DOI: https://doi.org/10.29298/rmcf.v12i64.847

Article

Supervivencia y crecimiento de *Pinus engelmannii* Carr. en una reforestación por micorrización y fertilización

Survival and growth of *Pinus engelmannii* Carr. in a reforestation from mycorrhization and fertilization

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Resumen

En un vivero forestal, a la planta se le brindan los cuidados e insumos necesarios para favorecer su calidad; con la finalidad de que tenga mejores oportunidades durante su desarrollo inicial. El objetivo del presente estudio fue evaluar la supervivencia y crecimiento en diámetro y altura de *Pinus engelmannii* en una reforestación, con base en la incorporación de inoculantes micorrícicos comerciales en la etapa de preacondicionamiento en vivero, y de fertilizante de lenta liberación al momento de plantar. Se evaluaron seis tratamientos: inoculante endomicorrícico con esporas de *Glomus*, inoculante ectomicorrícico con esporas de *Amanita rubescens*, *Amanita* sp., *Lactarius indigo*, *Ramaria* sp. y *Boletus* sp., e inoculante ectomicorrícico con esporas de *Pisolithus tinctorius* y *Scleroderma citrinum*, combinado con y sin fertilizante granulado *Multicote* 8® de lenta liberación (8-9 meses) (11 N-28 P_2O_5-11 K_2O+ micronutrimentos). Las variables respuesta registradas fueron: supervivencia y causa de mortalidad, diámetro del tallo y altura. Los resultados indicaron que la supervivencia disminuyó paulatinamente, y a los 12 meses varió de 57 a 83 % entre tratamientos. En las variables diámetro y altura existieron diferencias significativas de los tres a los 12 meses. Los tratamientos con inoculante ectomicorrícico sin fertilización tuvieron mayor supervivencia, mientras que los tratamientos con inoculante y fertilizante mostraron mayor incremento en diámetro y altura. Se concluye que la inoculación controlada repercutió en la supervivencia, y la fertilización tuvo efecto sobre el crecimiento en campo de *P. engelmannii*.

Palabras clave: Crecimiento, fertilizante de lenta liberación, ectomicorriza, *Pinus engelmannii* Carr., reforestación, supervivencia.

Abstract

In a forest nursery, seedlings are provided the necessary care and inputs to promote its quality, in order to have a greater chance of survival and growth during their initial stages. The objective of this study was to assess survival and growth in diameter and height of *P. engelmannii* in a reforestation task, based on the incorporation of commercial mycorrhizal inoculants in the preconditioning stage in the nursery and slow release fertilizer at the planting time. Six treatments were evaluated: endomycorrhizal inoculant with spores of *Glomus*; ectomycorrhizal inoculant with spores of *Amanita rubescens*, *Amanita* sp., *Lactarius indigo*, *Ramaria* sp. and *Boletus* sp.; ectomycorrhizal inoculant with spores of *Pisolithus tinctorius* and *Scleroderma citrinum*; combined with and without slow release Multicote 8® granular fertilizer (8-9 months) (11 N-28 P_2O_5-11 K_2O + micronutrients). The response variables recorded were: survival and cause of mortality, stem diameter and height. Survival was found to decrease gradually and at 12 months it ranged from 57 % to 83 % between treatments, with statistical differences; there were significant differences in the diameter and height variables from three to 12 months. The treatments with ectomycorrhizal inoculant without fertilization had greater survival, while the treatments with inoculant and fertilizer showed greater increase in diameter and height. It is concluded that controlled inoculation had an impact on survival, while fertilization had an effect on the growth of *P. engelmannii* in the field.

Key words: Growth, slow release fertilizer, ectomycorrhiza, *Pinus engelmannii* Carr., reforestation, survival.

