Growth responses of *Quercus fusiformis (Fagaceae)* to ectomycorrhizal inoculation with *Boletus luridellus*

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Key words: *Quercus fusiformis*, seedlings, *Boletus luridellus*, inoculation, effectiveness.

Abstract: Four strains of the ectomycorrhizal *Boletus luridellus* were used to inoculate *Quercus fusiformis* seedlings in pots in order to compare growth response effectiveness. *Boletus luridellus* is an edible fungus that forms ectomycorrhiza with *Quercus fusiformis* and other oak species in temperate forest in northeastern México. The effects of inoculation with the four strains of *B. luridellus* on growth of *Q. fusiformis* seedlings under greenhouse conditions were evaluated. The ANOVA analysis for percentage of mycorrhizae, seedling height, root collar diameter, roots system length, aerial fresh & dry weight, roots system fresh & dry weight showed significant differences ($p<0.05$) between the treatments used (i.e. strains) and seedling growth was greater in inoculated treatments than in the non-inoculated control. Strain no. 4 showed to be the best inducing growth response in oak seedlings under greenhouse condition. The inoculation of *Q. fusiformis* seedlings with *B. luridellus* is ecologically important and could be interesting as a forestry alternative for urban places in northern Mexico.

Zusammenfassung: Vier Stämme des Ektomykorrhizapilzes *Boletus luridellus* wurden verwendet, um kleine Sämlinge von *Quercus fusiformis* in Töpfen zu beimpfen und die Wirksamkeit der Wachstums-

förderung durch die Ektomycorrhiza zu vergleichen. *Boletus luridellus* ist ein essbarer Pilz, der Ektomykorrhizen mit *Quercus fusiformis* und anderen Eichenarten in Wäldern des gemäßigten Klimas im Nordosten Mexikos bildet. Es wurden die Auswirkungen der Inokulation mit den vier Stämmen von *B. luridellus* auf das Wachstum von *Q. fusiformis*-Sämlingen unter Gewächshausbedingungen bewertet. Die ANOVA-Analyse für den Prozentsatz der Mykorrhiza, die Sämlingshöhe, den Durchmesser des Wurzelhalses, die Länge des Wurzelsystems, das Frisch- und Trockengewicht der Sämlinge ohne Wurzel und das Frisch- und Trockengewicht des Wurzelsystems zeigte signifikante Unterschiede ($p < 0,05$) zwischen den verwendeten Stämmen. Das Wachstum der Sämlinge war bei den beimpften Pflanzen größer als bei der nicht beimpften Kontrolle. Stamm Nr. 4 erwies sich als derjenige, der die besten Wachstumsreaktionen bei Sämlingen von Eichen unter Gewächshausbedingungen hervorrief. Die Beimpfung von *Q. fusiformis*-Sämlingen mit *B. luridellus* ist ökologisch wichtig und könnte als forstwirtschaftliche Alternative für städtische Gebiete in Nordmexiko interessant sein.

Species of the family *Boletaceae* are very important in forest ecosystems because they form ectomycorrhizal associations with many species of gymnosperms and angiosperms in different vegetation types and many edible species are consumed by people in different regions of the world (ORTÍZ-SANTANA & al. 2007, GARIBAY-ORIJEL & al. 2009, AGREDA & al. 2010, WU & al. 2016, GELARDI 2020). The ectomycorrhizal associations of *Boletaceae* have been reported from *Fagaceae, Pinaceae, Ericaceae, Dipterocarpaceae, Caryophyllaceae* and many others, and only a few species are considered saprotrophic (MIKOLA 1973; ARORA 1986, 2008; TEDERSOO & al. 2010; SITTA & DAVOLI 2012; DENTINGER & SUZ 2014; WU & al. 2016; ALVAREZ & al. 2017; GARZA & al. 2018; GELARDI 2020). Ectomycorrhizal fungi are very important in forests since they promote nutrient uptake for their host partners. Their isolation and subsequent colonization practices in greenhouse and nursery conditions promotes seedling growth (MITCHELL & al. 1984, WILCOX 1990, MARROQUÍN & GARZA 1999, GARZA & al. 2018).

