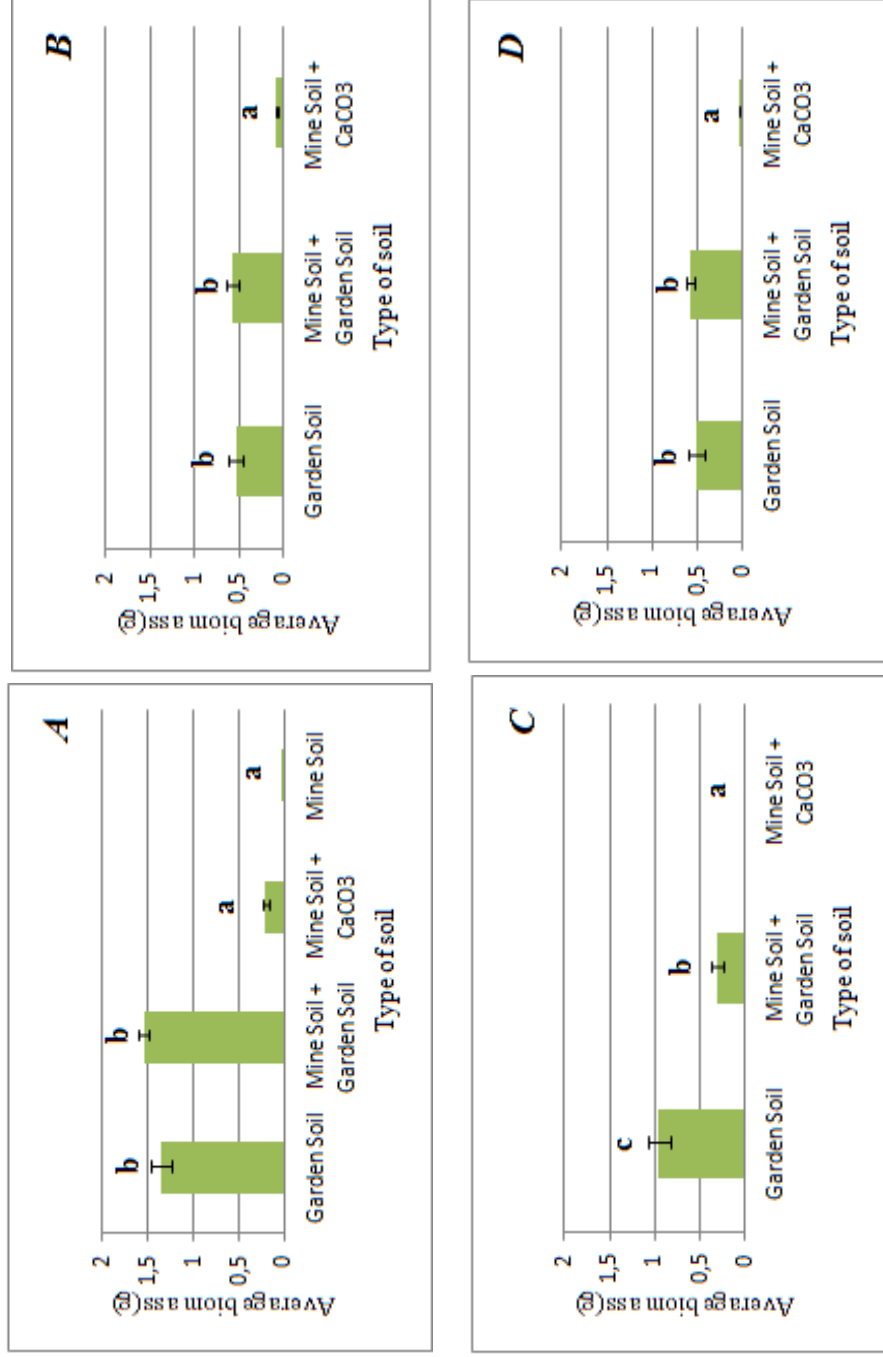
Figure 18 – Average aboveground biomass ± SE of the four species in the treatments at the end of the experiment. *Lolium perenne* (A), *Dactylis glomerata* (B), *Medicago sativa* (C) and *Trifolium subterraneum* (D). Different letters above the bars mean significant differences between each soil type after ANOVA analysis.

Statistical differences in total plant biomass were found for the four types of soil (Table XIV). Total plant biomass was higher in the Garden Soil, followed by Mine Soil + Garden Soil, Mine Soil + CaCO$_3$ and Mine Soil (Figure 19).

Table XIV: Results of One-way ANOVA for biomass of the four species.

	F	p
Type of soil	177.5528	<0.0001
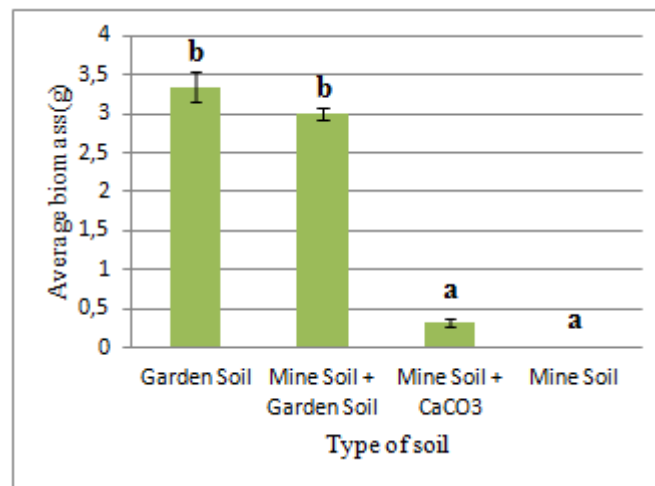
Figure 19: Average total aboveground biomass ± SE for each type of soil at the end of the experiment. Different letters above the bars mean significant differences between each soil type after ANOVA analysis.

4. DISCUSSION

The abandoned mining areas have a strong environmental and visual impact on the landscape and efforts to attenuate those impacts are very important. The establishment of plants in mine soils is very difficult due to the harsh conditions of the soil, namely low pH, low nutrients and high levels of toxic heavy metals. Thus a good vegetation cover could take years to achieve. This process can be accelerated through the addition of nutrients and/or increase of pH, which reduces the solubility of heavy metals. These amendments, by ameliorating plant growth conditions, also make plants less susceptible to diseases (Akthar and Malik, 2000).

To evaluate the effect of soil amendments in the establishment of new plants we have collected mine soil and mix it with garden soil, to add nutrients, or mix it with $CaCO_3$, as liming agent, to increase the pH.

Soil pH is related to plant survival, biomass production and affect metal bioavailability. Clemente *et al.,* 2003 concluded the effective role of liming agents in the control of soil pH and the reduced mobility of heavy metals by organic matter.

The effect of available heavy metals on plants depends on factors as quantity and availability, soil properties, environmental factors as pH and the plant species capacity to support the toxicity (Ross, 1994) while in soil the bioavailability is most influenced by pH, CEC, organic matter and clay content (Prasad and Hagemeyer, 1999). Acid pH increases the availability due to the "higher affinity of hydrogen ions for negative charges on colloids, thus competing with the metals ions of these sites, thus releasing metals" (Prasad and Hagemeyer, 1999).

The species tested in this experiment were two grasses, *Lolium perenne* and *Dactylis glomerata,* and two legumes, *Trifolium subterraneum* and *Medicago sativa.* Grasses are pioneers and usually adapted to adverse conditions, with an important role in protecting soil from erosion (Hubbard, 1954). Legumes, through the process of

nitrogen fixation, are important in the enrichment of soil with nitrogen, a very important nutrient for plants (Wilson *et al.,* 1982; Marschner, 1990; Snapp *et al.,* 2005).

4.1 Individual plant species performance

Mine soil results confirmed what was discussed in "Part I: Seed bank assessment", that is, the main reason for the lack of plant cover in mine soils is probably not the absence of seeds, but the difficulty of seeds to germinate and develop in these soils. None of the four species survived until the end of the experiment, probably due to the low pH, low contents of nutrients and high levels of toxic metals. However, there were differences among the species tested. Considering only the start of the data collection, without considering the 5 weeks when seeds were sown and seedlings were allowed to develop, *L. perenne* remained until week 5, being the species with more capacity to support those conditions, and showing a good potential in revegetation plans of mining areas. All the other three species germinated but the seedlings only survived two weeks, in the case of the two legumes, and one week in the case of *D. glomerata,* the other grass tested.

Lolium perenne, M. sativa and *T. subterraneum* grew significantly better on Garden Soil than in the other three soils. *Dactylis glomerata* had no significant differences in growth between Garden Soil and Mine Soil + Garden Soil. Concerning the number of leaves, *L. perenne* developed a significantly higher number on Garden Soil while *D. glomerata* had no significant differences between Garden Soil and Mine Soil + Garden Soil. *Medicago sativa* and *T. subterraneum,* depending on the week, had more leaves on Garden soil and Mine Soil + Garden soil. In general, the final plant biomass was higher in the Garden Soil followed by Mine Soil + Garden Soil and Mine

Soil + CaCO$_3$. *Dactylis glomerata* and *M. sativa* had no significant differences in biomass in Garden Soil and Mine Soil + Garden Soil.

Along the seven weeks of data collection, grasses tended to reach a plateau in terms of height and number of leaves, while the two legumes showed a slow increase in the beginning and after two weeks, showed an exponential increase of height and leaves. This can be related to a characteristic growth form of grasses and legumes. It is noteworthy that the difference between the growth curves of *T. subterraneum* in all treatments was smaller, when compared with the other three species. Somehow the Garden Soil and Garden Soil + Mine Soil did not improve the growth of *T. subterraneum* as much as it has improved the growth of the other species, particularly the grasses *L. perenne* and *D. glomerata*. This indicates that *T. subterraneum* is not so demanding in terms of nutrients, a property useful in poor nutrient soils.

Summarizing, in the Garden Soil the four species grew better, with higher height, number of leaves, and biomass, as expected. Garden soils are rich in organic matter and are prepared to have a good balance of nutrients to stimulate plant growth (Roldán *et al.,* 2006). The best amendment was the "Mine Soil + Garden Soil" for all the four species. This is probably related with two causes, the increase in the amount of nutrients and the increase of pH, from 3.7 of the mine soil to 7.07 of the mixture 1:1 of mine soil and garden soil. Thus, on one side we are giving more nutrients for plant growth, but also, by increasing the pH, the solubility and bioavailability of toxic heavy metals is reduced.

4.2 Plant species performance in a mixture of species

On Mine soil *L. perenne* was the only species which survived until the end of the experiment, while in the individual experiment, although it was the species which survived longer, after 5 weeks all individuals died. This can be related with the fact that the pots used for the mixture of species had a higher volume of soil, allowing a better growth of the roots of *L. perenne*. In fact, comparing the height of *L. perenne* after 7 weeks of growth, in the individual and mixture experiment, it showed, on average, 25 and 40 cm height, respectively. This was also observed for *D. glomerata* in the Garden Soil, although this species did not survive in the mine soil. Grasses develop a very intricate root system and probably need more volume of soil to increase their aboveground growth. The height of the two legumes was similar in the small and large pots, when grown in the Garden Soil, and both did not survive in the mine soil.