Fecha de recepción/Reception date: 29 de agosto de 2020
Fecha de aceptación/Acceptance date: 4 de noviembre de 2020

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Introduction

The forest area in Mexico is 138 million hectares, 34 million of which are forests. There are 94 species of conifers, 43 of which are endemic and 20 belong to the *Pinus* genus, whose habitat is in the state of *Durango* (Gernandt and Pérez-de la Rosa, 2014; Pérez and Ceja-Romero, 2019; Conafor, 2019), where *Pinus engelmannii* Carr. naturally grows, as well as in the states of *Chihuahua, Sinaloa, Zacatecas, Sonora*, and in the United States of America, in southeastern Arizona and the extreme southwest of New Mexico (Ávila-Flores *et al.,* 2016). In spite of the extensive forest areas and the abundant biodiversity that exists at the national level, adverse factors of natural and anthropogenic origin cause deforestation and degradation of these ecosystems.

To counteract deterioration, reforestations are part of several alternatives considered for that ending (Prieto and Goche, 2016). However, of the programs carried out between 2006 and 2014, survival one year after planting was 43 %; drought (42.4 %), inappropriate planting date (9.7 %), low plant quality (9.2 %), competition with vegetation (7.6 %) and grazing (7.3 %) were determined as the most important causes of mortality (Prieto *et al.,* 2016a; Prieto *et al*., 2016b). Conafor (2019) stated that from 2013 to 2017, the average survival increased to 59 % due to an improvement in the production process, transportation and planting of the forest material.

For 2019 the national plant production was 79 million plants of 43 species, distributed in 157 nurseries (Conafor, 2019).

The NMX-AA-170-SCFI-2016 Mexican standard indicates the guidelines for producing forest plants in nurseries, with the minimum specifications that the plant material must meet, prior to their planting in the field; for *Pinus engelmannii*, it refers to ≥5 mm diameter, an age of 10 to 18 months and the presence of mycorrhiza (Secretaría de Economía, 2016). However, most of the forest nurseries in Mexico have not included the application of ectomycorrhizal inoculants in their plant production programs for reforestation (Pérez-Moreno *et al.*, 2020), since each nursery produces plants based on the environmental conditions, available resources and technical knowledge possessed by those responsible for said activity, which affects the variability of the morphological characteristics of the produced plant (Burney *et al.,* 2015).

Mycorrhizal symbiosis in forest plant production favors seedling nutrition, provides greater resistance to drought and damage caused by diseases (Pera and Parladé, 2005). Although it is known that the species of the Pinaceae group form ectomycorrhizae, when they establish in open sites, some species of the genus *Pseudotsuga, Abies* and *Tsuga* can generate endomycorrhizae (Cázares and Trappe, 1993); however, it has also been established that fertilization can inhibit mycorrhization (Baltasar *et al*., 2007; Salgado *et al.,* 2009; Salcido-Ruiz *et al.,* 2020); so the compatibility depends on the degree of susceptibility of the fungus to fertilization (Trappe, 1977; Brundett *et al.*, 1996).

The aim of the present work consisted on the assessment of the survival as well as the growth in diameter and height of *Pinus engelmannii* in a reforestation, incorporating commercial mycorrhizal inoculants in the preconditioning stage in the nursery and slow release fertilizer at the time of planting. It was based on the hypothesis that the application of mycorrhizal inoculants in the nursery and fertilization during this process favor both responses of the individuals.

Materials and Methods

Study area

The plantation was established in the *Otinapa ejido* of the town with the same name, at *Durango* municipality, in the state of *Durango*, Mexico; it is located between 24°03'52.2" N and 105°01'31.1" W, at an altitude of 2 350 m.

The average annual temperature is 14.6 °C, with a minimum of 6.1 °C and a maximum of 21.1 °C; the mean annual rainfall was 667 mm. The predominant vegetation is composed of various species of the *Pinus* genus, among which are *P. engelmannii, P. cooperi* Blanco, *P. leiophylla* Schltdl. *et* Cham., *P. teocote* Schiede ex Schltdl., as well as *Juniperus* sp. (Prieto *et al.,* 2018; González, 2019). The soil has a sandy-loam texture (54 % sand, 28 % silt and 18 % clay), with an average pH of 6.9, electrical conductivity of 0.41 mS cm^{-1} and organic matter of 1.11 %.