Boletes have been reported from all over the world and molecular genetic studies are used to identify species from complicated taxonomic groups like the *Boletus edulis* complex (SMITH & THIERS 1971, BOTH 1993, MARROQUÍN & GARZA 1999, BESSETTE & al. 2000, GARZA & al. 2001, KUO 2018, MUÑOZ 2005, BINDER & HIBBET 2006). Some are economically important, e.g. *Boletus edulis* BULL., *B. aereus* BULL., and *B. pinophilus* PILÁT & DERMEK and they represent important income for collectors and processing companies in several countries of the world, e.g. Canada, France, Italy, Mexico, Portugal, Spain, and USA (VILLARREAL & al. 1995; SMITH & READ 1997; ÁGUEDA & al. 2006; GARIBAY-ORIJEL & al. 2009; GARZA & al. 2009, 2011; AGREDA & al. 2010; SITTA & DAVOLY 2012).

In Mexico, many Bolete species form ectomycorrhizae with different host species in temperate forests, oak woodland as well as in tropical and cloud forests (GARCÍA 1999, GARCÍA & GARZA 2000, DE LA FUENTE & al. 2018). Mycorrhizae are very important for nutrient transportation, and they are required to produce seedlings and restore oak and pine forests, but they are frequently overlooked by foresters (HASKING & GEHRING 2005, SOUTHERWORTH & al. 2009). So far, many Boletes have been reported from Mexico growing in different conditions, e.g. ecological, environmental, soil, vegetation and altitudes and molecular genetic studies are required in order to update the information in the country (GARCÍA & GARZA 2000). Mexico has a high diversity of oak species, and they have many species of ectomycorrhizal fungi associated but there are only a few studies regarding inoculation of oaks seedlings with ECM Bolete species (DIAZ & al. 2009, CHUNG & al. 2010, GARZA & al. 2018). In this study growth responses

of *Quercus fusiformis* seedlings inoculated with four strains of *Boletus luridellus* were investigated.

Materials and methods

Collection of fruiting bodies. Specimens of *Boletus luridellus* (MURRILL) MURRILL were collected from two localities, one at the municipality of Santiago (1 strain), and the other at the municipality of Linares (3 strains), both in the state of Nuevo Leon, Mexico (Fig. 1). Morphological characteristics of basidiomata were described according to LARGENT (1986), GARCÍA (1999), BESSETTE & al. (2000, 2016) and LODGE & al. (2004). The colors for the taxonomic description are based on KORNERUP & WANSCHER (1978). Microscopic characters were observed and measured using a Primo Star CARL ZEISS microscope and samples were processed using KOH (5 %) and MELZER´s reagent.

