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SEEDLING GROWTH OF RAINFOREST SPECIES INOCULATED WITH ARBUSCULAR MYCORRHIZAL FUNGI: AN ANALYSIS OF THE SIZE FRAGMENT EFFECT

[CRECIMIENTO DE PLÁNTULAS DE ESPECIES DE LA SELVA HÚMEDA INOCULADAS CON HONGOS MICORRIZÓGENOS ARBUSCULARES: UN ANÁLISIS DEL EFECTO DEL TAMAÑO DEL FRAGMENTO]

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SUMMARY

Deforestation is a process that brings as a consequence strong environmental problems in tropical rain forests. Restoration of damaged areas can accelerate succession process and improve seedling performance. One way to reach this objective is to inoculate them with native arbuscular mycorrhizal fungi. This study analyzed the effect of mycorrhizae inoculation on seedling survivorship and growth of two tree species, *Pleuranthodendron lindenii* (light demanding) and *Pimenta dioica* (shade tolerant) in shaded greenhouse and field conditions in the region of "Los Tuxtlas", Veracruz. We applied three inoculation treatments, without mycorrhizal inoculum (control), mycorrhizal inoculum from small fragments, and inoculum from large fragments. We analyzed survivorship and relative growth rates for height and diameter. For both species, significant differences ($p<0.05$) in growth rates in height and diameter were found for inoculum origin and time, as well as their interaction. The highest mean values corresponded to plants with inoculum from small fragments. Differences in survival among arbuscular mycorrhizal fungi treatments were significant only under shaded greenhouse conditions. The results are discussed in terms of life history traits and environmental conditions.

Keywords: Arbuscular mycorrhizal fungi; fragmentation; growth; tropical rain forest; *Pleuranthodendron lindenii; Pimenta dioica***.**

RESUMEN

La deforestación genera fuertes problemas ambientales en las selvas húmedas. La restauración de las áreas dañadas puede acelerar el proceso sucesional y mejorar el desempeño de las plántulas. Una forma de alcanzar este objetivo es inoculándolas con hongos micorrizógenos arbusculares. Este estudio analizó el efecto de la inoculación en la supervivencia y crecimiento de plántulas de dos especies arbóreas, *Pleuranthodendron lindenii* (demandante de luz) y *Pimenta dioica* (tolerante a la sombra), bajo condiciones de un exclusorio y de campo en la región de "Los Tuxtlas", Veracruz. Aplicamos tres tratamientos de inoculación, sin micorrizas (control), con inóculo de fragmentos pequeños y con inóculo de fragmentos grandes. Analizamos las tasas relativas de crecimiento en altura y diámetro, y la supervivencia. En ambas especies, tanto el origen del inóculo como el tiempo generaron diferencias significativas ($p<0.05$) en altura y diámetro; mientras que las interacciones origen del inóculo×zona y zona×tiempo fueron diferentes. Los valores promedio más altos correspondieron a las plantas con inóculo de los fragmentos pequeños. Las diferencias en supervivencia entre los tratamientos de hongos micorrizógenos arbusculares fueron significativas ($p<0.05$) solamente en el exclusorio. Los resultados son discutidos en términos de las características de historia de vida y las condiciones ambientales.

Palabras clave: Hongos micorrrizógenos arbusculares; fragmentación; crecimiento; selva húmeda; *Pleuranthodendron lindenii; Pimenta dioica***.**

INTRODUCTION

Fragmentation of tropical forests due to deforestation has carried out severe biodiversity loss and ecological interaction breakage, as well as depletion in economic and social activities related to these forests (Laurance, 1999; Kareiva and Marvier, 2007).

Deforestation disrupts the continuous forest in several patches, or fragments (Guevara *et al*., 2004b). Fragmentation of landscape is a complex process that produces abiotic and biotic changes that depend on fragment size, shape and arrangement in landscape (Bennett and Saunders, 2010). These changes lead to reduction and isolation of original populations, resulting in extinction population rate increase and colonization population rate decrease (MacArthur and Wilson, 1967), as well as ecological interaction shifts (Lienert, 2004; Mangan *et al*., 2004).