Plant production

The vegetative material was reproduced in the "*Praxedis Guerrero*" forest nursery, of the Department of Natural Resources and Environment of the Government of the State of *Durango*, Mexico; It is located at kilometer 12.5 of the *Durango-El Mezquital* highway (23°56'58.3" N and 104°34'07.4" W), at an altitude of 1 890 m.

The plant was produced in black rigid polyethylene tubes with internal root guides, with a 4 cm upper diameter, 21 cm long and 165 mL. As a substrate, a mixture based on peat (48 %), composted pine bark (22 %), vermiculite (16 %) and agrolite (14 %) was used. During the preparation of the substrate, 4 kg m^{-3} of Multicote 8®, a slow release granulated fertilizer (8-9 months), with the composition 11N-28P_2O_5-11K_2O + micronutrients were added. The seed came from a seed stand, located at 2 465 m altitude, in *San Miguel de Cruces, San Dimas, Durango*.

Site preparation and planting

The selected site was five years without tree cover, due to an extreme drought in 2012 and subsequent damage in 2013 by bark cutters, which caused tree mortality. Three years before planting, the soil was plowed with an agricultural tractor, through the passage of a subsoiler, with which lines 40 cm deep were marked in the center of the planting line and 3 m apart with a Truper® mod. TP20ME of 20 m measuring tape. In each line, with a Truper® mod. 18631 *talacho*, vines of 25 to 30 cm in diameter and depth were made, separated at 1.0 m (this measurement was made with an Anvil® mod. 96408 tape measure). The plantation covered an area of 600 m^2. The 11-month-old plant was established in the field on August 11th, 2018, with an average diameter of 6.5 mm and height of 8 cm, acceptable morphological characteristics for *P. engelmannii*, based on the NMX-AA-170-SCFI-2016 standard (Secretaría de Economía, 2016). Measurements of these variables were made with a Truper® model 14388 calibrator and a Pilot® model RAS-30 graduated ruler, respectively.

Treatments and experimental design

Six treatments were evaluated, with three inoculants and two fertilization conditions (Table 1). In the mycorrhization factor, one month before planting, the vegetative material was inoculated with three commercial products: A) Bio Bravo®, B) Ecto-Myc® and C) MycorTree Ecto-injectable®, the first two are of national origin, while the third is imported; all three products contained spores. Inoculants A and B were applied 10 mL plant^{-1}, while for C, 2 mL plant^{-1} were added; the doses applied were based on the recommendations of the assessed products. In relation to fertilization, two conditions were established: planted with 7 g of fertilizer and without fertilizer; Granulated fertilizer Multicote 8® was applied with the 11N-28P_2O_5-11K_2O + micronutrients composition, placing it at the bottom of the strain and covering it with a layer of 5 cm of soil, to avoid direct contact with the roots.

Table 1. Assessed treatments in survival and growth of *Pinus engelmannii* Carr. in the field.

Treatment	Description
IA	Inoculant A (10 mL plant^{-1}) and without fertilization
IB	Inoculant B (10 mL plant^{-1}) and without fertilization
IC	Inoculant C (2 mL plant^{-1}) and without fertilization
IA+F	Inoculant A (10 mL plant^{-1}) and fertilization of 7 g plant^{-1}
IB+F	Inoculant B (10 mL plant^{-1}) and fertilization of 7 g plant^{-1}
IC+F	Inoculant C (2 mL plant^{-1}) and fertilization of 7 g plant^{-1}

IA= Arbuscular mycorrhizal inoculant with *Glomus* spores; IB = Ectomycorrhizal inoculant with *Amanita rubescens* Pers., *Amanita* sp., *Lactarius indigo* [Schwein] Fr., *Ramaria* sp. and *Boletus* sp. Spores; IC = Ectomycorrhizal inoculant with *Pisolithus tinctorius* (Pers.) Coker *et* Couch y *Scleroderma citrinum* Pers.spores; F = Slow release Multicote 8® granulated fertilizer (8-9 months), with the 11N-28P_2O_5-11K_2O+ micronutriment composition.