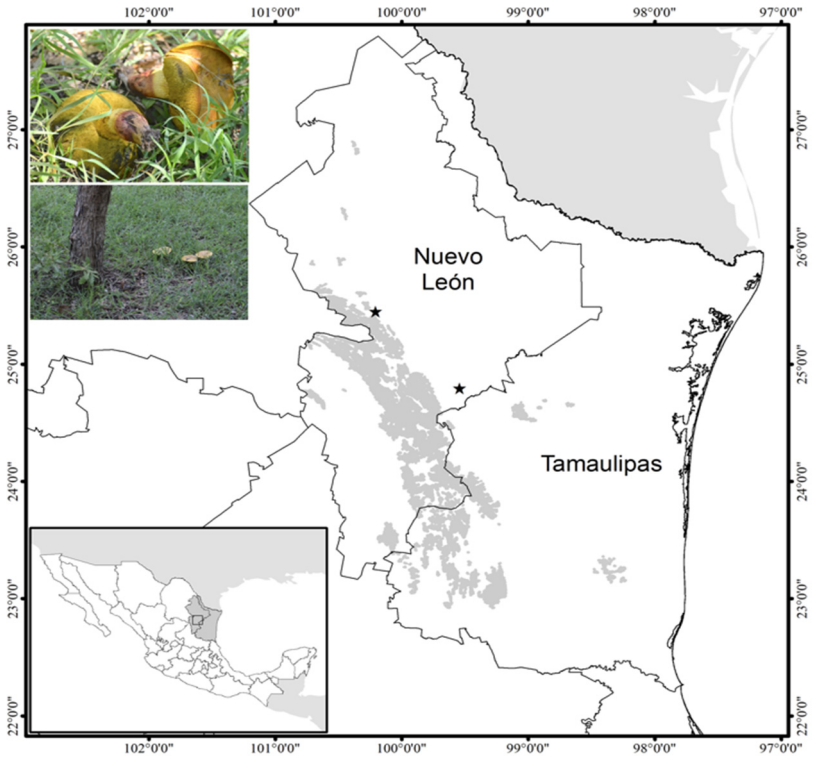


Fig. 1. Locations (marked with asterisks) where fruiting bodies of *Boletus luridellus* were collected for isolation of strains at the municipalities of Santiago (above), and Linares (below).

Culturing *in vitro*. Isolation of strains was carried out from young firm specimens, context tissues were taken with an axenic scalpel and placed into rows on solid Melin Norkrans medium adjusted to pH 6.5 in Petri dishes (DIAZ & al. 2009; GARZA & al. 2011, 2018). Plates were incubated (Shellab model LI20) at 25 °C in the dark for two months.

Production of inoculum. Inoculum for the four strains used was prepared using wide mouth flasks of 500 ml filled up with vermiculite and peat moss (4:1 in volume) and 70 % of liquid Melin Norkrans medium was included (MITCHELL & al. 1984; GARZA et al. 2014, 2018). Flasks were sterilized at 120 °C for 40 min and left to cool, inoculation for each independent strain was performed using one Petri dish of a newly obtained solid culture. Inoculated flasks were incubated in the dark for a period of 5 weeks at 25 °C and were shaken weekly to accelerate mycelium growth and colonization of substrate.

Seed germination. Oak seeds were collected from local *Quercus fusiformis* SMALL trees and were surface sterilized with H_2O_2 at 30 % for 15 min, then washed three times in deionized sterile water to eliminate any excess of hydrogen peroxide. Germination was carried out in wide mouth flasks with water agar 1.6 % and they were placed in an incubator at 25 °C in the dark for five weeks.

Production of inoculum. Inoculum for each strain was made using 50 % inoculant previously washed with sterile deionized water to eliminate extra carbohydrates from the medium, and 50 % sterile soil mixture made from (1:1:1) in volume of peat moss, vermiculite, and worm compost. Germinated acorns were placed with the root system into the inoculant-soil substrate 500 ml pots; 50 seedlings per treatment were used and each strain corresponded to a treatment and a non-inoculated control. Treatments were placed separately in the greenhouse for 12 months at 26 °C and 60 % humidity and they were watered every third day.

Sampling of seedlings. Measurements of seedling variables were carried out in the laboratory using 10 randomly selected seedlings per treatment. Variables measured per treatment were percentage of mycorrhizae, seedling height, root collar diameter, fresh and dry weight of aerial part and root system, as well as root length.

Percentage of mycorrhizae. Measurement was carried out using ten randomly selected seedlings per treatment. The whole root systems were washed with distilled water for eliminating soil particles. Mycorrhizal root tips were counted using a stereoscopic microscope (ZEISS, Stemi 2000Ci). Morphology of mycorrhizae was observed in thin handmade sections to see the HARTIG net and to measure the fungal mantle (BRUNDRETT & al. 1996, GARZA & al. 2001). Measurement of percentage of mycorrhizae followed (MARTINEZ & al. 2009).

$$\text{Percentage of mycorrhizal colonization} = \frac{\text{No. of mycorrhizal roots} * 100}{\text{No. of roots observed}}$$

Statistical analysis. Results were analyzed using SPSS version 20 and ANOVA and a comparison of media (p≤) were carried out in order to know the significant differences of variables measured to the seedlings and their relationship with the strains used.