Fragmentation effects have been well studied on animal and plant communities (Debinski and Holt, 2000; Matthies *et al*., 2004; Helm *et al*., 2006). In contrast, in Neotropical forests there are few works that analyze how fragmentation modifies arbuscular mycorrhizal fungi (AMF) community structure and functionality (Mangan *et al*., 2004).

AMF form an important ecological relationship with a great number of plants (Siqueira *et al*., 1998; Zangaro *et al*., 2003). This mutualistic association has been proven to increase plant biomass and mineral concentration in plant tissue (Smith and Read, 2008; Varma, 2008), to stimulate flower, fruit, and pollen production in some plant species (Daft and Okusanya, 1973), and to increase cytokinins on leaves and roots (Allen *et al*., 1980), in exchange fungi receive the required carbohydrates for their existence.

Incipient data point at AMF colonizing capability differs depending on their origin site because AMF distribution in time and space is not haphazard, and it can change according to physical, biological and ecological characteristics of each place (Johnson and Wedin 1997; Allen *et al*., 1998; Picone, 2000; Mangan *et al*., 2004; Violi *et al*., 2008). As a consequence, we hypothesized that AMF communities from small fragments should be different in structure and functionality, as well as their effects on plants, compared to those from large fragments.

Given seedling vulnerability, inoculation with AMF can be essential to improve survival and growth, as it has been demonstrated in several studies (Kiers *et al*., 2000; van der Heijden, 2004; Zangaro *et al*., 2005). However, plant species response to AMF species will depend upon plant and AMF identity (van der Heijden, 2002), and plant life history (Siqueira *et al*., 1998), as a consequence, each species of AMF can exhibit different affinities and impacts on seedling fitness (van der Heijden *et al*., 1998a; Kiers *et al*., 2000; Bever *et al*., 2010).

Plant life histories in tropical rain forest have been classified according to their role in natural regeneration and their light requirements. There are basically two large groups, light demanding and shade tolerant species. The former comprises plants that need high light intensities during its whole life cycle and have relative growth rates and resource requirements higher than those of shade tolerant species. On the contrary, shade tolerant species can germinate and live under shade for several years, their relative growth rates and resource requirements are lower than those of light claimants (Martínez-Ramos, 1994).

Following the previous ideas, our main goal was to assess the effects of different origin AMF inocula on seedling growth of two native tropical rain forest species with contrasting life histories under shaded greenhouse and field conditions. Because the responses of plants to AMF presence is determined by their life history and identity of AMF species, we hypothesized that growth and survival of shade tolerant species (TS) will be higher with inoculant from large forest fragments, while species light demanding (LD) will respond more positively to inoculant from small fragments.

This study contributed to the development of more accurate models of restoration in deforested areas, in order to develop strategies to accelerate the regeneration process of Los Tuxtlas tropical rain forest. Our hypothesis is that, in the long term, changes in AMF community from small fragments will occur, and this will have an effect on their functionality, favoring those plant species with the highest functional complementarity or affinity, *i.e*. light demanding species in small fragments, and shade tolerant species in large fragments.

MATERIAL AND METHODS

The Los Tuxtlas region is located in the coastal plain of the Gulf of Mexico, at the south part of the State of Veracruz (Guevara *et al*., 2004a). At low altitudes, tropical rain forest dominates (Miranda and Hernandez-X., 1963), most of it is concentrated into remnant fragments of different sizes which are mainly surrounded by a matrix of secondary vegetation and/or cattle ranching lands (Ibarra-Manriquez *et al*., 1997). Af(m)w"(i')g is the main climate type with a mean annual precipitation of 4,084 mm, and 25 °C as mean annual temperature (data taken from the Los

Tuxtlas Tropical Biology station, belonging to the period from 1996 to 2008). In particular, our shaded greenhouse was located at Los Tuxtlas Tropical Biology station (LTTBS), a research center of tropical biology under the Universidad Nacional Autónoma de México protection and we carried out our field transplants in two small fragments, 5 and 7 ha in size (Figure 1). All sites are part of the Los Tuxtlas National Biosphere Reserve.

