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Article

Lupinus response to chemical scarification and two culture media in *in vitro* propagation

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Abstract

In Mexico, the genus *Lupinus* has a great richness of species and its seeds generally show dormancy. The objective was to evaluate the effect of chemical scarification in two culture media on seeds of three species of *Lupinus*. The research was conducted in the tissue culture laboratory of the Tabasco *Campus* of the College of Postgraduates. The following was used: three times (20, 30 and 40 min) of immersion in 98% sulfuric acid, seeds of three species of *Lupinus*, *L. campestris*, *L. exaltatus*, and *L. montanus*, and MS and Gamborg culture media with and without Fe-EDTA. The variables evaluated were germination percentage, root length, stem length, and number of leaves. The results showed that there was a significant interaction between the treatments evaluated, the immersion time in sulfuric acid influenced the germination of the seeds. The highest germination percentage (83) was achieved in a 30 min immersion for *L. exaltatus*. In contrast, the germination percentage was 63 for *L. campestris*, and 12 for *L. montanus*. In the Gamborg culture medium with Fe-EDTA, the germination percentage was 82 and without Fe-EDTA, it was 72 for *L. exaltatus*, followed by *L. campestris* and *L. montanus*. The growth variables evaluated showed high values in the Gamborg culture medium without the addition of Fe-EDTA; in the opposite case, the variables analyzed showed increases in the values obtained. In the MS culture medium with and without Fe-EDTA, the values found were lower compared to the Gamborg culture medium.

Keywords:

dormancy, fabaceae, scarification, tissue culture.

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Introduction

The genus *Lupinus* belongs to the order Fabales, family Fabaceae, and tribe Genisteae, widely spread worldwide. It has a rich diversity of species that are divided into two large groups: 13 species from the northern Mediterranean and two from East Africa (Grether, 2005). There is no exact number of taxa in this genus; Planchuelo (1999) indicates about 1 700 species, but Clements *et al*. (2005) mention at least 600. The genus *Lupinus* L. includes a dynamic group of species characterized by their palmately compound leaves and papilionaceous flowers in terminal clusters. The plasticity to adapt to different environments, the ease of undergoing genetic changes, and phenotypic variability makes the delimitation of species a very difficult task, which led to nomenclatural confusions (Planchuelo, 1999; Eastwood *et al*., 2008; Planchuelo, 2022).

In Mexico, wild species of this genus are distributed throughout most of the national territory, with a high concentration in the Sierra Madre Occidental and the Trans-Mexican Volcanic Belt (Ruiz-López *et al*., 2006), where there is a high diversity of species (Bermúdez-Torres *et al*., 2009). The genus is represented by annual, biennial or perennial, herbaceous, shrubby, and tree plants, with alternate and stipulated leaves, usually palmately compound, with 4 to 17 leaflets (Wolko *et al*., 2010).

In their wild form, all species of *Lupinus* contain alkaloids which are toxic substances that give a bitter taste to the grain and green parts of plants (Mera, 2016), which led to the search for specimens without alkaloids (Australian Government, 2013). Currently, four species are grown in different parts of the world (*L. albus* L., *L. angustifolius* L., *L. luteus* L., and *L. mutabilis* Sweet), the grains of which represent an important source of protein for human and animal food (Nuñez, 2021).

The seeds of wild species of *Lupinus* show physical dormancy, associated with the hardening of the seed coat, so they are impermeable to both water and oxygen (Rodríguez and Rojo, 1997; Pablo-Pérez *et al*., 2013; Sánchez-Soto *et al*., 2017). Generally, the germination of seeds with a hard testa is erratic and the seedlings are fragile, so for their propagation, it is necessary to know the factors that influence the dormancy and germination of these seeds.

To break the physical dormancy in seeds, the technique used is scarification, which consists of making the seed testa permeable to stimulate the imbibition of water (Medina-Sánchez and Lindig-Cisneros, 2005; Matoor *et al*., 2019). Among the most common methods are those that use chemical and mechanical treatments. Studies related to *L. campestris*, *L. exaltatus*, and *L. montanus* indicate that scarification with sulfuric acid promotes germination; however, the response is variable and depends on the species (Acosta-Perscástegui and Rodríguez-Trejo, 2005; Gutiérrez *et al*., 2010; Garduza-Acosta *et al*., 2020). This legume is characterized by its high content of protein, oil, and alkaloids, as well as a source of protein in animal feed (Águila *et al*., 2018). Nevertheless, species of the genus *Lupinus* have seeds with a testa that is hard and impermeable to moisture and oxygen, so they require scarification treatments to induce softening and thus germination (Australian Government, 2013).