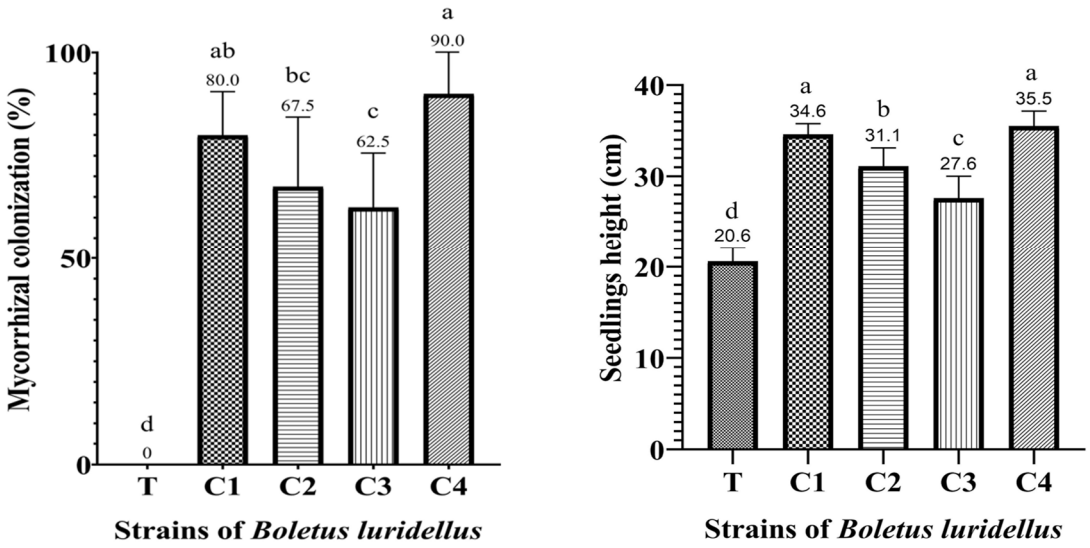
Results

The growth responses of *Quercus fusiformis* seedlings inoculated with four strains of *Boletus luridellus* are summarised in Tab. 1 and Figs. 2–9.

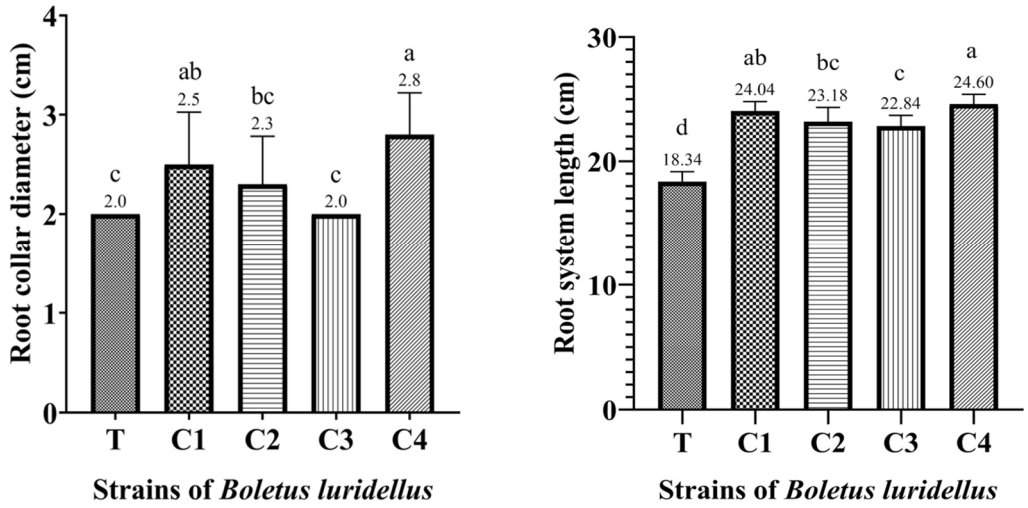
Tab. 1. Growth responses of *Quercus fusiformis* seedlings to inoculation with four strains of *Boletus luridellus* (strains C1–C4 + control). Parameters: *1* mycorrhizal colonization (%), *2* height (cm), *3* root collar diameter (cm), *4* root system length (cm), *5* aerial fresh weight (g), *6* aerial dry weight (g), *7* root system fresh weight (g), *8* root system dry weight (g). Small letters indicate significance.