The distribution of the plants was carried out in an experimental design of complete random blocks. Each treatment had four replications and 10 plants per experimental unit.

Assessed variables and statistical analysis

Survival and the cause of death were evaluated every three months for one year, under the following categories: A) Drought, complete plants and no physical damage; B) Grazing, plants broken by trampling or browsing; C) Noxious fauna, partial or total disappearance of the plant coupled with the appearance of a mound of earth in the place where it was planted. In addition, the diameter at the base of the stem was recorded with a Truper® digital vernier and the height of the plants, measured with a conventional graduated ruler. An analysis of variance was performed for each variable and in the cases in which there were statistical differences, Duncan's means comparison tests ($p \leq 0.05$) were performed using the statistical program SAS™ version 9.2 (SAS, 2009). The percentage values of survival and cause of death were transformed with the arcsine function and square root.

Results and Discussion

Survival and mortality

Survival at three months ranged from 92.5 to 100 %, at six months from 85 to 100 %, at nine months from 77 to 97 %, and at 12 months from 58 to 83 %; however, only statistical differences ($p \leq 0.05$) were found between treatments at nine and 12 months. At nine months, the IA, IC and IC + F treatments were located in the upper statistical group with more than 94 % survival, while in the final evaluation, the IC was better and the IA + F treatment obtained the lowest value (Table 2).

Table 2. Survival of *Pinus engelmannii* Carr. for 12 months, based on the use of inoculants in the preconditioning and fertilization stage at the time of planting.

Treatment	Survival (%)			
	3 months	**6 months**	**9 months**	**12 months**
IA	100 ±0.0 a	100 ±0.0 a	97.5±0.1 a	72.5±0.05 abc
IB	97.5±0.1 a	92.5±0.1 a	87.5±0.1 ab	77.5±0.03 ab
IC	100 ±0.0 a	97.5±0.1 a	95.0±0.1 a	82.5±0.06 a
IA+F	92.5±0.1 a	85.0±0.1 a	77.5±0.1 b	57.5±0.05 c
IB+F	97.5±0.1 a	92.5±0.1 a	85.0±0.1 ab	60.0±0.04 bc
IC+F	100 ±0.0 a	100 ±0.0 a	95.0±0.1 a	72.5±0.12 abc

IA = Arbuscular mycorrhizal inoculant with *Glomus* spores; IB = Ectomycorrhizal inoculant with *Amanita rubescens* Pers., *Amanita* sp., *Lactarius indigo* [Schwein] Fr., *Ramaria* sp. and *Boletus* sp. spores; IC = Ectomycorrhizal inoculant with *Pisolithus tinctorius* (Pers.) Coker *et* Couch and *Scleroderma citrinum* spores; F = Slow release Multicote 8® granulated fertilizer. ±Standard error of the mean. Means with different letters in the same column indicate differences between treatments (Duncan, $p \leq 0.05$).

After nine months, there was a general decrease in survival, with lower percentages in the IA + F and IB + F treatments, which coincided with the dry season, normally from February to May. The highest survival, 12 months after planting, occurred in the IC and IB treatments, which lacked fertilization and were inoculated with ectomycorrhizae; in these treatments, mycorrhization could have given the plant a greater chance of survival, as the fungi improve plant growth and favors greater tolerance to drought stress (Kipfer *et al.,* 2010).