On the other hand, *in vitro* culture can be an important tool for the multiplication of *Lupinus* species and provides aseptic material from cell cultures and tissues *in vitro* for physiological and agronomic studies of these species. In *L. montanus*, it has been observed that the culture of the epicotyl in MS medium with 3 µM IAA and 1 µM BA has shown an increase in the number of stems and their height (Ramírez-González *et al*., 2015). Therefore, the present work aimed to evaluate the effect of chemical scarification and two culture media on seeds of three species of *Lupinus*.

Materials and methods

Plant material

The research was carried out in the tissue culture laboratory of the Tabasco *Campus* of the College of Postgraduates. Mature pods of *L. campestris* Schltdl & Cham, *L. montanus* Kunt, and *L. exaltatus* Zucc. were collected in the municipalities of Chalchicomula de Sesma and Tlachichuca in the state of Puebla, Mexico, located at 19° 04' north latitude; 97° 19' west longitude, and an altitude of 3 442 m.

Revista Mexicana de Ciencias Agrícolas
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The seeds were separated from the pods, dried at room temperature, and stored for approximately three months at 4 °C in plastic containers for germination analysis. Figure 1 shows the seeds of the three species under study.

Figure 1.*Lupinus* seeds. a) *L. campestris*; b) *L. exaltatus* and c) *L. montanus*, used to evaluate the effect of chemical scarification and two culture media.

Scarification and seeding

Before applying the scarification treatments, the percentage of viability of the seeds was evaluated with the use of 0.3% tetrazolium chloride (Salazar-Mercado *et al*., 2020) to determine the germinative capacity (viability of the seeds). Scarification was performed with 98% sulfuric acid (H_2SO_4).

Scarification treatments

T_0= control, T_1= 20 min immersion in sulfuric acid, T_2= 30 min immersion in sulfuric acid, T_3= 30 min immersion in sulfuric acid.

T_0= testigo, T_1=20 min de inmersión en ácido sulfúrico, T_2= 30 min de inmersión en ácido sulfúrico, T_3= 30 min de inmersión en ácido sulfúrico.

T_0 + *L. campestris*	T_1 + *L. campestris*	T_2 + *L. campestris*	T_3 + *L. campestris*
T_0 + *L. exaltatus*	T_1 + *L. exaltatus*	T_2 + *L. exaltatus*	T_3 + *L. exaltatus*
T_0 + *L. montanus*	T_1 + *L. montanus*	T_2 + *L. montanus*	T_3 + *L. montanus*

The culture media used were (MS), proposed by Murashige and Skoog (1962); Gamborg *et al*. (1968).

Germination treatments

Gamborg + Fe-EDTA + *L. campestris*	Gamborg + *L. campestris*
Gamborg + Fe-EDTA + *L. exaltatus*	Gamborg + *L. exaltatus*
Gamborg + Fe-EDTA + *L. montanus*	Gamborg + *L. montanus*
MS + Fe-EDTA + *L. campestris*	MS + *L. campestris*
MS + Fe-EDTA + *L. exaltatus*	MS + *L exaltatus*
MS + Fe- EDTA + *L. montanus*	MS + *L. montanus*

Agar Agar® (Sigma) was used as a gelling agent at a rate of 5 g L^{-1}. The pH was adjusted to 5.8. Sterilization was performed in a CV300 autoclave (manufactured domestically) at a temperature

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of 121 °C and 1.2 kg cm^{-2} pressure for 15 min. For germination, the seeds scarified in the culture medium were placed in a Thermo Scientific™ Precision™ 818 growth chamber, programmed at a temperature of 20°,15 °C and with a photoperiod of 14 h light and 10 h dark.

Each scarification and growth treatment consisted of 20 replications (test tubes). A seed was placed in each tube. The experiments were replicated three times. During the 15 days of culture, the number of germinated seeds was evaluated and expressed as a percentage (%). During 45 days of culture, root length (cm, measured with a 30 cm ruler), stem length (cm, measured with a 30 cm ruler), and the number of leaves were evaluated.