Treatments	Parameters							
	1	2	3	4	5	6	7	8
Strain C1	80 ab	34.6 a	2.5 ab	24.04 ab	5.76 bc	2.69 c	6.85 c	1.68 bc
Strain C2	67.5 bc	31.1 b	2.3 bc	23.18 bc	5.33 c	2.09 d	5.91 d	1.29 cd
Strain C3	62.5 c	27.6 c	2.0 c	22.84 c	6.28 b	3.42 b	7.71 b	1.83 ab
Strain C4	90 a	35.5 a	2.8 a	24.60 a	7.24 a	3.90 a	8.35 a	2.11 a
Control	0	20.6 d	2.0 c	18.34 d	4.63 d	1.83 d	5.07 e	1.04 d

Results from the ANOVA for percentage of mycorrhizae showed significant differences ($p<0.05$) between treatments (Fig. 2). The treatments with strains 4 and 1 showed the higher percentage of mycorrhizae (90 % and 80 %, respectively) with a Tukey (P=0.05). These were followed by treatments corresponding to strains 2 and 3 with 65 % and 62 %, and the control had no mycorrhizae. In average all treatments showed a mean of 74.25 % of mycorrhizae (Tab.1).



Figs. 2, 3. Percentage of mycorrhizal colonization (Fig. 2, left) (mean) and seedling height (Fig. 3, right) (mean in cm) in inoculated seedlings of *Quercus fusiformis*. – Different letters indicate significant differences among treatment according to Tukey test ($p \leq 0.05$).



Figs. 4, 5. Root collar diameter (Fig. 4, left) and root system length (Fig.5, right) (mean in cm) in inoculated seedlings of *Quercus fusiformis*. – Different letters indicate significant differences among treatment according to Tukey test ($p \leq 0.05$).

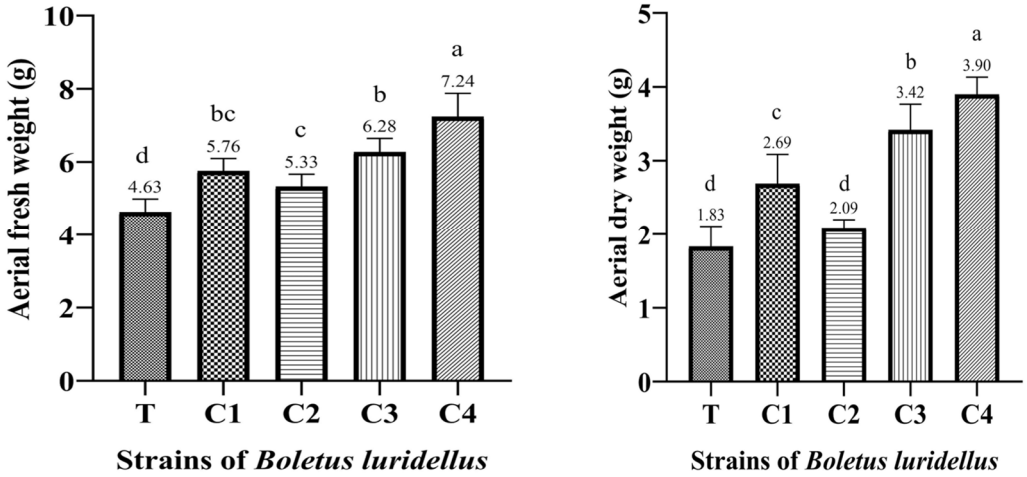
In addition, results from the ANOVA for seedling height, showed significant differences ($p<0.05$) between the strains (Fig. 3). The treatments with strains 4 and 1 showed the higher seedlings height ($\mu=35.5$ and $\mu=34.6$, respectively) with a Tukey ($P=0.05$). These were followed by treatments corresponding to strains 2 and 3 with 31 and 27.6 cm, respectively, and the control with 20.6 cm. In general, an average height of 32.2 cm was obtained for all the strains used of *B. luridellus*, compared to the control of 20.6 cm.