In general, *M. sativa* grew better on Garden Soil, *L. perenne* on Garden Soil and Mine Soil + Garden Soil and *D. glomerata* and *T. subterraneum* on Garden soil during the first weeks, ending the experiment with no differences between Garden Soil and Mine Soil + Garden Soil. Concerning the number of leaves, *M. sativa* had more leaves on the Garden Soil, *D. glomerata*, *T. subterraneum* and *L. perenne* on Garden Soil and Mine Soil + Garden Soil.

Summarizing, as in the former experiment, Garden Soil is the best soil and Mine Soil + Garden Soil the best amendment for plant growth. As in the previous experiment, *L. perenne* was the only species that could develop in the Mine Soil, although growing very little, confirming its potential to be used in revegetation programs of mining areas. However, all species tested increased their performance in terms of growth in the mixture of Garden soil with Mine Soil, being similar to the Garden Soil. Thus it is not

enough to increase the pH, as shown by the treatment of Mine Soil + $CaCO_3$, where all the species had a poor growth.

When compared with the individual pot experiment, in the mixture of species, the two grasses had a better growth in terms of height, while the legumes showed no differences between the two experiments. This somehow indicates that the competition among the species was not very strong - the legumes did not reduce or increase their growth parameters. The grasses could have beneficiated from the presence of legumes or could simply be related to the higher volume of the pots used for the mixture of species, with grasses having more soil to develop their roots. Nonetheless, the mixture of grasses and legumes in revegetation programs of mining soils is important because they represent two functional types of plants with different roles in the stability of soils. Grasses, with their highly developed root system can stabilize the soils and reduce erosion, while legumes can add up nitrogen to the soil, preparing the entrance of other plant species typical of later stages of succession (Tilman *et al.,* 1996; Sanchez *et al.,* 2001).

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PART III: GENERAL CONCLUSIONS

Mine soils have poor conditions for plant growth, namely low pH, low nutrients and high levels of toxic metals. The seed germination was very low in the mine soils of Touro's mine, while in the technosols, a mixture of different types of organic residuals placed on the surface of contaminated soils, germination and development of plants was much higher, being effective in stimulating vegetation cover of mine soils. The high content of nutrients present in these technosols has a fundamental role in the plant establishment and growth.

The seed bank present in the technosols probably comes from the surrounding areas of the Touro's mine. However, it is also important to screen different plant species, of different functional groups, for their ability to establish in contaminated soils. This database of local and common species is important to enrich the seed bank, in terms of quality and quantity, of mining areas, and not just being dependent on what is available around the mine area. Seed dispersion can be quite low, if the source of seeds is at some distance of the mining area, for example. In these cases, to improve vegetation growth, a mixture of seeds should be added to the area.

In the greenhouse experiment, the grass *L. perenne* was the only species that could grow, although little, in the mine soil. The other species that were tested, *D. glomerata*, *T. subterraneum* and *M. sativa*, could not survive and develop in those soils. However, the mixture of garden and mine soil was fundamental for a better performance of all species. Thus, besides screening plant species resistant to harsh soil conditions, for a better long term development of the plants, some properties of the soil must be improved, namely nutrients and a higher pH.

The physical mixture of garden soil (or technosols) with mine soils, involves heavy machinery, increasing the costs of this process. On the other hand, by just putting a layer of soil, with a seed bank, on top of mine soil, is probably less costly. Thus

initially, the seed germination and seedling establishment occurs in a 'safe soil'. However, as plants grow, roots can reach the layer of soil mine, with all the adverse characteristics. One interesting research would to evaluate if the initial layer is enough to sustain plant development, or if is necessary to regularly add new soil to mitigate the effects of the belowground layer of mine soil.

PART IV: APPENDICES
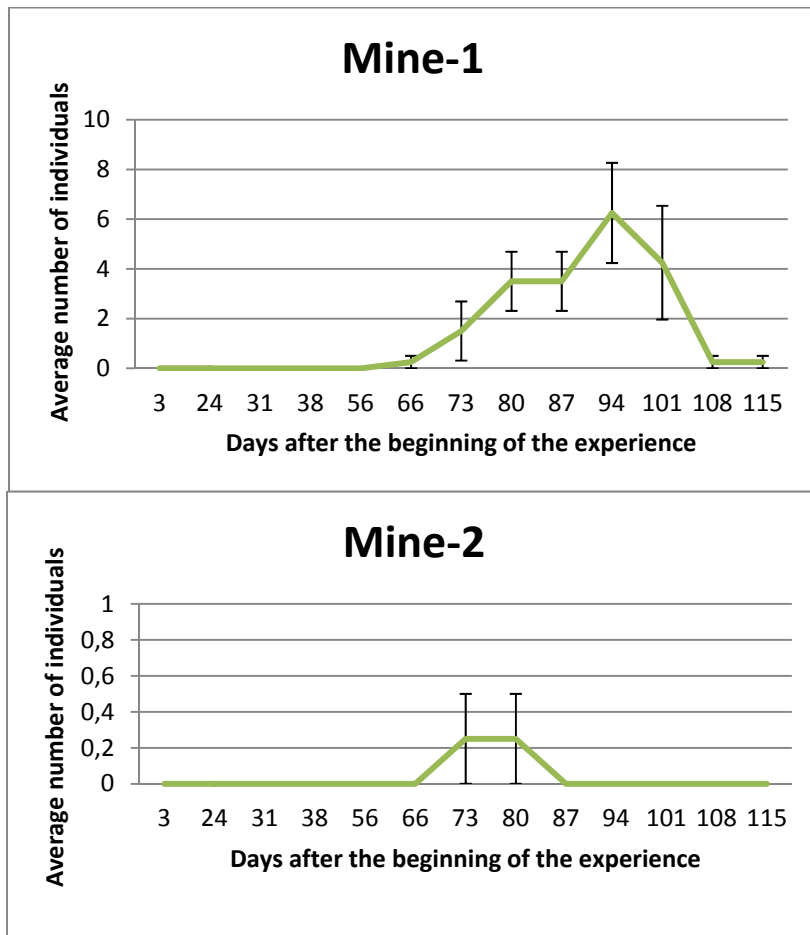
Figure 20: Average number of individuals ± SE in Mine-1 and Mine-2