Statistical analysis

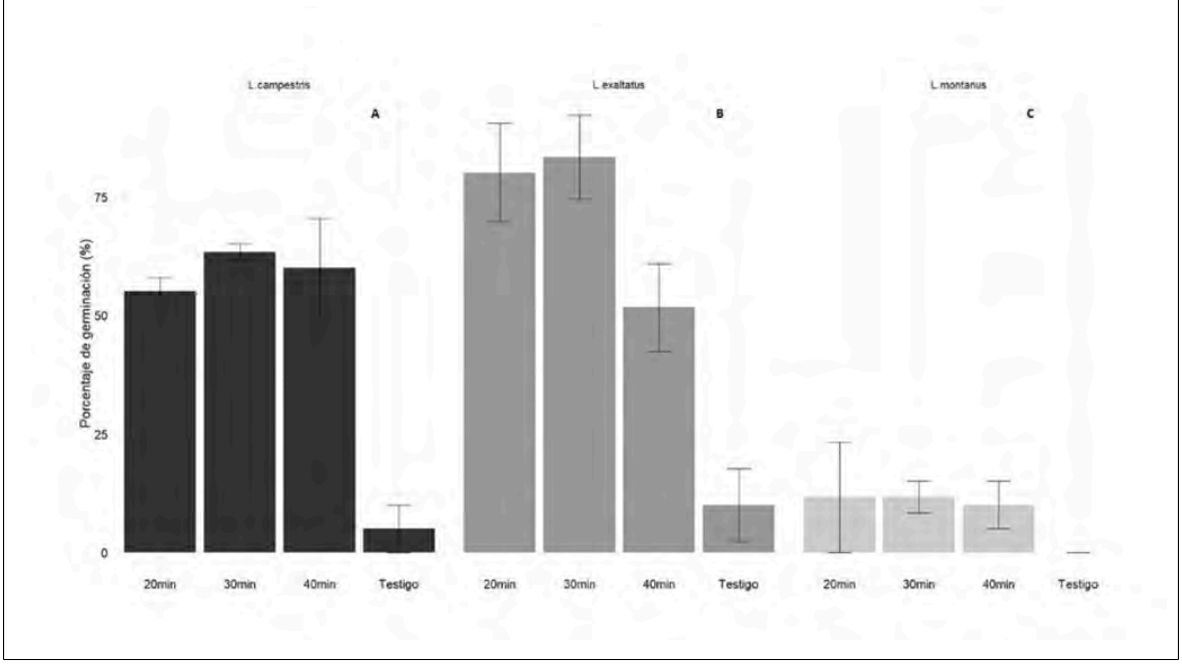
The experiments were conducted under a scheme of a completely randomized design. The experimental data of the evaluated variables were statistically processed by means of an analysis of variance with factorial arrangement. The comparison of the means was made according to Tukey's ranges ($p \leq 0.05$). All analyses were performed with the statistical software of R Core Team version 4.2.2. for Microsoft® Windows.

Results and discussion

Response to chemical scarification

The results showed statistical differences between the treatments evaluated. The interaction was significant (p-value= 0.003), the immersion time affected the germination of the seeds of the three species of *Lupinus* under study (Figure 2).

Figure 2. Germination percentage (%) of scarified seeds of three *Lupinus* varieties.



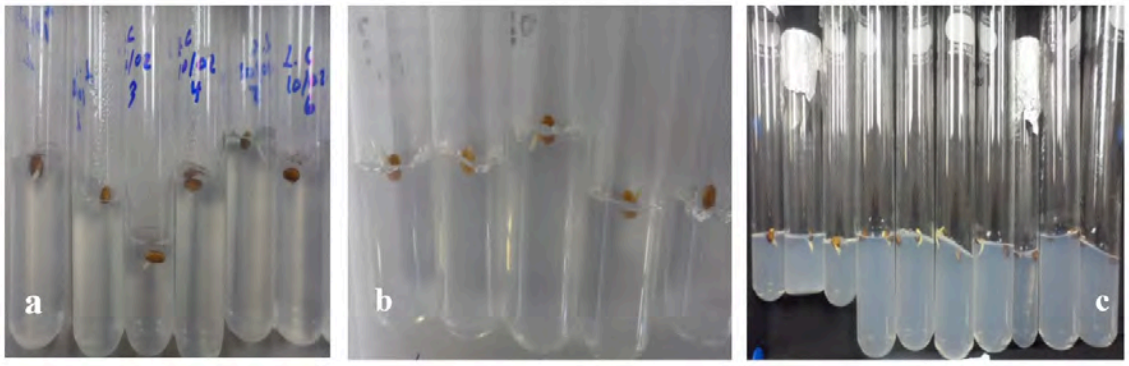
The seeds of the three species of *Lupinus* in the control treatment (without scarifying) showed low germination percentages. For the species *L. campestris*, germination percentages of 55, 63, and 60 were obtained; as the scarification time increased, the percentage of germinated seeds decreased.