Similarly, the ANOVA results for seedlings root collar diameter showed significant differences ($p<0.05$) between the strains used (Fig. 4). The treatments with strains 4 and 1 showed the higher root collar diameter ($\mu=2.8$ and $\mu=2.5$, respectively) with a Tukey ($P=0.05$). These were followed by treatments corresponding to strains 2 and 3 with 2 and 2.3 mm respectively and the control with 2.0 mm.

Furthermore, the ANOVA test for seedlings root system length showed significant differences ($p<0.05$) between the strains used (Fig. 5). The treatments with strains 4 and 1 showed the higher root system length ($\mu=24.6$ and $\mu=24.4$ respectively) with a Tukey ($P=0.05$). These were followed by treatments corresponding to strains 2 and 3 with 23.18 and 22.84 cm respectively and the control with 18.34 cm (Tab.1).

Also, the ANOVA results for seedlings aerial fresh weight showed significant differences ($p<0.05$) between the strains used (Fig. 6). The treatments with strains 4 and 3 showed the higher aerial fresh weight ($\mu=7.24$ and $\mu=6.24$ respectively) with a Tukey ($P=0.05$). These were followed by treatments corresponding to strains 1 and 2 with 5.75 and 5.33 g respectively and the control with 4.63 g. A shift in the general behavior observed for the other variables occurred for aerial fresh weight.

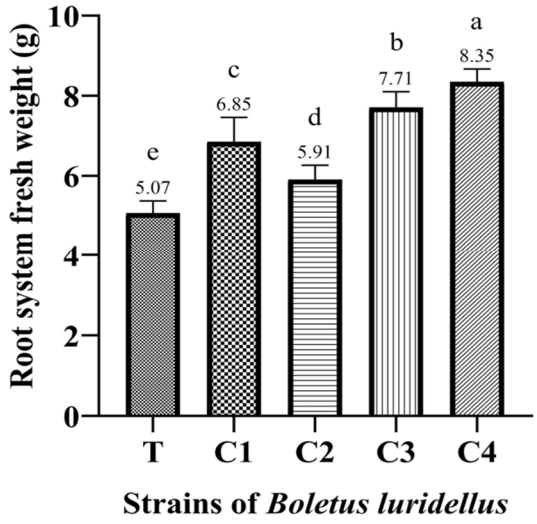
Similarly, the ANOVA analysis for seedlings aerial dry weight showed significant differences ($p<0.05$) between the treatments (Fig. 7). The treatments with strains 4 and 3 showed the higher aerial dry weight ($\mu=3.90$ and $\mu=3.42$) with a Tukey ($P=0.05$). These were followed by treatments corresponding to strains 1 and 2 with 2.69 and 2.09 g respectively and the control with 1.83 g. A shift in growth response was observed for aerial dry weight as well.