Selected species

Pleuranthodendron lindenii (Turcz.) Sleumer belongs to Tiliaceae family. It is an evergreen native tree species. It is common in secondary vegetation at Los Tuxtlas forest, mainly associated with gaps; we classified it as a light demanding (LD) species (Pennington and Sarukhán, 2005).

Pimenta dioica (L.) Merr. belongs to Myrtaceae family. It is an evergreen native tree that grows under shade; we classified it as a shade tolerant (ST) species (Pennington and Sarukhán, 2005).

Inoculum collection

In March 2005, we collected soil from the interior and most conserved zone of two large fragments (211 and 640 ha in size) and two small ones (5 and 7 ha) (Figure 1). Large fragments are little disturbed sites. Particularly, 640 ha fragment is the LTTBS and has never been managed or cleared. In contrast, small fragments have a higher LD species presence (Sánchez-Gallen, 2011). All four fragments were surrounded by cattle ranching lands.

In each of these fragments, we chose twenty five sampling points in a one-way field trip; each point was separated every two meters. Soil samples were taken from the first 20 cm in depth where we can find colonized roots by AMF, as well as mycelium and spores. After, we mixed all soil samples from the same size fragment group. Rocks and pieces of coarse roots were removed.

Subsequently, we placed collected soil in pots containing trap plants of various forest species, and after six month we characterized inoculum from each of the fragment groups (Luna, 2009). Identification was conducted by MSc. Laura Hernández-Cuevas, she based her results on current descriptions of different manuals (Schenk and Pérez 1988) and the International culture collection of arbuscular mycorrhizal fungi website (http://invam.caf.wvu.edu/Myc_Info/Taxonomy.species.html), following Schüßer and Walker (2010) classification.