DOI: https://doi.org/10.29312/remexca.v16i2.3437

For its part, for *L. exaltatus*, the germination percentages were 80, 83, and 52 in the sulfuric acid immersion times of 20, 30, and 40 min, respectively. The species *L. montanus* showed germination percentages of 12 and were the lowest for the three immersion times.

This shows that the acid managed to soften the hard testa of the seeds of the studied species, allowing their permeability, which influenced the response to germination of seeds of *L. campestris* and *L. exaltatus*, which presented the highest germination percentages among the species evaluated. Figure shows that the seed embryos in the treatments evaluated did not show damage. (Figure 3)

Figure 3. Visual assessment of seeds scarified with 98% sulfuric acid and time: a) *L. campestris* (30 min); b) *L. exaltatus* (30 min) and c) *L. montanus* (40 min).



The *in vitro* differential response to chemical scarification of the *Lupinus* seeds studied may be due to differences in the constitution of the tissues that make up the testa of this species, the conditions that prevailed during their maturation, or the response to the culture medium (Clements *et al*., 2005). Through scarification, the seed cracks and the coat softens, which allows the entry of water, oxygen, light and consequently, germination is activated (Medina-Sánchez and Lindig-Cisneros, 2005; Sánchez-Soto *et al*., 2017).

The results obtained in seeds of *L. montanus* differ from those obtained by Acosta-Perscástegui and Rodríguez-Trejo (2005) in seeds of the same species but collected in the Cumbres del Ajusco National Park in Mexico City at 3 200 masl, where with 15 min of exposure to sulfuric acid, they achieved 100% germination in Petri dishes with wet paper. In contrast, for *L. campestris*, Gutiérrez *et al*. (2010) obtained 50% germination with an immersion time in sulfuric acid of 90 minutes, results lower than those achieved in this research.

For *L. elegans* Kunth, another species presents in Mexico, germination percentages between 88 and 91 have been observed when the seeds were scarified with sulfuric acid in an immersion time of 30 or 60 min (Medina-Sánchez and Lindig-Cisneros, 2005; Corona *et al*., 2007). In the case of *L. exaltatus*, the germination percentages obtained were much higher than those obtained by Garduza-Acosta *et al*. (2020), with the same sulfuric acid for 15 min. However, the response to chemical scarification indicates that the seeds of the different species of wild *Lupinus* studied have hard or poorly permeable testas, which influences their dormancy and limits their agronomic development (Pablo-Pérez *et al*., 2013).

Response to culture medium

The results showed significant differences (*p*-value= 0.019) in the treatments when two culture media were used: Gamborg (B5) and Murashige and Skoog (MS) containing Fe-EDTA. Germination percentages of 55, 82, and 31 were achieved for the seeds of *L. campestris*, *L. exaltatus*, and *L. montanus*, respectively. On the other hand, in the MS culture medium, germination percentages decreased and were 45, 37, and 9 for L. campestris, L. exaltatus and L. montanus, respectively (Table 1).

DOI: https://doi.org/10.29312/remexca.v16i2.3437

Table 1. Germination percentage (%) of three *Lupinus* species evaluated in two culture media: Gamborg (G-CFe) and MS (MS-CFe).

Culture medium	Variety		
	L. campestris	*L. exaltatus*	*L. montanus*
MS-Fe	45	37	9
Gamborg-Fe	55	82	31

In this study, when Fe-EDTA was not added to the Gamborg (B5) and MS culture media, the best percentage (72) of germination was for *L. exaltatus* in the Gamborg culture medium. Nonetheless, seeds of *L. campestris* and *L. montanus* obtained a better response in the germination percentage, of 56 and 28, respectively, in the MS culture medium without the addition of Fe-EDTA compared to the Gamborg culture medium under the same conditions (Table 2).

Table 2. Germination percentage (%) of three *Lupinus* species evaluated in two culture media: Gamborg (G-SFe) and MS (MS-SFe).