throughout the experiment. The scale used on the graphs is different.
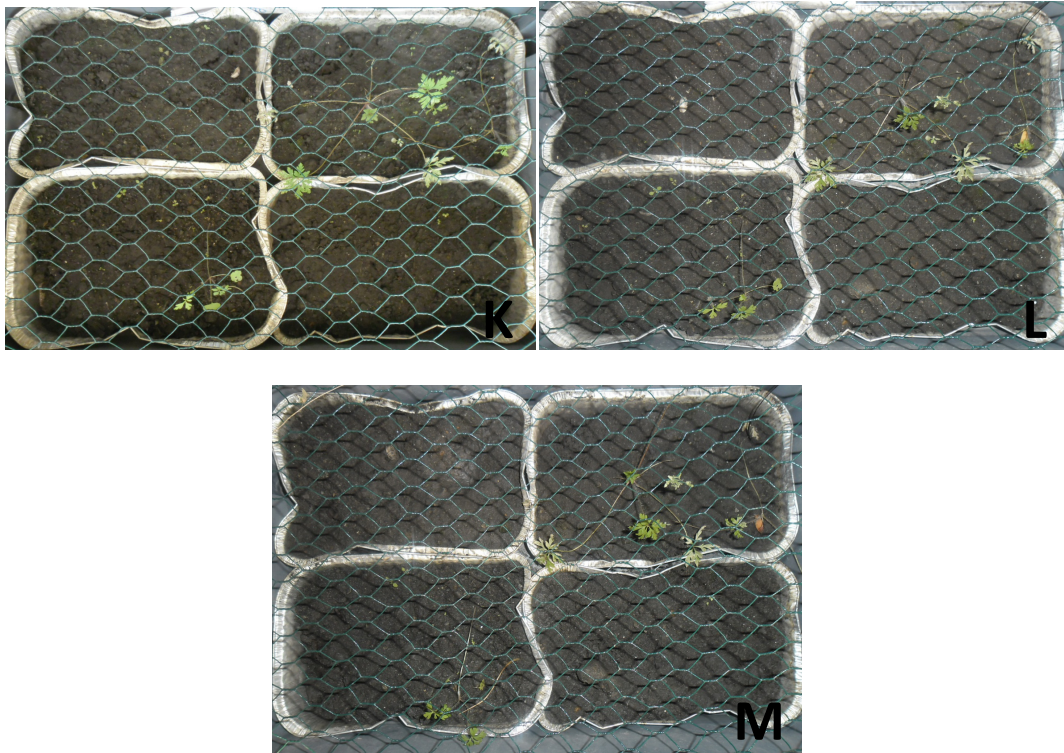
Appendix II
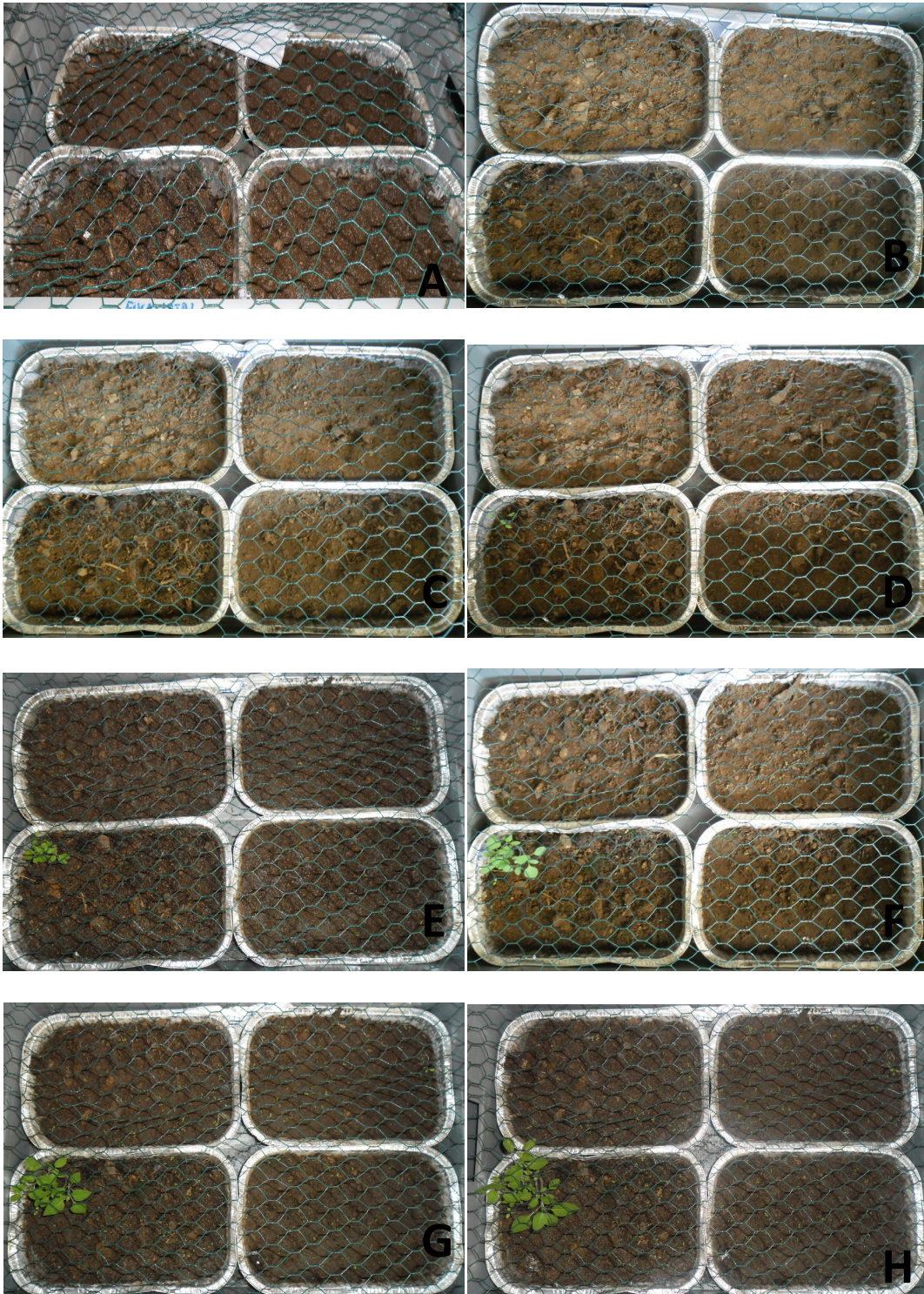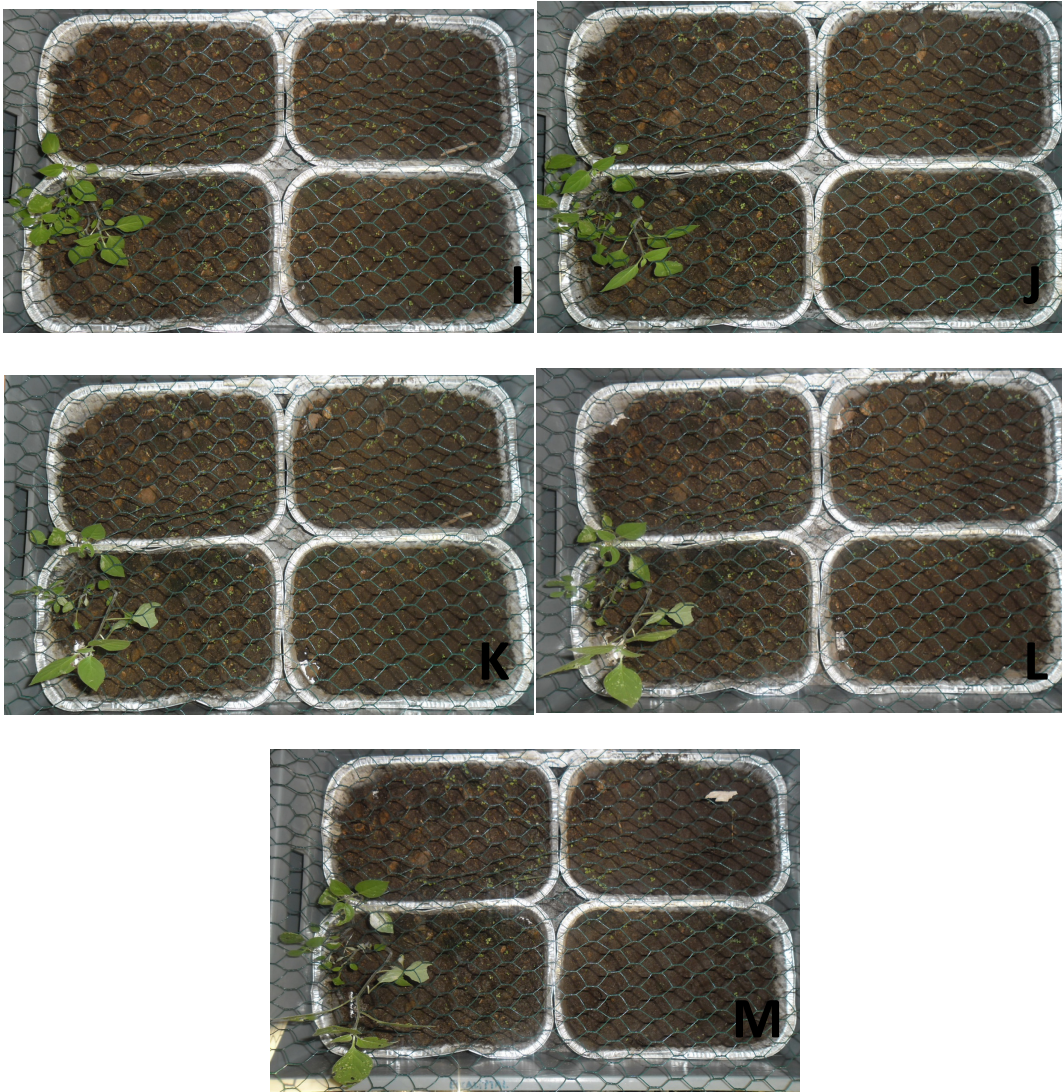
Figure 21: Photographs of the trays showing the evolution of the plant cover in "Control" on day 3 [A], 24 [B], 31 [C], 38 [D], 56 [E], 66 [F], 73 [G], 80 [H], 87 [I], 94 [J], 101 [K], 108 [L] e 115 [M].
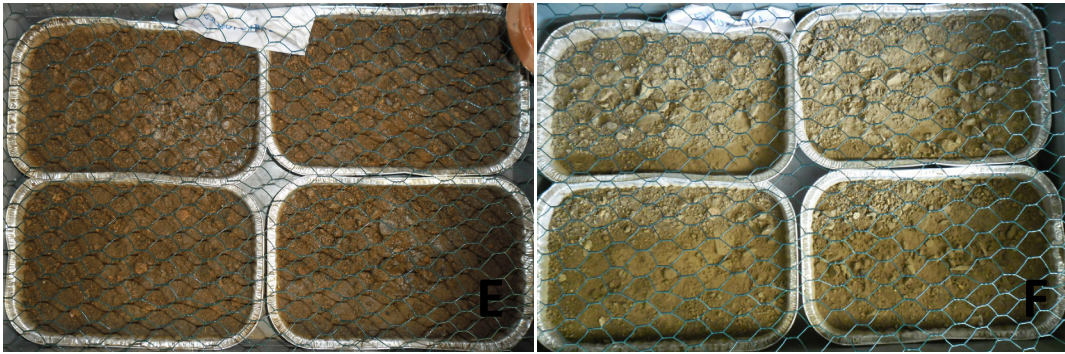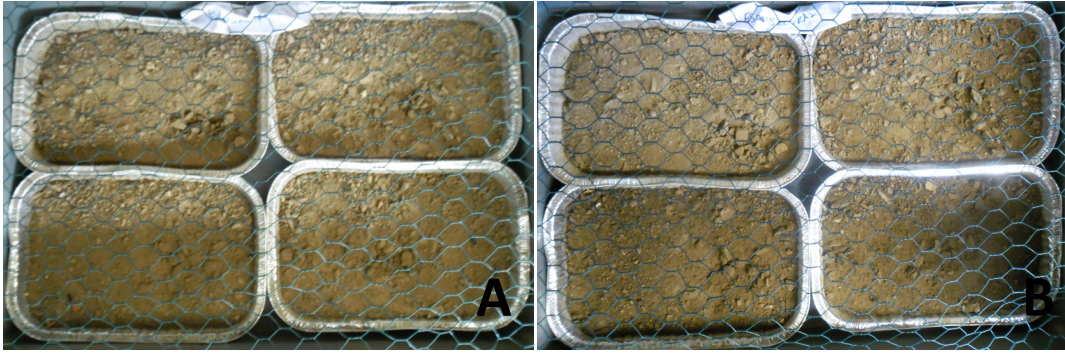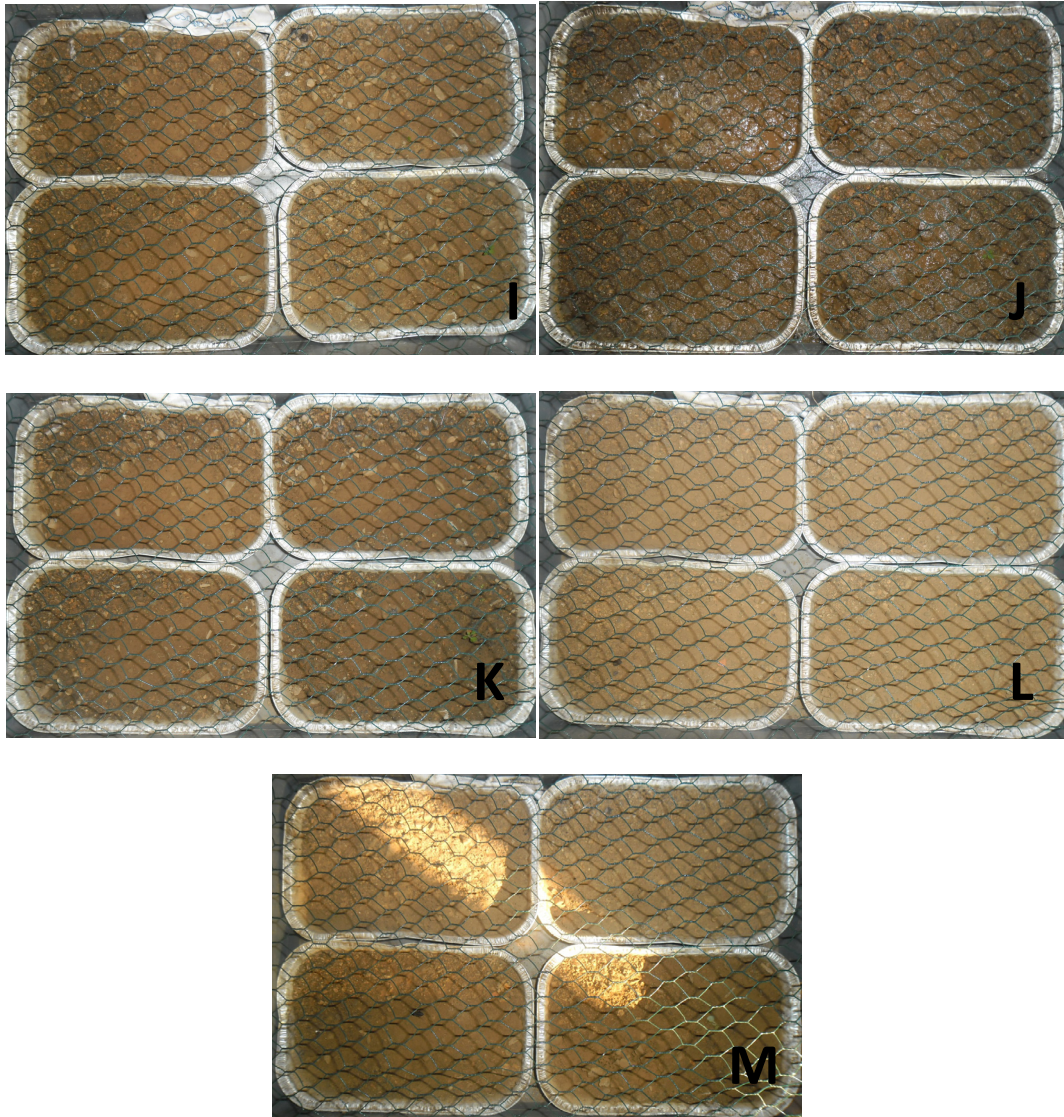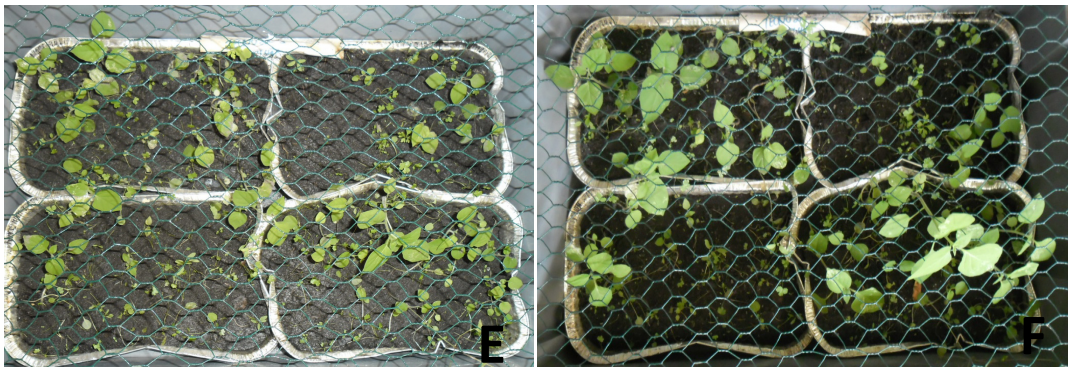