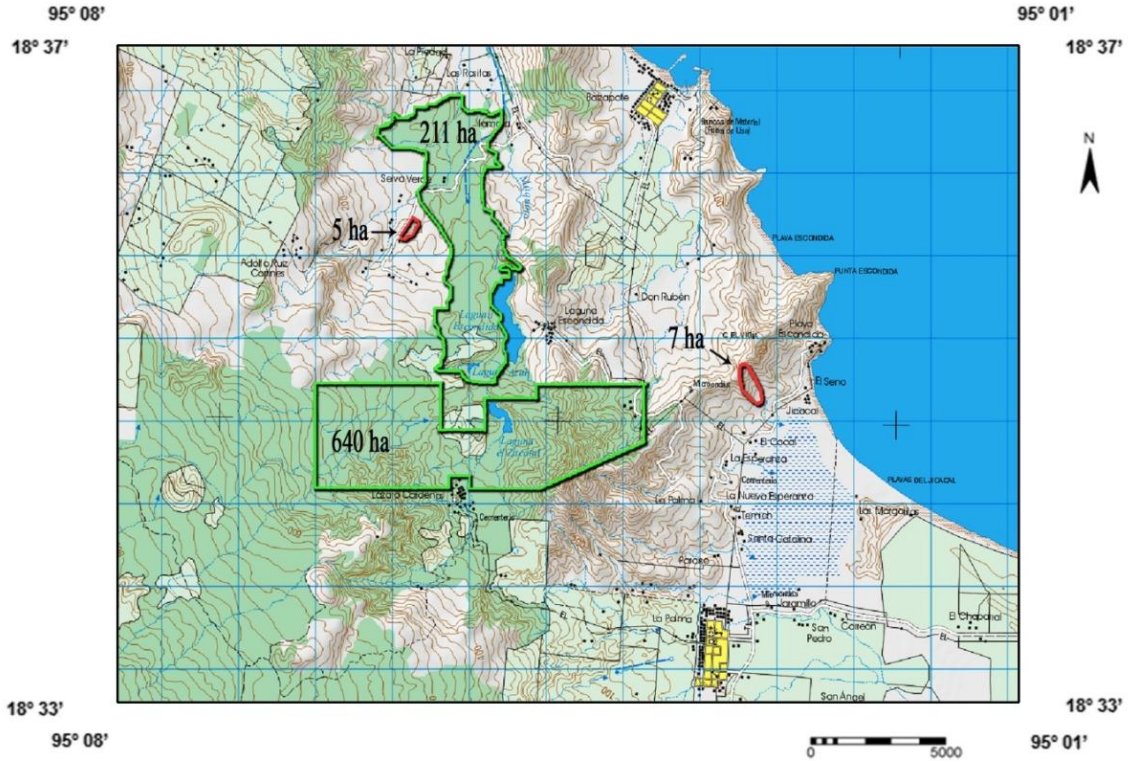

Figure 1. Fragment location at Los Tuxtlas region, Veracruz, Mexico (modified from the INEGI (2000) topographic chart, key e15 a63, New Victory scale 1: 50 000). 640 ha Fragment = LTTBS. We show fragments 5 ha and 7 ha, where plants were transplanted.

Small fragment inoculum had an average of 8.7 spores g^{-1}, we identified a total of 11 AMF species. *Glomus tenebrosum* was the predominant one and *Acaulospora morrowiae*, *G. flavisporum*, *Sclerocystis rubiformis*, and *A. mellea* were only found in this type of inoculum. Whereas, in large fragments the average number of spores per gram was 9.7. We recorded a total of 15 species of AMF, with an abundance of *A. scrobiculata* and eight exclusive species (*A. foveata*, *G. aggregatum*, *Claroideoglomus claroideum*, *Sclerocystis sinuosa*, *Scutellospora gilmorei*, *A. laevis*, *Ambispora leptoticha*, and *Gigaspora decipiens*). Shared species by both inocula were *A. scrobiculata*, *A. spinosa*, *G. tenebrosum*, *Funneliformis geosporum*, *G. microaggregatum*, *F. verruculosum*, and *Redeckera fulvum*.

Experimental

The experiment considered the inoculum origin factor with three levels: 1) without AMF (M-), 2) with AMF from large fragments (MLF), and 3) with AMF from small fragments (MSF).

Prior to starting the experiment, in December 2004 we obtained seeds of the two above mentioned species by collecting fruits from 10 different trees located inside the forest of Los Tuxtlas. We washed all seeds with tap water and submerged them in a 3% chlorine solution during 10 min to disinfect them.

Simultaneously, in laboratory, we steam-sterilized soil from the forest and sand in an autoclave, for 1 hour at 90 °C. This procedure was repeated twice with a 24-hour period in between. After this, we filled pots with a sterile soil-sand (1:1) mixture and we sowed in the disinfected seeds, all pots were kept in the LTTBS shaded greenhouse.

After three months of growth, in March 2005, we randomly selected 420 seedlings for each species and transplanted to 2 kg black plastic bags. The bags were filled with sterilized soil and sand mixture (3:1). Sterilization process was the same as described above. We placed 50 g of fresh soil from the trap pots as the inoculum around each seedling belonging to large or small fragments per bag. The bags were tagged according to species name and inoculum type (treatment).

Three months later, in July 2005 (rainy season) we transplanted a total of 90 seedlings to the field, in the border zone of two small fragments (5 and 7 ha in size), 45 for each species, 15 plants per treatment. The mean *Pleuranthodendron* seedling height (± SD) was 17.7 cm (±3.4), 21.4 cm (±5.3), and 21.7 cm (±4.6), for M-, MLF and MSF, respectively. Mean height (± SD) for *Pimenta* per treatment was 9.9 cm (±1.9) (M-), 9.8 cm (±1.6) (MLF), and 9.4 cm (±1.8) (MSF).

Simultaneously, we left 150 seedlings for each species, 50 for each treatment in the shaded greenhouse of the LTTBS, they were randomly placed into three blocks for each species, following the applied inoculation treatments (M-, MLF, or MSF) to avoid any contamination among treatments. Also, we rotated them every month to assure that seedlings were growing under similar light conditions, environmental temperature and humidity were not controlled, and they were consistent with those outside the shaded greenhouse.

All surviving plants were harvested nine months later (March 2006). And to confirm AMF colonization, we took roots from two plants of every treatment and species, we stained them following Phillips and Hayman technique (1970) modified by Hernández-Cuevas et al. (2008) and estimated total colonization percentage.