Culture medium	Variety		
	L. campestris	*L. exaltatus*	*L. montanus*
MS-Fe	56	53	28
Gamborg-Fe	54	72	18

The lower response of the evaluated species to the MS environment may be due to the fact that this medium has one of the most negative osmotic potentials (Cárdenas and Villegas, 2002), which makes it difficult for water to enter the seed. This osmotic and toxic effect due to the presence of salts can inhibit germination. The presence of salts in the medium decreases the water potential, causing a lower availability of water for the seeds, so that they must generate sufficient osmotic potential to improve the water status of the embryos and allow their growth (Goycokic and Saavedra, 2007).

Osmotic stress could also enhance the synthesis of ABA, which is one of the main causes of dormancy in seeds (Raghavendra *et al*., 2010). In the case of the response of *L. montanus* to the MS medium added with Fe, it could be due to a lower tolerance of this species to a greater accumulation of ions from the MS medium compared to that of Gamborg, since when Fe-EDTA was removed, there was an increase in the germination percentage of this species.

L. montanus seeds contain 73.7 mg L^{-1} of Fe (Pablo-Pérez *et al*., 2013) and lupin species and genotypes differ in germination and growth in response to variations in pH and microelement content in the culture medium (Tang *et al*., 1992; Sánchez-Soto *et al*., 2017). Low germination in MS has also been observed when compared to other basal media that differ in composition and have lower concentration of salts, such as WPM, Anderson and White's (Bueno *et al*., 2009).

For example, the maximum germination values of MS-containing media suggest that the germination of *U. molinae* Turkczaninou is very sensitive to the presence of salts in the medium; the inhibitory effect of this culture medium is evident even at low concentrations, such as using ⅛ of the MS culture medium (Rodríguez *et al*., 2014). Regarding the growth variables, the results showed statistically significant differences (p-value= 0.0018< α for the variable of root length, a p-value= 0.0126< α for the variable of number of leaves, and a p-value= 0.0197< α for stem length).

When the MS culture medium contained Fe-EDTA, root length, stem length, and number of leaves decreased in all three varieties evaluated. In contrast, when the culture medium did not contain Fe-EDTA, the evaluated variables increased their values in the three varieties: *L. campestris*, *L. exaltatus*, and *L. montanus* (Figure 4).

DOI: https://doi.org/10.29312/remexca.v16i2.3437

Figure 4. rowth variables of three *Lupinus* varieties. Root length (cm), stem length (cm) and number of leaves in the MS culture medium (MS-CFe and MS-SFe).

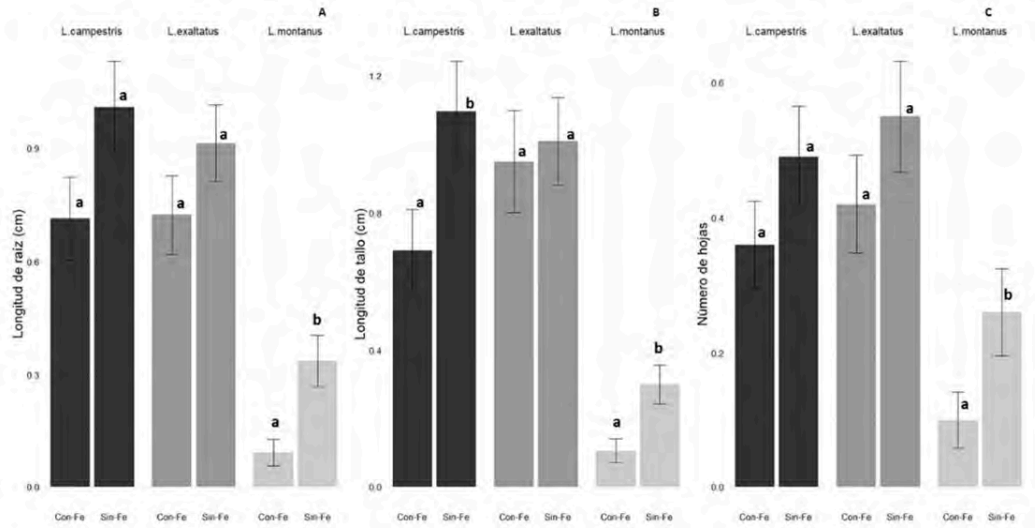
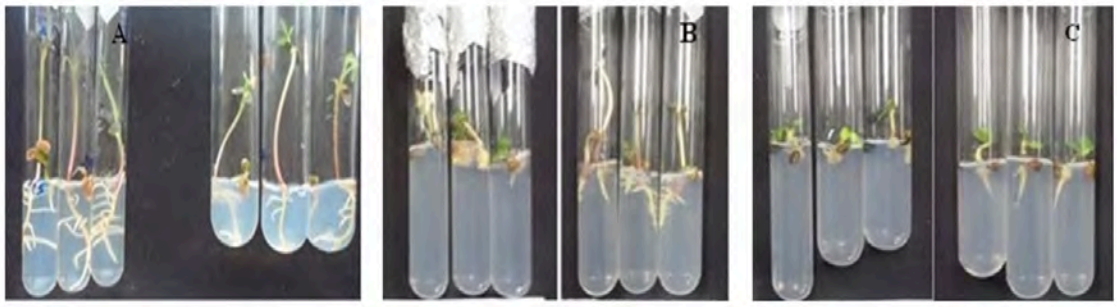