Figure 22: Photographs of the trays showing the evolution of the plant cover in "Mine-1" on day 3 [A], 24 [B], 31 [C], 38 [D], 56 [E], 66 [F], 73 [G], 80 [H], 87 [I], 94 [J], 101 [K], 108 [L] e 115 [M].
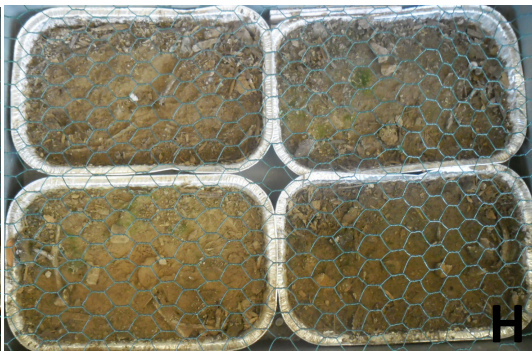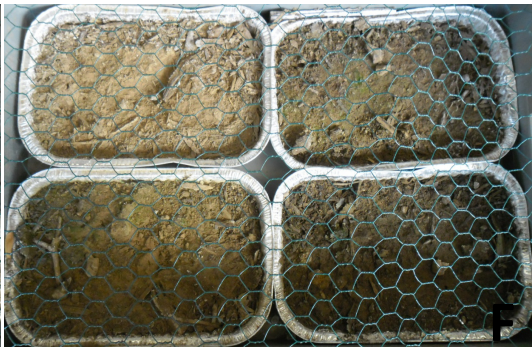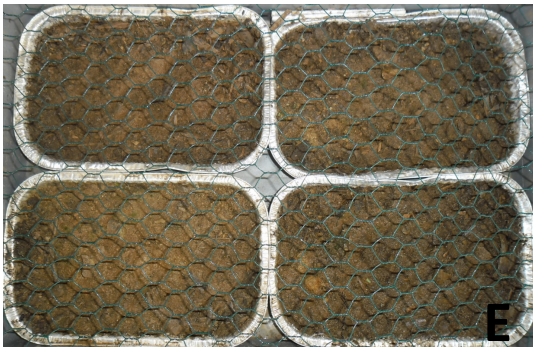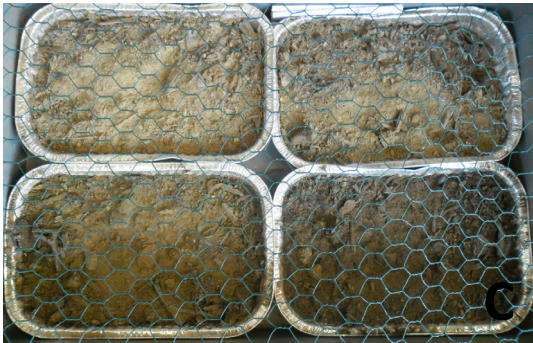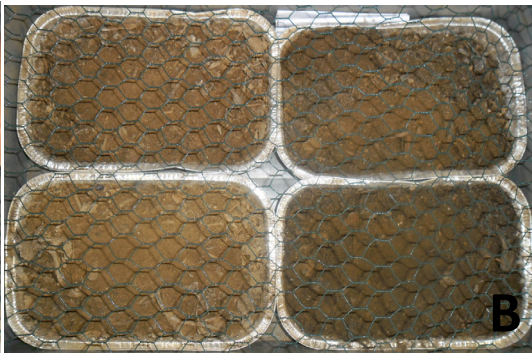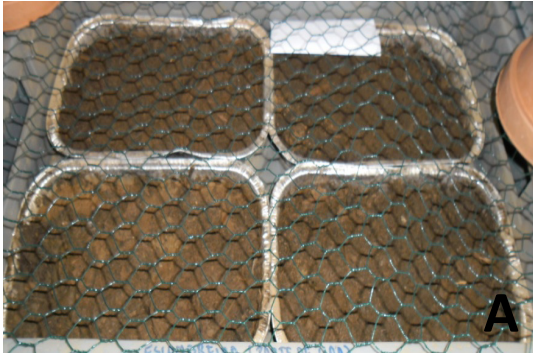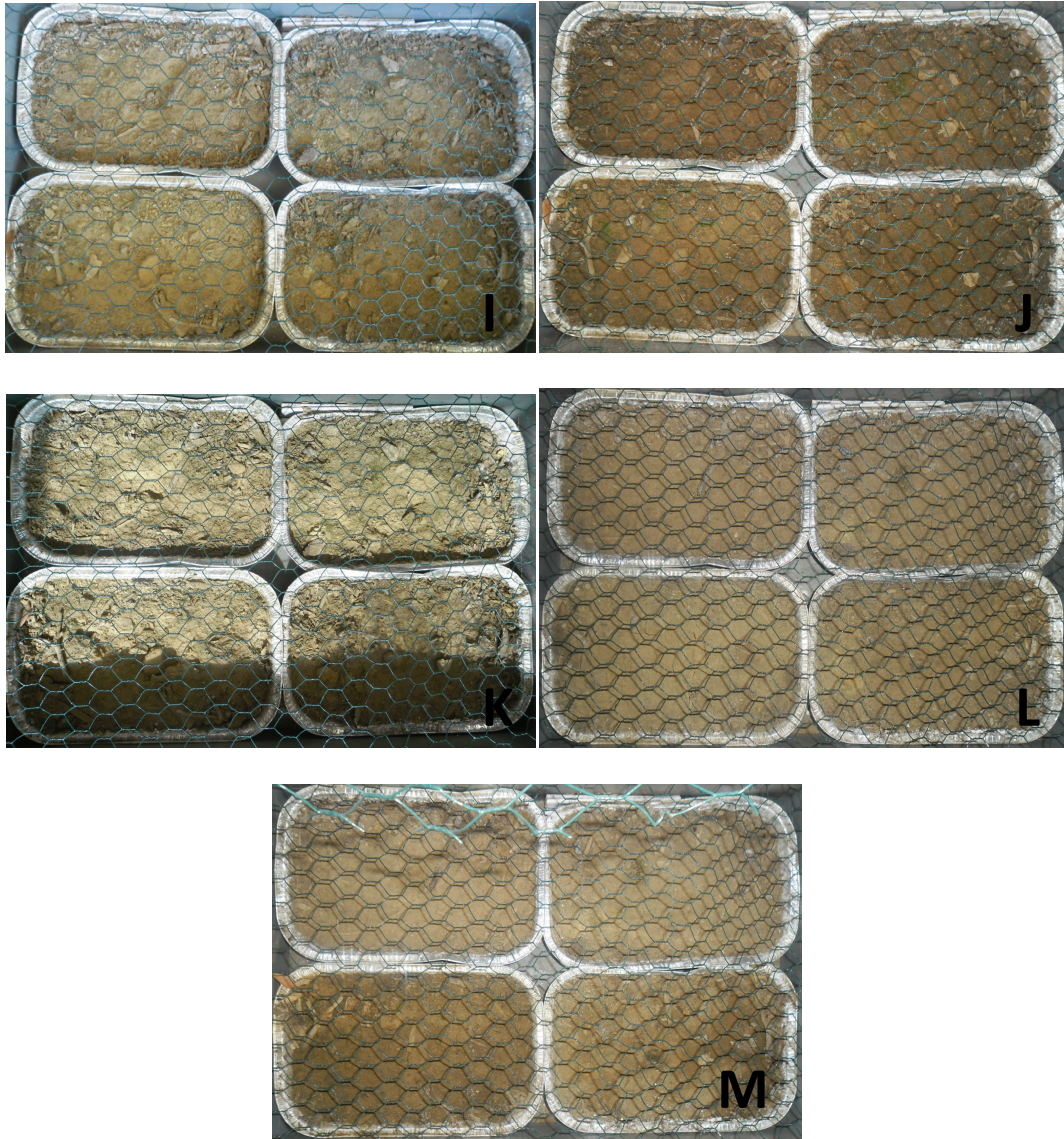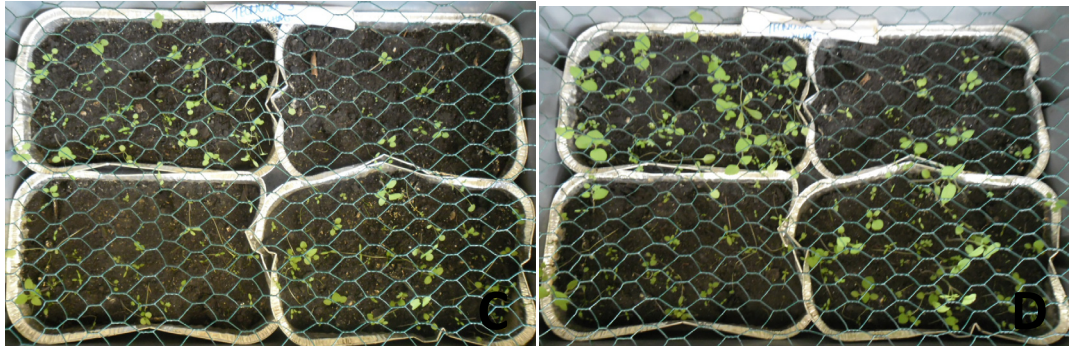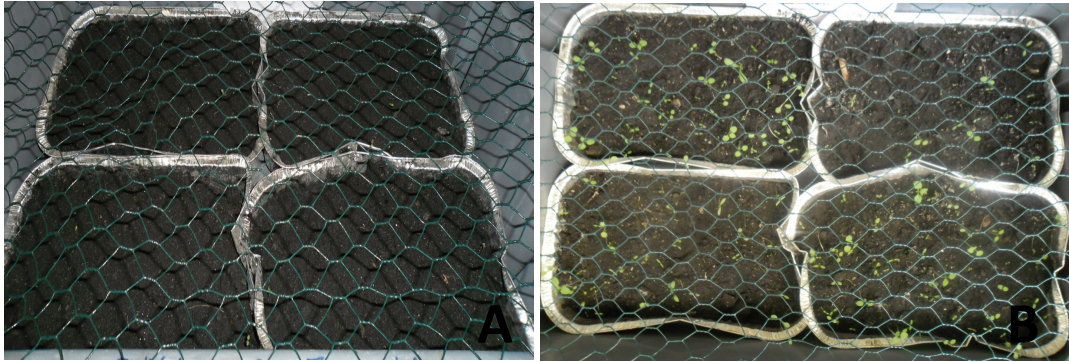