Data analysis

Every month, we measured total height and diameter at the base of 15 randomly chosen seedlings per species and treatment, in shaded greenhouse and field. Similarly, survival was recorded counting the number of total seedlings that remained alive every month, per species per treatment. We repeated these measures until January 2006.

Growth analysis

We calculated relative growth rates based in height (cm cm^{-1} day^{-1}) and diameter (mm mm^{-1} day^{-1}) following a functional growth analysis (Hunt, 1982):

$$RGR = \frac{H \text{ or } D_{t2} - H \text{ or } D_{t1}}{t_2 - t_1}$$

Where H= Heigh, D= diameter, t_1 = number of days elapsed after previous data collection, and t_2 = number of days elapsed after next data collection.

We transformed RGR diameter data by natural logarithm and RGR height by arcsine in order to reach normal distribution and variance homogeneity (Zar, 2009). We analyzed treatment mean differences by using repeated measure analysis of variance (ANOVA). And, only when we obtained significant differences, in order to discriminate among different treatment levels we applied a Tukey analysis (Montgomery, 1991). An analysis of covariance was not necessary to perform because RGRs allow to weight initial differences among individuals. We used

STATISTICA 8 (Statsoft Inc., 2000) software for all analyses of variance.

Survivorship

We compared survivorship among inoculum treatments, separating field and shaded greenhouse sites with Peto and Peto test (Pyke and Thompson, 1986).

RESULTS

Growth analysis

Average relative growth rates (RGR) for *Pleuranthodendron lindenii* fluctuated between 0.01 and -0.001 cm cm^{-1} day^{-1}, and 0.07 and -0.004 mm mm^{-1} day^{-1}, for height and diameter, respectively. In *Pimenta dioica*, values were between 0.008 and -0.005 cm cm^{-1} day^{-1}, and 0.06 and -0.002 mm mm^{-1} day^{-1} for height and diameter, respectively.

Inoculum origin and time factors, for both species and response variables had significant differences among levels, while their interaction was not significant; on the contrary, zone factor was only significant for *Pleuranthodendron* (Table 1).

Both species, *Pleuranthodendron*, and *Pimenta* had the highest significant mean values in height and diameter when are inoculated with fungi from large fragments (MLF) (Table 1).

However, even when inoculum origin is an important factor for both species growth, when we analyzed interactions among factors, shaded greenhouse interacting with any date of collection and type of inoculum had the highest plant response (Figure 2 and 3) for both species.

Mean total colonization percentage was higher in MLF treatment for both species (*Pleuranthodendron* 52% (±5) and *Pimenta* 46% (±7)). While M- had *Pleuranthodendron* 15% (±5) and *Pimenta* 23% (±7).

Survivorship

Survival individual number comparisons among AMF inoculum treatments had significant differences ($p<0.05$) only under shaded greenhouse conditions. For both species, we observed the lowest survivorship value with inoculum from MSF, while control and large fragment inoculum treatments had the same trend (Figure 4).

DISCUSSION

Our hypothesis was that growth, measured in terms of height and diameter, and survival of *P. lindenii* (light demanding species) would reach their highest values with inoculum from small fragments (MSF). On the contrary, we expected that *P. dioica* would have higher relative growth rates and survival with inoculum from large fragments (MLF) than with MSF. Our results partially supported this hypothesis, since both *Pleuranthodendron* and *Pimenta* growth was benefited, at some time, from both inocula.

We did not find a clear relationship among inoculum origin and species life history. This indicates that AMF presence is important for both species growth but not all the time, this perhaps is related with changes of plant physiological needs or energetic costs involved with the relationship, because even when several studies characterized arbuscular mycorrhiza as a mutualistic interaction, sometimes, depending on environmental conditions it could turn parasitic, where fungi parasites plant due to an excess of plant carbon investment (Johnson *et al*., 1997), and this can be happening in the field, where environmental conditions are so stressing that plants can barely produce carbon and allocate it to its main functions.