Figure 5 shows the differences in the growth of the three *Lupinus* varieties evaluated when the MS culture medium was with (left) and without (right) Fe-EDTA.

Figure 5. Growth and development of three *Lupinus* varieties. A) *L. campestris*; B) *L. exaltatus* and C) *L. montanus* in the MS culture medium (MS-CFe and MS-SFe) at 45 days of culture.



For growth variables, there were statistically significant differences in the Gamborg culture medium (Figure 6). When the culture medium contained Fe-EDTA, the response of the three *Lupinus* varieties was better than when it was not added.

Revista Mexicana de Ciencias Agrícolas

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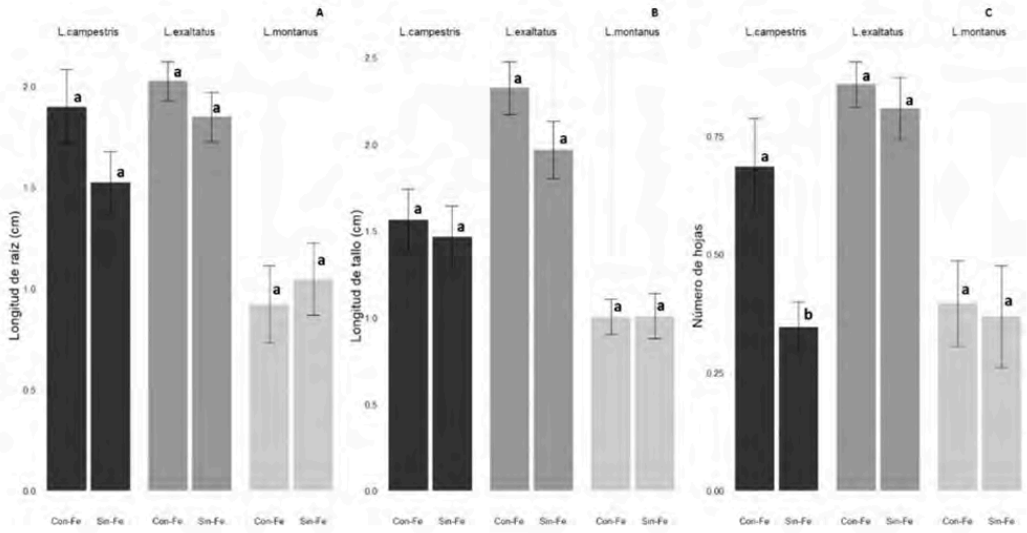
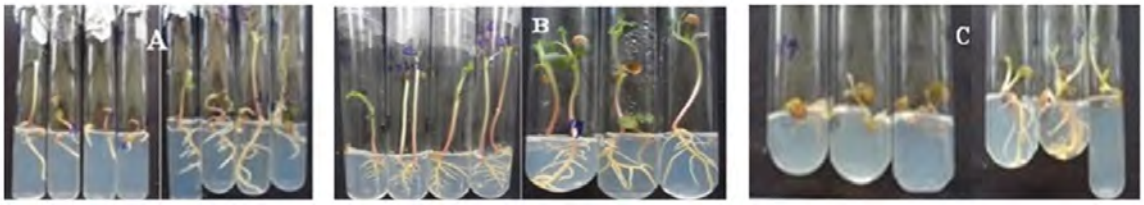
Figure 6. Growth variables of three *Lupinus* varieties. Root length (cm), stem length (cm) and number of leaves in the Gamborg culture medium (G-CFe and G-SFe).