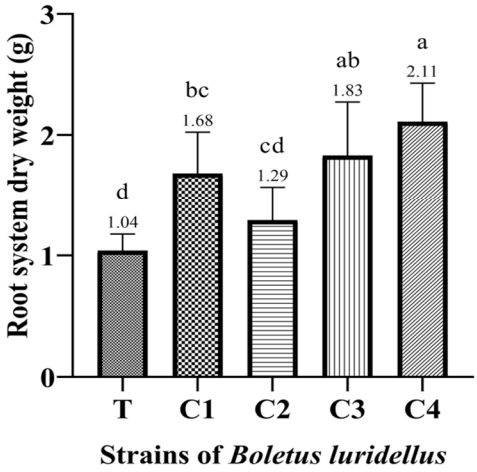


Figs. 6, 7. Aerial fresh weight (Fig. 6, left) and aerial dry weight (Fig. 7, right) (mean in g.) in inoculated seedlings of *Quercus fusiformis*. – Different letters indicate significant differences among treatment according to Tukey test ($p \leq 0.05$).

Results from the ANOVA for seedlings root system fresh weight showed significant differences ($p<0.05$) between the treatments (Fig. 8). The treatments with strains 4 and 3 showed the higher root system fresh weight ($\mu=8.35$ g and $\mu=7.71$) with a Tukey ($P=0.05$). These were followed by treatments corresponding to strains 1 and 2 with 6.85 and 5.91 g and the control with 5.07 g. A shift in the general behavior observed for the other variables occurred for root system fresh weight.

Finally, results from the ANOVA test for seedlings root system dry weight showed significant differences ($p<0.05$) between the treatments (Fig. 9). The treatments with strains 4 showed the higher root system dry weight ($\mu=2.11$ g) followed by treatments strains 3 and 1 with ($\mu=1.83$ g y $\mu=1.68$ g) with a Tukey ($P=0.05$). They were followed by treatment strains 2 with 1.29 g and the control with 1.04 g (Tab. 1). A shift in the general behavior observed for the other variables occurred for root system dry weight.





Figs. 8, 9. Root system fresh weight (Fig. 8, left) and root system dry weight (Fig. 9, right) (mean in g) in inoculated seedlings of *Quercus fusiformis*. – Different letters indicate significant differences among treatment according to Tukey test ($p \leq 0.05$).