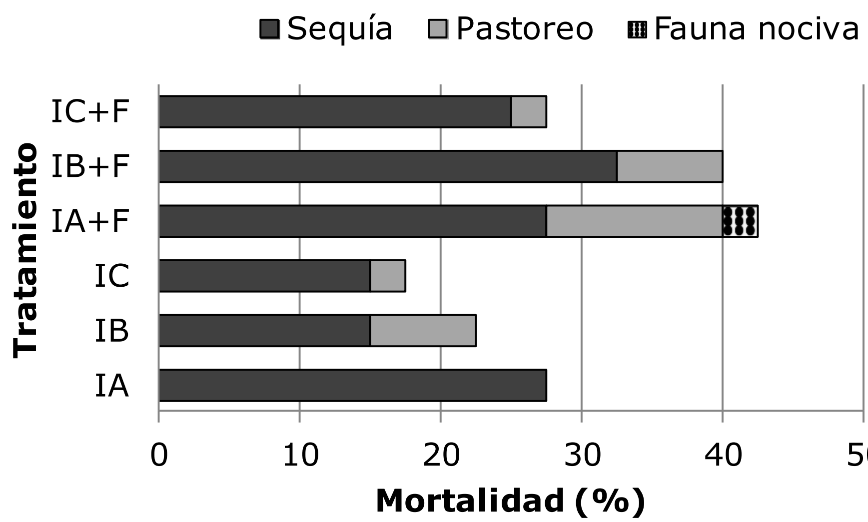
According to Mejía *et al.* (2015), forest plantations go through a critical stage after planting, where survival is affected in the first years. These results showed that the survival achieved was greater than the national average (59 %) at one year of planting (Conafor, 2019), but less than that obtained by González (2019) in a *P.*

engelmannii plantation in a neighboring site, who obtained an average survival of 85 % a year after planting.

At a site located less than 3 km from this study, Prieto *et al.* (2018) evaluated survival and growth in *P. engelmannii*. In this investigation, they handled plants with two initial diameters, 6.5 and 5.0 mm (similar to the diameter of the plants used in this essay), fertilized with slow release material (composition 18N-6P_2O_5-12K_2O+2MgO+ micronutrients), applied in doses of 7 g plant^{-1}. At 13 months after being planted, survival for the larger diameter specimens was 67.5 %. In this case, the IC treatment obtained a higher survival percentage (82.5 %), in which inoculation without fertilization could have influenced this result (López-Gutiérrez *et al*., 2018).

In regard to the dead saplings (30 %) one year after being planted, three causes associated with this fact were identified: drought (80 %), grazing (18 %) and noxious fauna (2 %); the higher mortality can be explained by the lack of moisture in the soil during the dry season, which, as it has a sandy-loam texture, it limits water retention, coupled with the low content of organic matter. It should be added that the habitat for the species under study is not ideal as it is a transition zone, with rains in summer and an annual rainfall of 667 mm, which is near the lower limit defined by Martínez (1992), from 500 to 1400 mm.

The factors associated with mortality had a different impact on the treatments (Figure 1). In the case of IB and IC treatments, a greater resistance to drought damage is observed, which can be explained by an effective ectomycorrhizal symbiosis, since it is the component they share and it is one of the functions that have been attributed to them (Gómez-Romero *et al*., 2015; Barroetaveña *et al*., 2016). In addition to this, the causes of mortality registered coincide with those reported by Prieto *et al*. (2016b) at the national level, of which drought is the most important.



Tratamiento = Treatment; *Mortalidad* = Mortality; *Sequía* = Drought; *Pastoreo* = Grazing; *Fauna nociva* = Noxious fauna.

Figure 1. *Pinus engelmannii* Carr. mortality at 12 months after planting based on the use of inoculants in the preconditioning and fertilization stage at the time of planting.

Although it has been documented that the *Pinus* genus predominantly establishes ectomycorrhizal symbiosis (Bücking *et al*., 2012), reference has also been made to the use of arbuscular mycorrhiza on *P. engelmannii* with which favorable reactions were obtained in the growth of the plant (Montes-Rivera *et al.,* 2001). The results of the present study for the IA treatment may indicate that an effective symbiosis was not established, because the time contemplated for the formation of the mycorrhizal association was possibly insufficient, especially in the case of a slow-growing species, compared to species shrubby or herbaceous, in which the formation of arbuscular mycorrhiza is faster (Brundett *et al.,* 1996).