Differences can be observed in the growth of the three *Lupinus* varieties evaluated in the Gamborg culture medium with (left) and without (right) Fe-EDTA (Figure 7).

Figure 7. Growth and development of three *Lupinus* varieties. A) *L. campestris*; B) *L. exaltatus* and C) *L. montanus* in the Gamborg culture medium (G-CFe and G-SFe) at 45 days of culture.



The results obtained are due to the fact that the MS culture medium has a high concentration of minerals, it is a rich and saline medium that can be harmful to certain species of plants, to avoid this, it is often used with the concentration of the complete microelements and the macroelements at half or three-quarters of the concentration originally described (Murashige and Skoog, 1962). The contents and source of ammonium differ in the two culture media used, ammonium ions decrease cell growth when concentrations exceed 2 mM. This reduction in growth rate is due to the inhibition of some enzymes in the Krebs cycle (Gamborg, 1970).

These results differ from those achieved by Karaguzel *et al*. (2004) in *L. varius* L., where they obtained seedlings with a height of 5.81 cm and root length of 7.5 cm after 12 days of cultivation in a plastic greenhouse with treatments of 14 and 16 hours and with the use of photoperiod lighting. The results obtained indicate that *L. varius* L. behaves as a long-day facultative plant. In *L. montanus*, seedling heights of 11.5 cm were reached when an auxin and a cytokinin were added to the culture medium (Ramírez-González *et al*., 2015).

This may be because of oxidative stress generated by an accumulation of excess Fe in the culture medium or the release of formaldehyde into the culture medium by photodegradation of Fe-EDTA (Molassiotis *et al*., 2003). In mutants of peas (*Pisum sativum* L.) with defects in the regulation of iron absorption, it was observed that Fe accumulates in ferritins and they precipitate this metal to deposit

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it in the form of electron-dense particles in the cytoplasm, mitochondria, and endoplasmic reticulum and that this constitutes a defense mechanism of the plant against the excessive accumulation of soluble Fe, which gives rise to oxidative stress (Becker *et al*., 1988; Lazarowski *et al*., 2022).

Culture media differ in salt composition; the osmotic stress caused by a salt-rich medium could cause the metabolism of plant tissues to stimulate the release of compounds that are easy to oxidize and give rise to phytotoxins (Turkan and Demiral, 2009). Fe is essential for cell growth and should be added to the culture medium in a concentration of 0.01 to 0.15 mM in the form of Fe-EDTA chelate since this form increases the solubility of iron (Llorente, 2000).

Fe can participate in reactions where free radicals and other reactive oxygen species that are toxic are released. This oxidative stress can lead to dysfunction of metabolic activities and DNA damage (Molassiotis *et al*., 2003). The Fe stress response varies among *Lupinus* species as the formation of cluster roots under Fe stress conditions depends on ion absorption mechanisms and the correlation of root ATPase activity and ion transport across the plasma membrane.

Marschner (1995) research reports that, in terms of the great capacity of the roots, it excretes protons and reduces Fe(III), and they resemble apical root zones that contain transfer cells. Certainly, *Lupinus* species such as *L. albus* L., *L. cosentinii* Guss., and *L. pilosus* L., which produce cluster roots, are much less sensitive to iron deficiency compared to *Lupinus* species that do not produce cluster roots, such as *L. angustifolius* and *L. luteus* (Tang and Robson, 1993; Tang *et al*., 1995; Planchuelo, 2022). In cluster roots of *L. albus* that develop under iron stress, the individual rootlets do not swell and reach less width than those that develop under phosphate stress.

Conclusions

Chemical scarification promoted *in vitro* germination of the evaluated wild *Lupinus* species. The highest germination percentage for *L. campestris* was 83, *L. exaltatus* 63, and *L. montanus* 12, and was obtained when the seeds were immersed in sulfuric acid for 30 min. The culture medium influenced the germination of the species under study. In *L. exaltatus*, the highest percentage of germination was found in the Gamborg culture medium with Fe-EDTA and without Fe-EDTA, where the best response was recorded in *L. exaltatus*.

For the growth variables of root length, stem length, and number of leaves, the best results were achieved in the Gamborg culture medium with Fe-EDTA when compared with the results achieved in the MS culture medium. Therefore, it was concluded that chemical scarification and immersion time influence seed germination, but it depends on the species. The culture medium contributes to germination without the addition of Fe-EDTA.

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