Figure 23: Photographs of the trays showing the evolution of the plant cover in "Mine-2" on day 3 [A], 24 [B], 31 [C], 38 [D], 56 [E], 66 [F], 73 [G], 80 [H], 87 [I], 94 [J], 101 [K], 108 [L] e 115 [M].
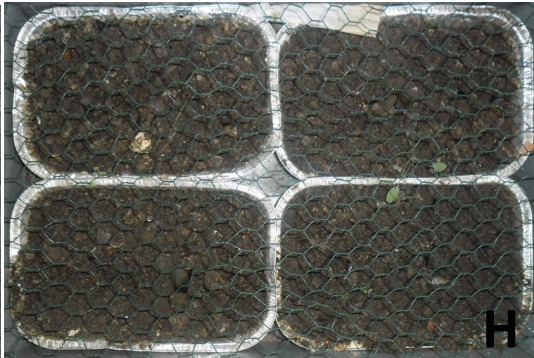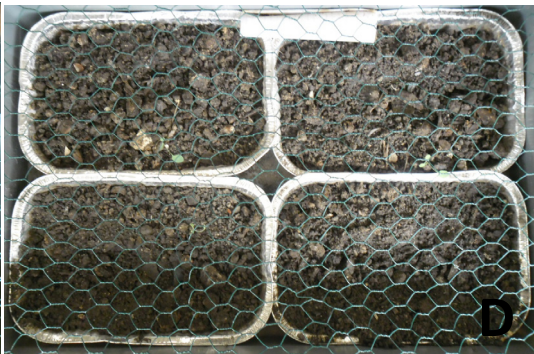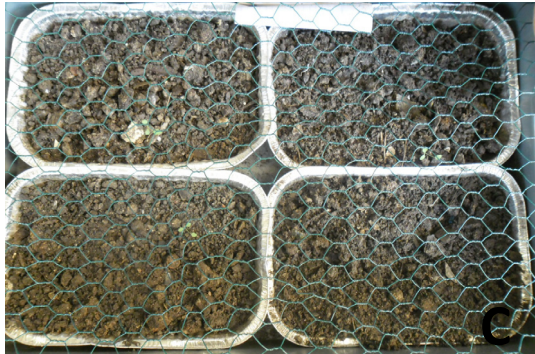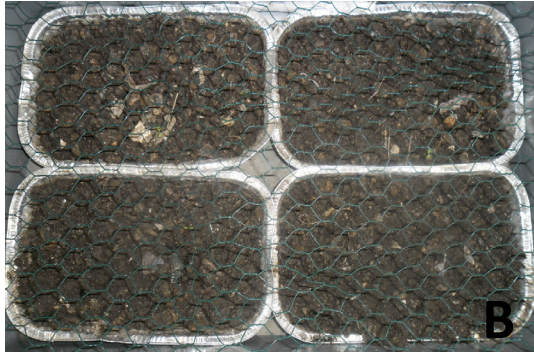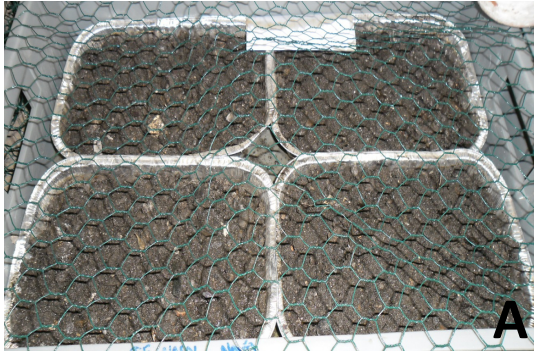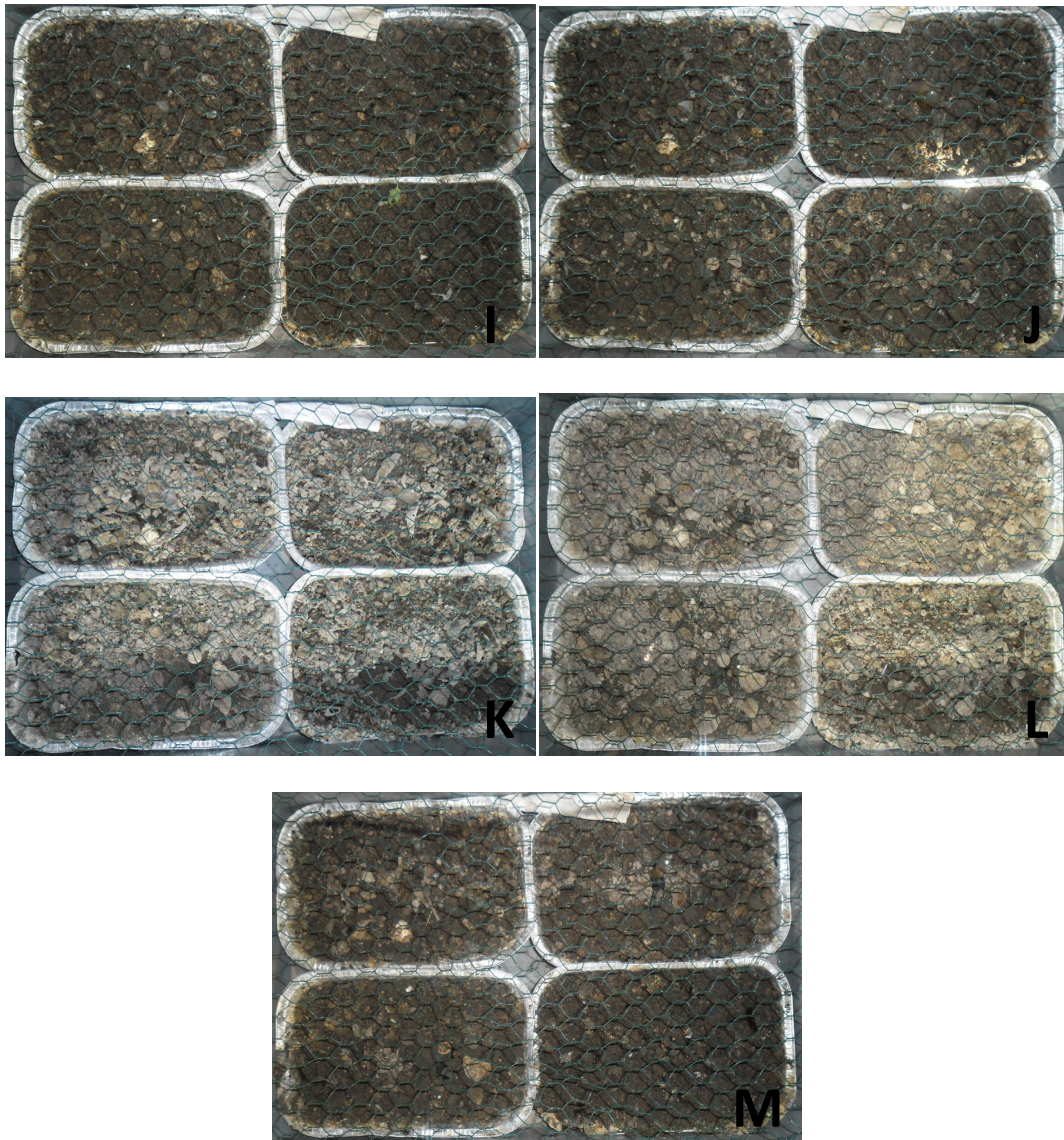
Figure 24: Photographs of the trays showing the evolution of the plant cover
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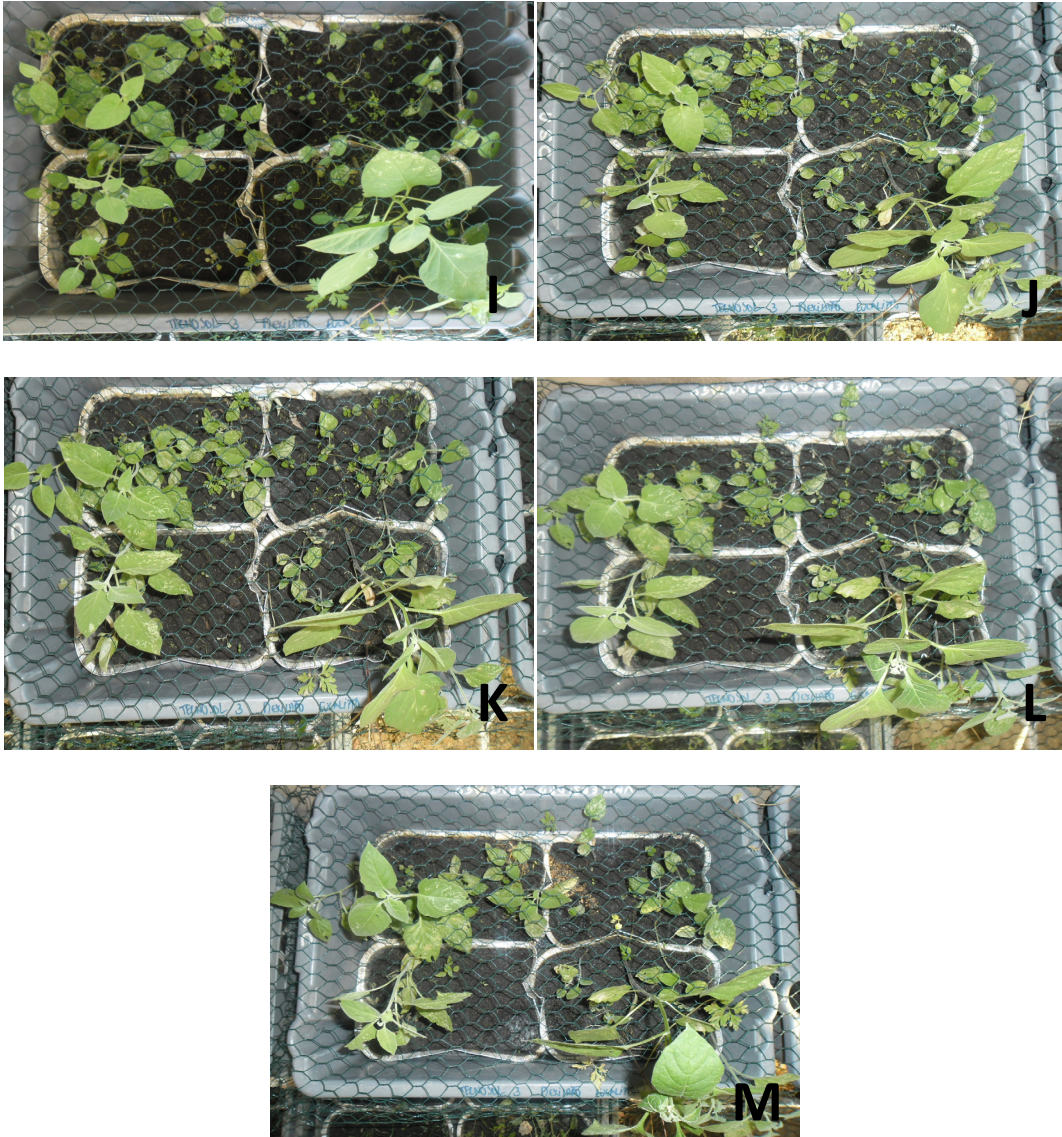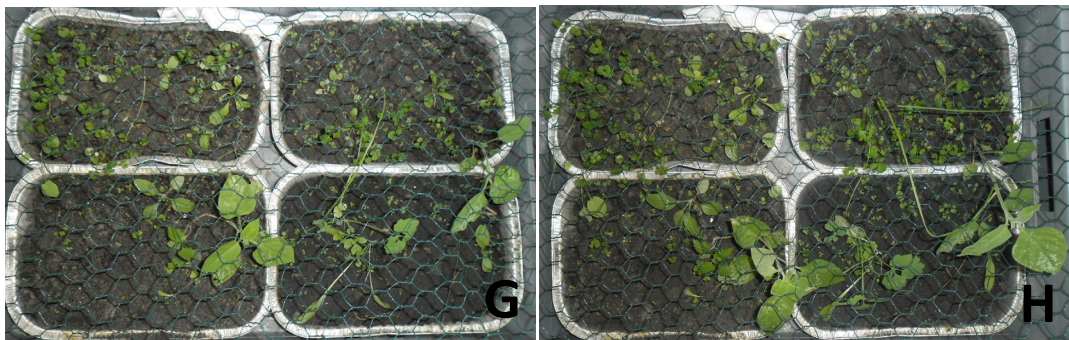[H], 87 [I], 94 [J], 101 [K], 108 [L] e 115 [M].