The fact that presence of AMF, regarding origin, favored higher growth rates has been shown in other studies with tropical forest native species (Allen *et al*., 2003; Fischer *et al*., 1994; Kiers *et al*., 2000; Zangaro *et al*., 2000). Fischer *et al*. (1994) found that inoculum from abandoned grasslands resulted in higher growth, compared with inoculum from lowland secondary forest, and Zangaro *et al*. (2000) found that early successional species had higher growth rates when they were inoculated with inoculum from an area dominated by pioneer species. Although Kiers *et al*. (2000) found that fungi are important for species growth, they reported that a tree pioneer species and a mature forest tree species grew much more with an inoculum from a late successional plant species (which in our case would correspond to MLF inoculum) and with inoculum from an early successional plant species (equivalent to our MSF inoculum), respectively. In the same terms, Allen *et al*. (2003) showed that early late successional seedlings had a higher benefit when they were inoculated with soil from an early successional site of deciduous tropical forest instead of using inoculum from a mature forest soil of the same forest.

Table 1. Summary of analyses of variance. We show F-value and significance value (p). We also highlight the highest and lowest mean values of the response variable for each factor.

Species	Response variable	Factor	F	p	Highest mean	Lowest mean
Pleuranthodendron lindenii	RGR in height	Inoculum origin (IO)	275.292	<0.001	MLF	MSF
		Zone	28.768	<0.001	Shaded greenhouse (SG)	Field
		Time	5.398	<0.001	Jul-Aug	Dec-Jan
		IO×Zone	11.370	<0.001	MLF×SG	MSF×Field
		IO×Time	0.788	>0.05	MLF×Jul-Aug	MLF×Dec-Jan
		Zone×Time	9.416	<0.001	SG×Jul-Aug	Field×Dec-Jan
		IO×Zone×Time	2.535	<0.001	MLF×SG×Jul-Aug	MLF×Field×Sep-Oct
	RGR in diameter	Inoculum origin (IO)	284.22	<0.001	MLF	MSF
		Zone	14.08	<0.001	Shaded greenhouse (SG)	Field
		Time	24.11	<0.001	Aug-Sep	Sep-Oct
		IO×Zone	8.96	<0.001	MLF×SG	M-×SG
		IO×Time	1.46	>0.05	MSF×Aug-Sep	MLF×Sep-Oct
		Zone×Time	4.60	<0.001	SG×Aug-Sep	Field×Sep-Oct
		IO×Zone×Time	1.55	>0.05	MLF×SG×Aug-Sep	MLF×Field×Sep-Oct
Pimenta dioica	RGR in height	Inoculum origin (IO)	432.159	<0.001	MLF	M-
		Zone	1.825	>0.05	Shaded greenhouse (SG)	Field
		Time	3.819	<0.001	Aug-Sep	Dec-Jan
		IO×Zone	4.188	<0.01	MLF×SG	M-×Field
		IO×Time	1.304	>0.05	M-×Oct-Nov	M-×Nov-Dec
		Zone×Time	4.675	<0.001	SG×Jul-Aug	Field×Sep-Oct
		IO×Zone×Time	0.597	>0.05	M-×SG×Jul-Aug	M-×Field×Oct-Nov
	RGR in diameter	Inoculum origin (IO)	423.53	<0.001	MLF	M-
		Zone	2.90	>0.05	Shaded greenhouse (SG)	Field
		Time	7.89	<0.001	Aug-Sep	Sep-Oct
		IO×Zone	4.44	<0.01	MLF×SG	M-×Field
		IO×Time	0.87	>0.05	M-×Oct-Nov	M-×Nov-Dec
		Zone×Time	1.80	>0.05	SG×Aug-Sep	Field×Dec-Jan
		IO×Zone×Time	1.75	<0.05	MLF×SG×Aug-Sep	MLF×Field×Dec-Jan