In fertilized treatments (IA + F, IB + F and IC + F), a possible effect of inhibition of mycorrhiza formation by fertilization is also observed. In this regard, it has been pointed out that increasing this procedure can decrease mycorrhizal colonization (Brundett *et al.,* 1996; Salgado *et al*., 2009), as occurred in *Pinus engelmannii*, where plants inoculated in the nursery with native fungi (*Amanita rubescens* Pers., *Amanita* sp., *Lactarius indigo* [Schwein] Fr., *Ramaria* sp. and *Boletus* sp.) plus 3 g L^{-1} of controlled release fertilizer, had a higher Dickson quality index and a higher percentage of mycorrhizal colonization, in relation to a plant fertilized with a dose of 6 g L^{-1} (Salcido-Ruiz *et al.,* 2020).

The causes of mortality external to the components of each treatment, such as grazing and noxious fauna, had an impact on 20 %. According to Prieto *et al.* (2016b), physical damage to plants by the presence of livestock can be fatal due to trampling or browsing; in this experiment, the affected specimens were trampled and failed to recover. On the other hand, the damage caused by *Pappogeomys castanops* Baird (1852) (gopher) was also fatal, as Gunn *et al.* (2016) stated they are burrowing rodents that consume plants or parts of them, which, being of human interest, are classified as a pest that requires control. However, the importance of the dynamics of gophers has been demonstrated for the conservation of other species that use their gallery system as refuge sites (Rosas-Espinoza *et al*., 2014), so their benefit or harm is in terms of the study object.

Stem diameter

Statistical differences ($p \leq 0.05$) were found between treatments in the four evaluations carried out, with the treatments with the mycorrhizal condition plus fertilization (IA + F, IB + F and IC + F) presenting the best results (Table 3). The final average increase in the treatments with fertilization was 10 mm, while without fertilization (IA, IB and IC) was 6.9 mm.

Table 3. Stem diameter of *Pinus engelmannii* Carr. during 12 months, based on the use of inoculants in the preconditioning and fertilization stage at the time of planting.

Treatment	Stem diameter (mm)			
	3 months	6 months	9 monthss	12 months
IA	8.7±0.2 b	10.5±0.3 b	12.4±0.4 b	14.3±0.4 b
IB	8.6±0.3 b	10.0±0.3 b	11.2±0.3 c	12.6±0.3 c
IC	8.5±0.2 b	10.1±0.3 b	11.7±0.3 bc	13.2±0.4 bc
IA+F	10.7±0.3 a	13.6±0.3 a	14.9±0.4 a	16.6±0.5 a
IB+F	11.2±0.3 a	13.7±0.4 a	14.9±0.4 a	16.5±0.5 a
IC+F	10.7±0.3 a	12.9±0.4 a	14.8±0.4 a	16.3±0.5 a

IA = Arbuscular mycorrhizal inoculant with *Glomus* spores; IB = Ectomycorrhizal inoculant with *Amanita rubescens*, *Amanita* sp., *Lactarius indigo*, *Ramaria* sp. and *Boletus* sp. spores; IC = Ectomycorrhizal inoculant with *Pisolithus tinctorius* and *Scleroderma citrinum* spores; F = Slow release Multicote 8® granulated fertilizer. ±Standard error of the mean. Means with different letters in the same column indicate differences between treatments (Duncan, p≤0.05).

These results were superior to those of Mejía *et al*. (2015), who evaluated forest plantations in the state of *Durango*, and recorded an average diameter of 13 mm for a three-year-old *P. engelmannii* plantation. All treatments evaluated one year after planting exceed this reference, with the exception of treatment IB. However, for Prieto *et al*. (2018) and González (2019) the diameter increases were higher (11.2 to 14.3 mm and from 15.6 to 17.9 mm, respectively) in 13 and 12 months old *P. engelmannii* plantations in sites close to the one of this study, since those that were fertilized with Multicote® in doses of 7 and 10 g plant^{-1} at the time of planting.