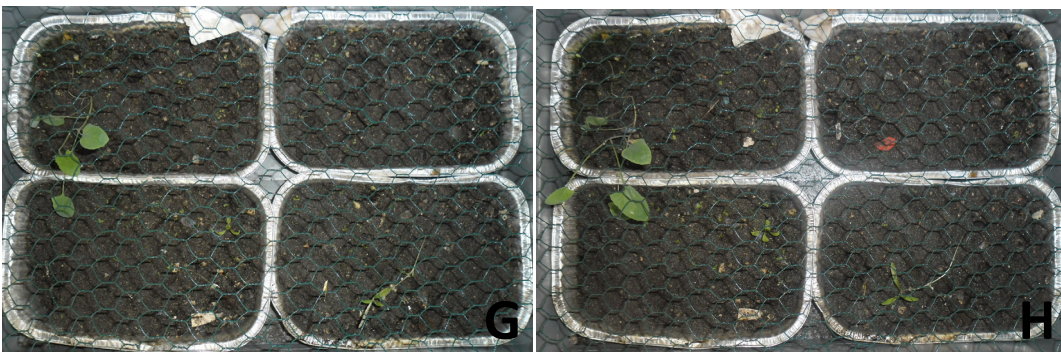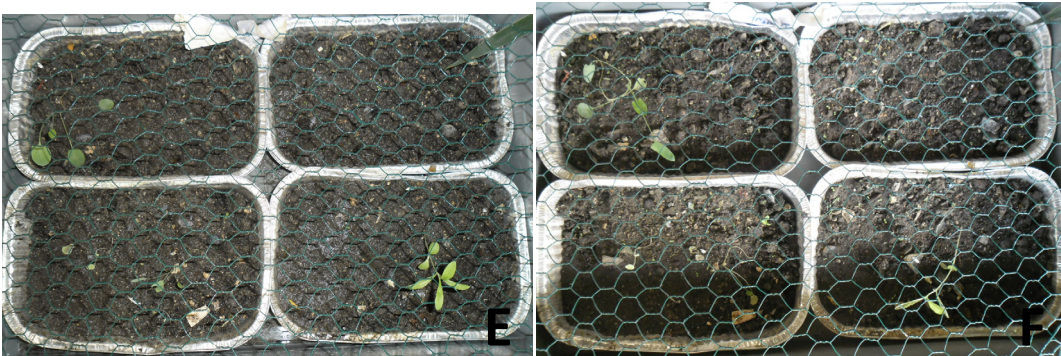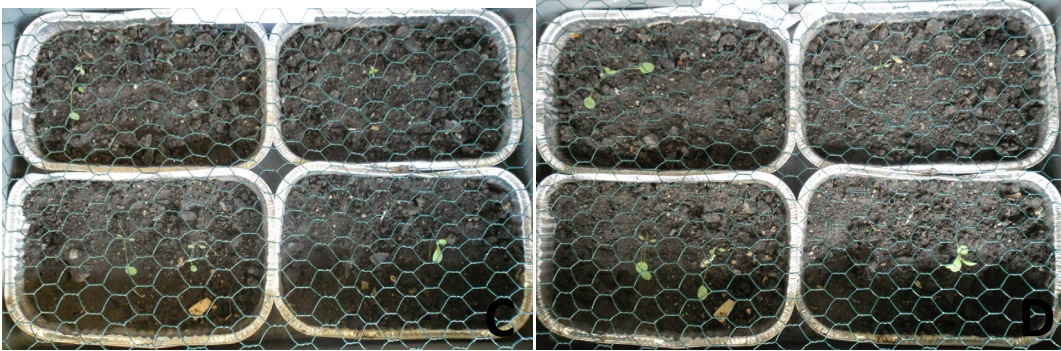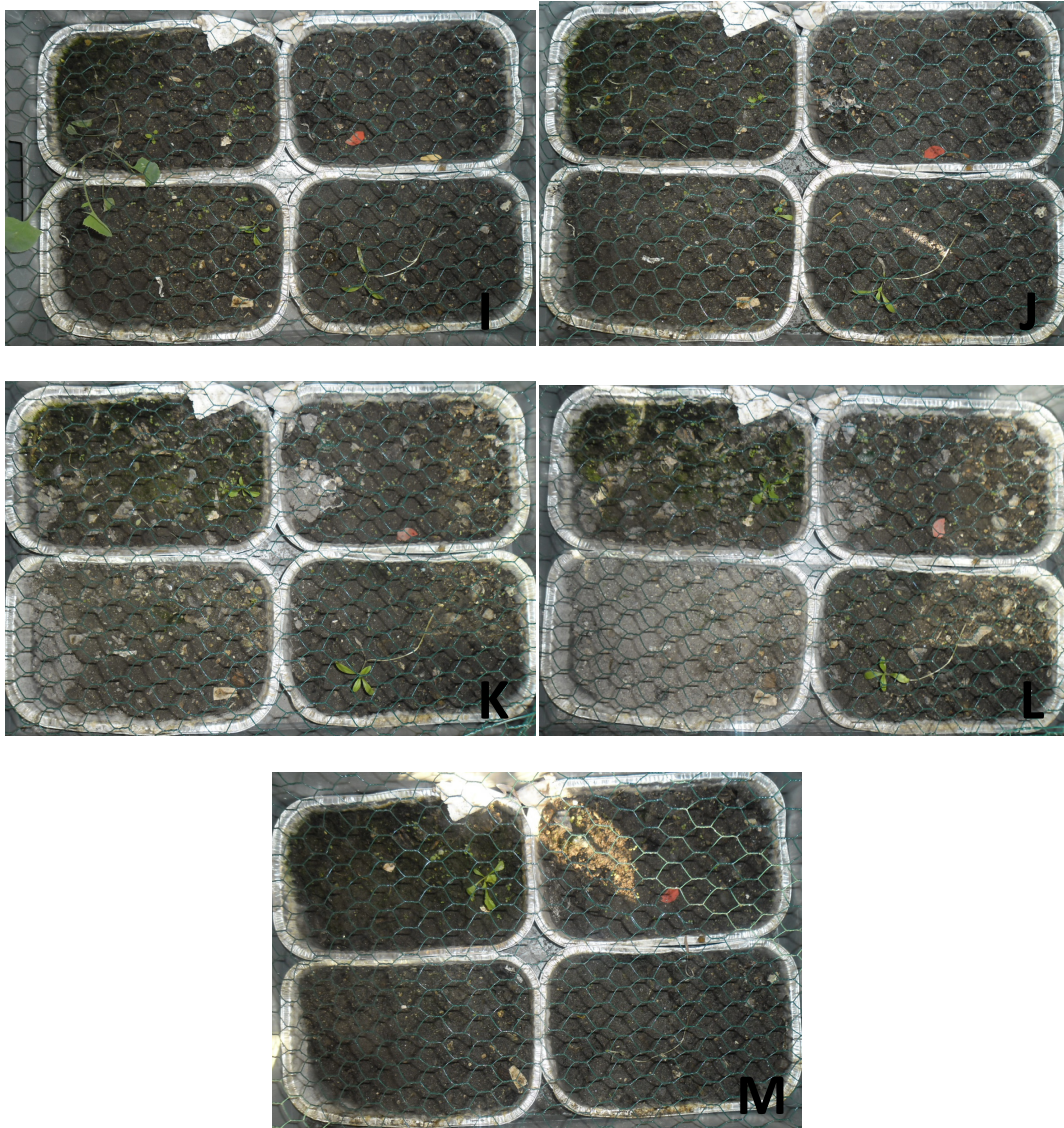
Figure 25: Photographs of the trays showing the evolution of the plant cover in "Tec-1" on day 3 [A], 24 [B], 31 [C], 38 [D], 56 [E], 66 [F], 73 [G], 80 [H], 87 [I], 94 [J], 101 [K], 108 [L] e 115 [M].
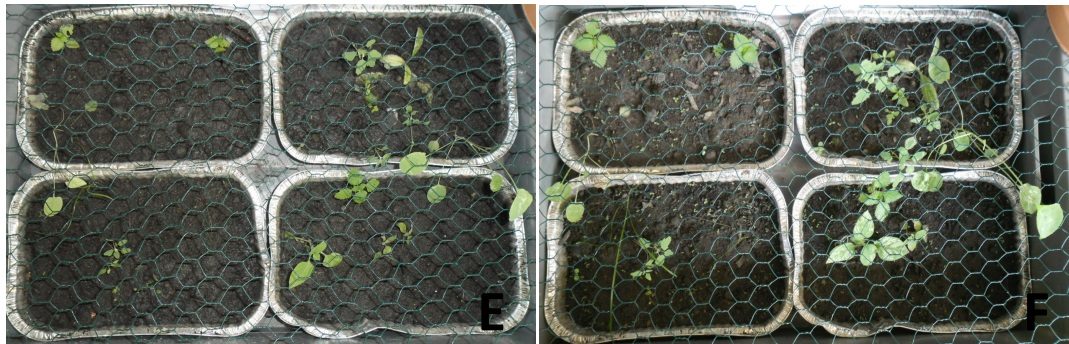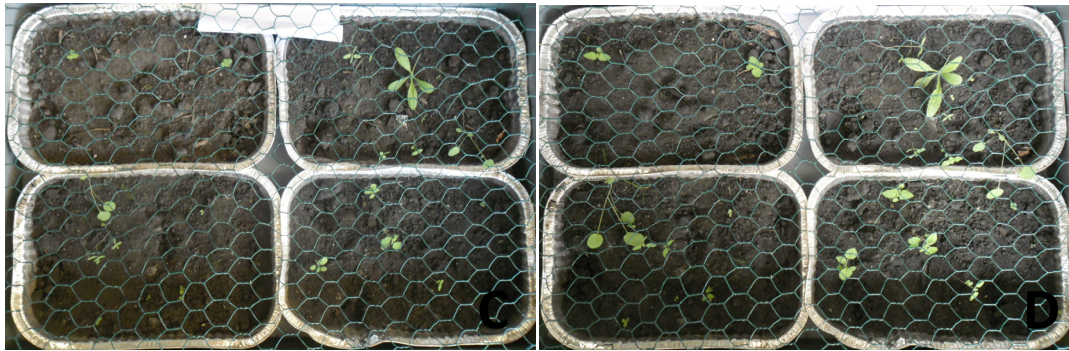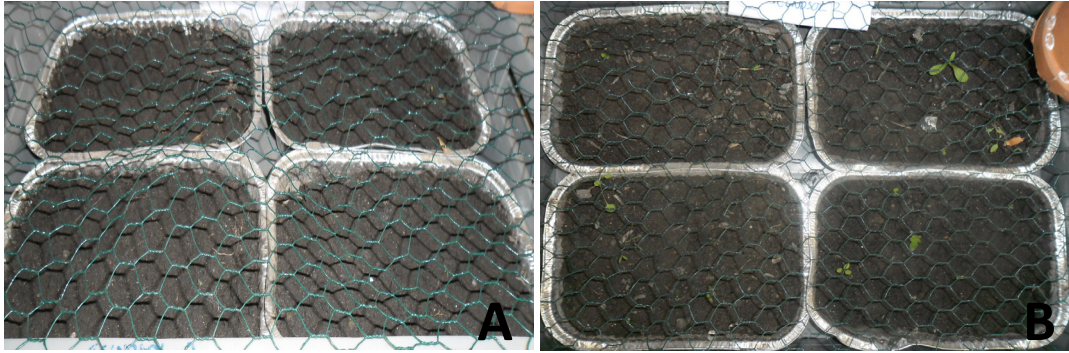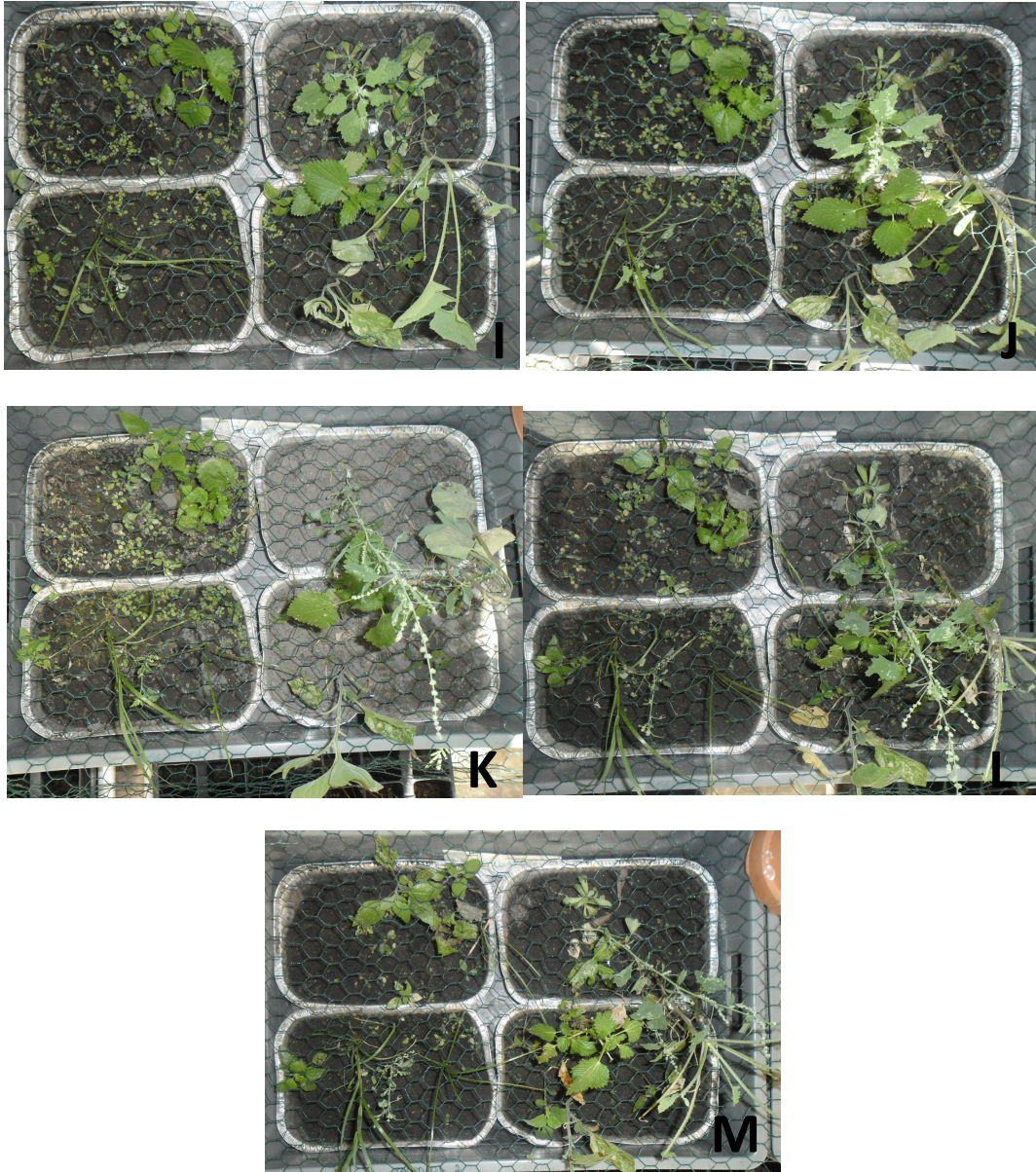