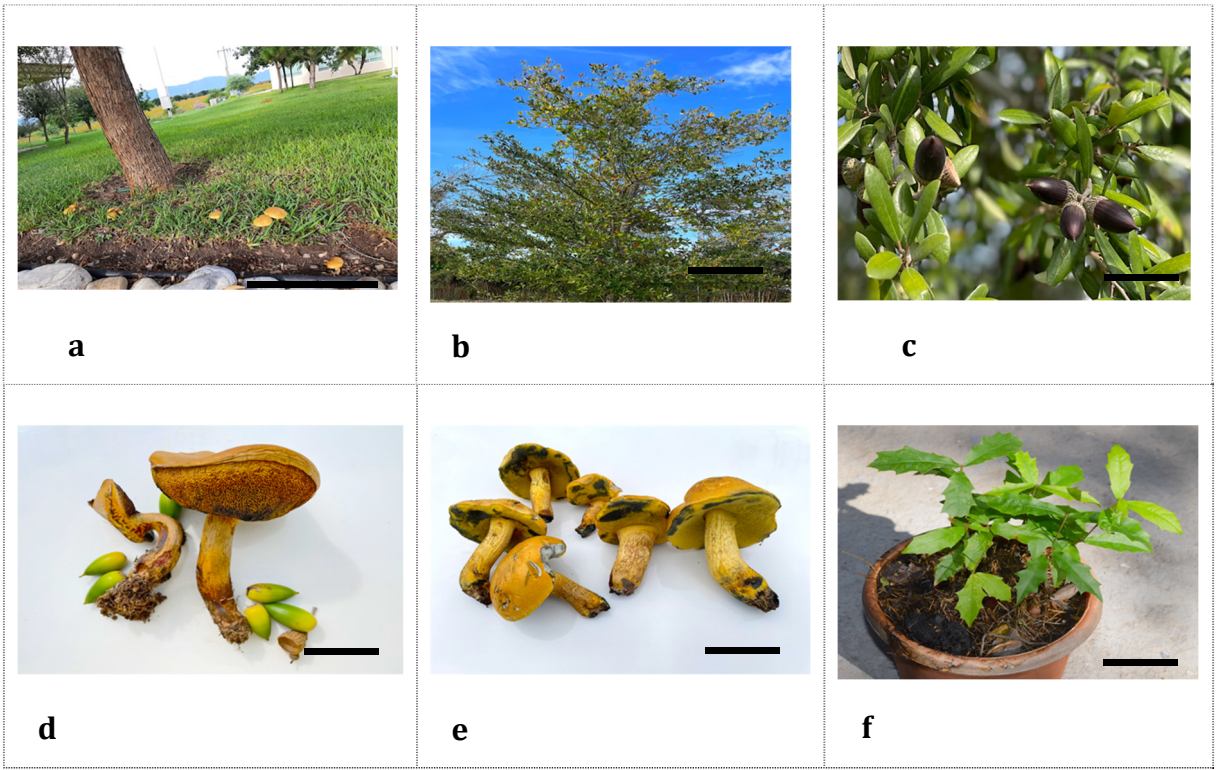


Fig. 10. *a–c.* Growth of *Boletus luridellus* with *Quercus fusiformis* inoculated trees in urban gardens showing acorn production. *d-f.* Close up of fruiting bodies and acorns collected in urban garden and inoculated seedlings in pots. Scale bars: a = 1 m; b = 1.5 m; c = 5 cm. d = 5 cm; e, f = 10 cm.

Discussion

Results show that there were significant differences regarding general growth responses of *Quercus fusiformis* seedlings to inoculation with the four strains of *Boletus luridellus* and the non-inoculated control always showed lower values. These results agree with

the conclusions of MITCHELL & al. (1984) regarding the test of fungal ecotypes before selecting for inoculation procedures in container grown seedlings. DEVINE & al. (2009) found that inoculation of white oak seedlings improved shoot and root growth responses. HASKINS & GEHRING (2005) also suggest that inoculated seedlings have better field establishment when selecting the appropriate mycorrhizal fungal inoculum. Strain growth responses in seedlings were similar to previous results from *in vitro* experiments (GARZA & al. 2018); strain 4 showed the best effectiveness regarding the growth responses obtained in live oak seedlings under greenhouse condition. Strains 1, 2 and 3 also showed that their mycelial growth *in vitro* was like the growth responses found for root collar diameter and root system length variables under greenhouse conditions (GARZA & al. 2018). Early mycorrhizal development shows increased growth responses in live oak seedlings, as was also reported by DIXON & JOHNSON (1992).

The pattern of results found under greenhouse conditions for percentage of mycorrhizae was also like those obtained previously for strain growth *in vitro* (GARZA & al. 2018). Fresh and dry weight of aerial and root system behave different from the patterns of strains obtained for percentage of mycorrhizae. These differences might be due to the genetic variations regarding enzymatic activities and nutrients translocation of each strain. Thus, results for seedlings growth responses found for all variables measured might not always be linked to the high percentage of mycorrhizae formed (MITCHELL & al. 1984). Some mycorrhizal fungi might not produce a high percentage of mycorrhizae but still be able to induce better growth responses due to their high translocation level. Selection of strains to be used for production of oak seedlings under greenhouse conditions is very important to obtain best seedling growth responses and better results can also be expected when seedlings are out planted in the field (HASKINS & GEHRING 2005, SOUTHWORTH & al. 2009).

It can be concluded that the four strains of *Boletus luridellus* used in this study formed ectomycorrhizae and showed significant growth responses of inoculated *Quercus fusiformis* seedlings. All strains formed mycorrhizae and induced different growth responses in the inoculated seedlings. Although a general pattern of seedlings growth responses was found for some variables the exception occurred for the fresh and dry weight of aerial part and radicular system.

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