Diameter has been recorded as a good predictor of plant survival in the field (Prieto *et al.,* 2018), hence the NMX-AA-170-SCFI-2016 stipulates minimum values required for each species (Secretaría de Economía, 2016). However, in this investigation, the treatments that produced more outstanding diameters did not correspond to the highest survival rates; this could be explained by the arguments of Grossnickle and

Macdonald (2017), who indicate that when the diameter is not related to survival in the field, it may be due to the stressful conditions of the site, where the importance of mycorrhizal symbiosis stands out.

Height

The results showed statistical differences (p≤0.05) between some treatments in the four evaluations. In all cases the plants with the highest data were obtained in some of the treatments with ectomycorrhizal inoculant (IB or IC) plus fertilization (F) (Table 4).

Table 4. Height of *Pinus engelmannii* Carr. during 12 months after planting.

Treatment	Height (cm)			
	3 months	**6 months**	**9 months**	**12 months**
IA	8.4±0.2 ab	8.6±0.2 ab	8.9±0.3 ab	9.2±0.3 b
IB	8.3±0.2 ab	8.5±0.2 ab	8.9±0.2 ab	9.6±0.2 ab
IC	7.9±0.2 b	8.1±0.2 b	8.4±0.3 b	9.4±0.3 ab
IA+F	8.0±0.2 ab	8.7±0.2 ab	9.0±0.3 ab	9.7±0.3 ab
IB+F	8.5±0.2 ab	8.9±0.2 a	9.1±0.2 ab	10.1±0.3 a
IC+F	8.7±0.3 a	8.9±0.2 a	9.2±0.2 a	9.7±0.3 ab

IA = arbuscular mycorrhizal inoculant with *Glomus* spores; IB = Ectomycorrhizal inoculant with *Amanita rubescens*, *Amanita* sp., *Lactarius indigo*, *Ramaria* sp. and *Boletus* sp. spores; IC = Ectomycorrhizal inoculant with *Pisolithus tinctorius* and *Scleroderma citrinum* spores; F = Slow release Multicote 8® granulated fertilizer. ± Standard error of the mean. Means with different letters in the same column indicate differences between treatments (Duncan, p≤0.05).

The final average increase in the treatments with fertilization was 1.8 cm, while in individuals without fertilization (IA, IB and IC) it was 1.4 cm. González (2019) recorded a final average increase of 5.8 cm for *P. engelmannii* seedlings at 12 months of planting, in which slow release fertilizer was also used, only at four months and in higher doses (10 g plant^{-1}).

The NMX-AA-170-SCFI-2016 standard does not consider the minimum height in the plant quality evaluation of *P. engelmannii* (Secretaría de Economía, 2016), because it is a species that in its initial stage is characterized by production abundant needles that can measure more than 30 cm, so their growth is cespitose and this causes a similar increase in height (Ávila-Flores *et al*., 2014; Rosales *et al.,* 2015).

Conclusions

The survival percentage was significantly affected in the treatments added with controlled release fertilizer and the main cause of plant mortality was drought. A positive effect of mycorrhization was perceived on the survival of the plant during the stage of its establishment in the field. The addition of slow-release fertilizer at the time of planting had an important influence on the growth of the plant, expressed in the diameter of the stem and its height.

Acknowledgements

To Conacyt for the support of the postdoctoral stay of the first author in the *Programa Institucional de Doctorado en Ciencias Agropecuarias y Forestales* of the *Universidad Juárez del Estado de Durango*. Mexico.

Conflict of interest

The authors declare no conflict of interests.

Contribution by author

Silvia Salcido Ruiz: execution of the research, data analysis, verification of results and review of the manuscript; José Ángel Prieto Ruíz: supervision of the research, methodological design, verification of results, field support and review of the manuscript; Enrique Santana Aispuro: supply of study materials, field support and review of the manuscript; Jorge Armando Chávez Simental: field support and review of the manuscript; Rosa Elvira Madrid Aispuro: statistical analysis and review of the manuscript.

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