Figure 26: Photographs of the trays showing the evolution of the plant cover in "Tec-2" on day 3 [A], 24 [B], 31 [C], 38 [D], 56 [E], 66 [F], 73 [G], 80 [H], 87 [I], 94 [J], 101 [K], 108 [L] e 115 [M].
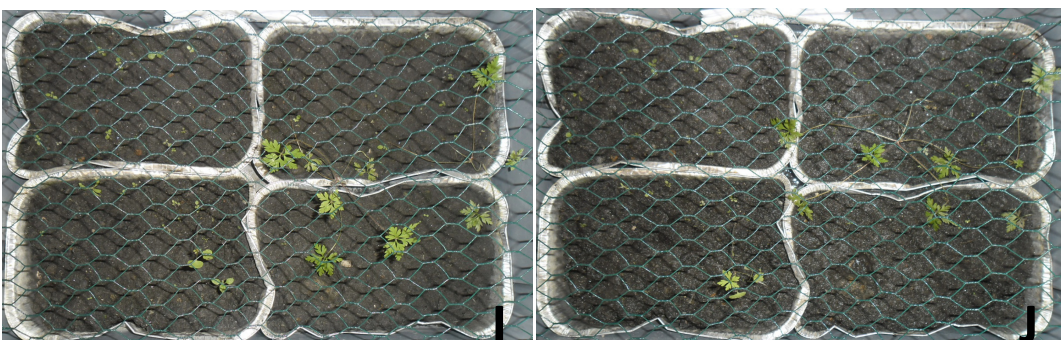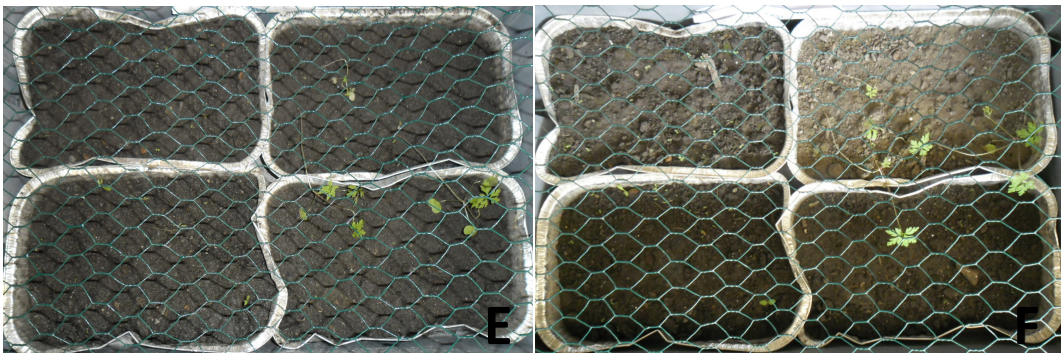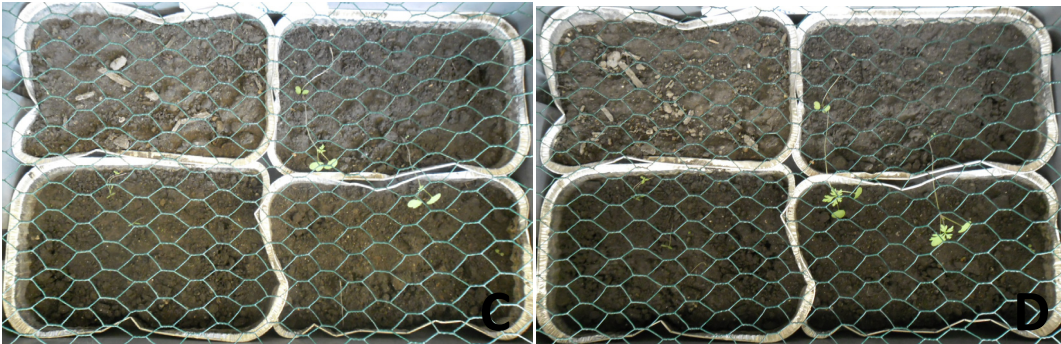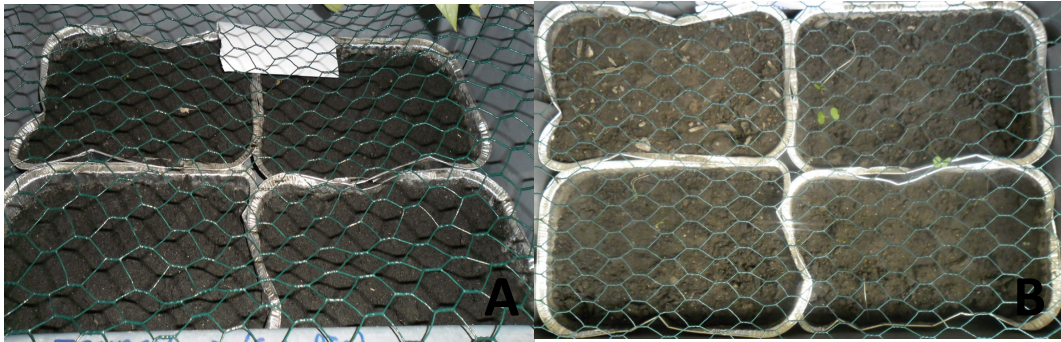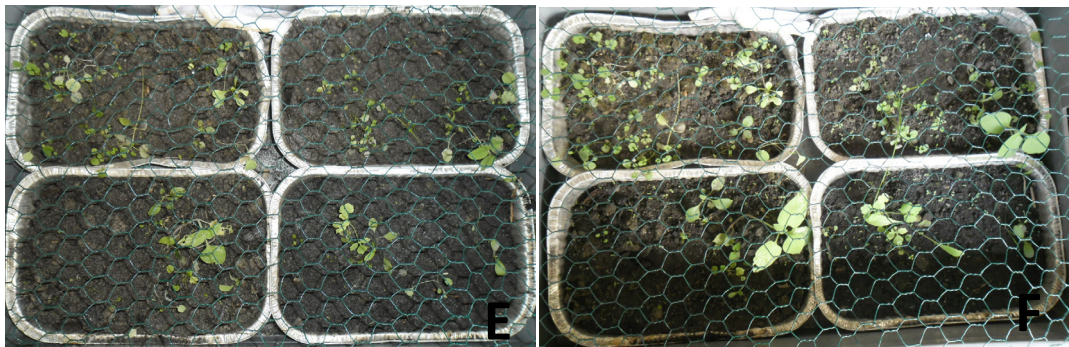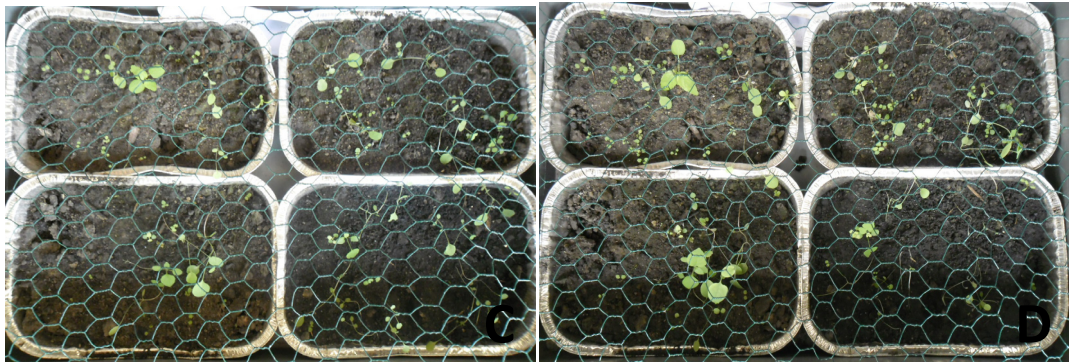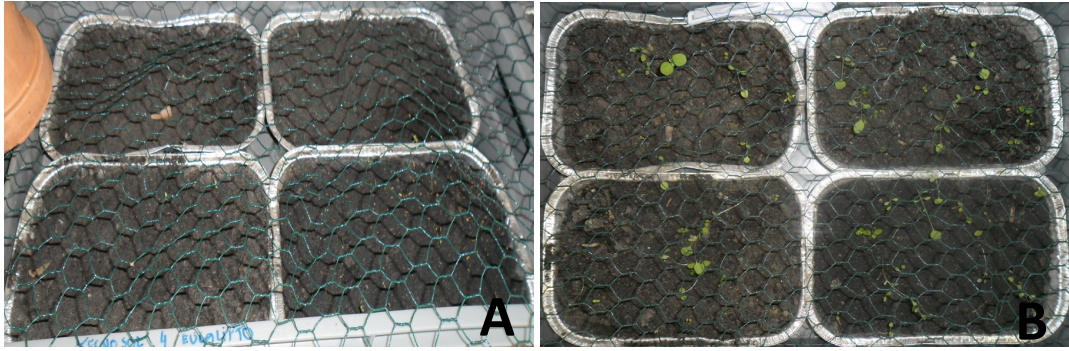
Figure 27: Photographs of the trays showing the evolution of the plant cover in "Tec-2E" on day 3 [A], 24 [B], 31 [C], 38 [D], 56 [E], 66 [F], 73 [G], 80 [H], 87 [I], 94 [J], 101 [K], 108 [L] e 115 [M].

Figure 28: Photographs of the trays showing the evolution of the plant cover in "Tec-3" on day 3 [A], 24 [B], 31 [C], 38 [D], 56 [E], 66 [F], 73 [G], 80 [H], 87 [I], 94 [J], 101 [K], 108 [L] e 115 [M].
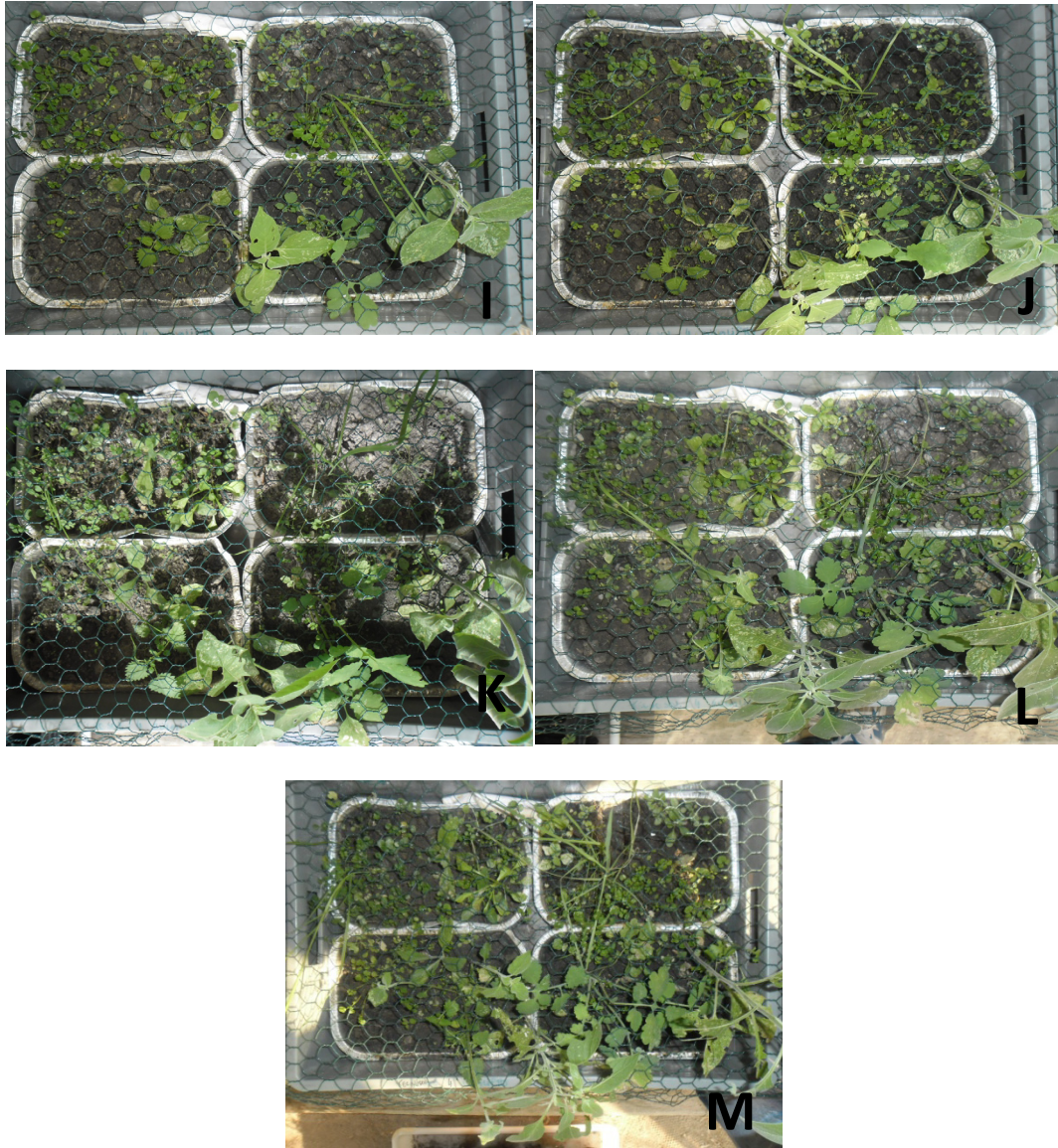
Figure 29: Photographs of the trays showing the evolution of the plant cover in "Tec-4" on day 3 [A], 24 [B], 31 [C], 38 [D], 56 [E], 66 [F], 73 [G], 80 [H], 87 [I], 94 [J], 101 [K], 108 [L] e 115 [M].