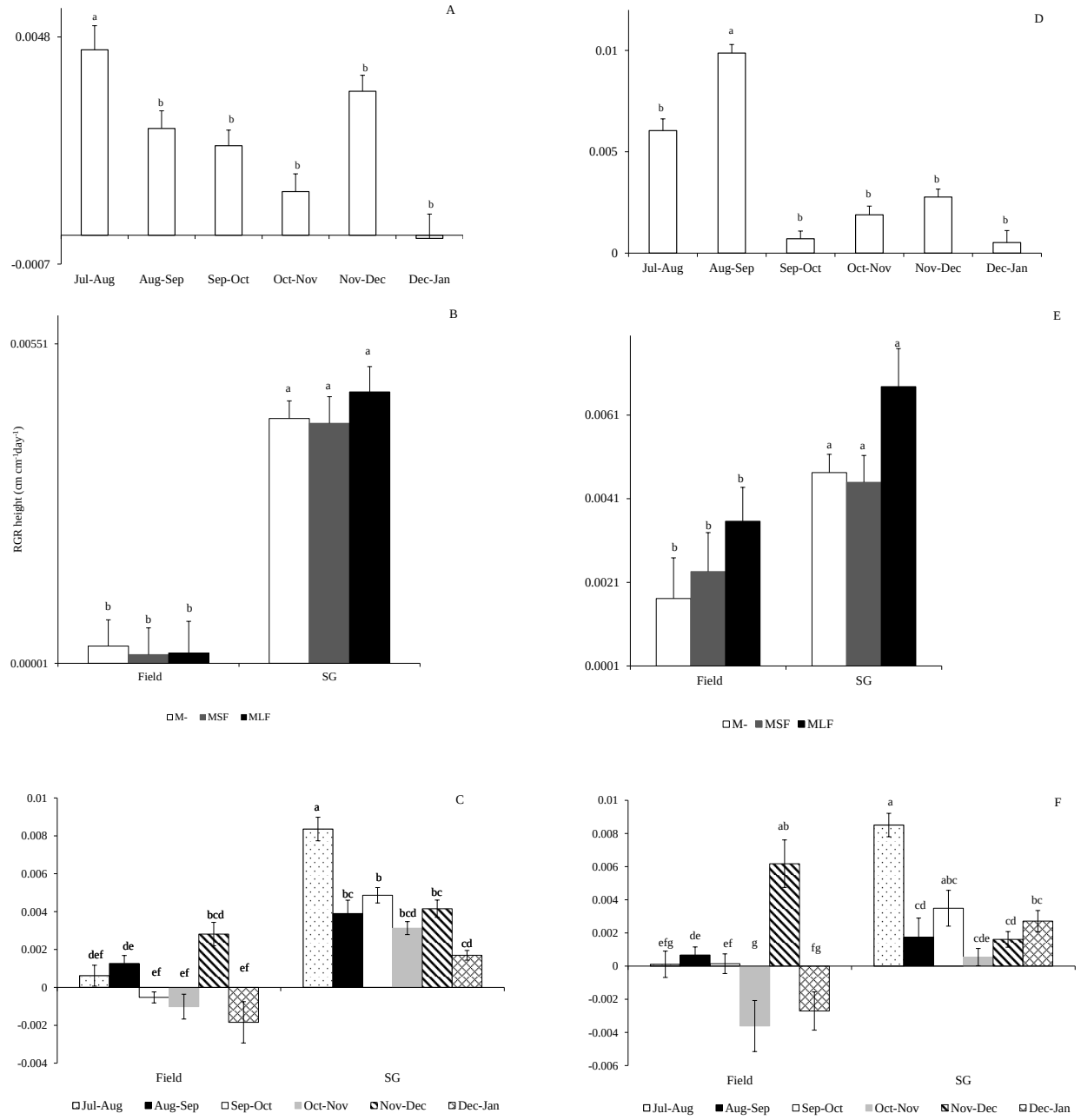

Figure 2. Mean height relative growth rates according to time (A, and D), and interactions among factors, inoculum origin (IO)×Zone (B, and E), and Zone×Time (C, and F). A, B, and C correspond to *Pleuranthodendron lindenii* while D, E and F correspond to *Pimenta dioica*. Different letters indicate significant differences according to Tukey test ($p < 0.05$). Zone: field and shaded greenhouse (SG).

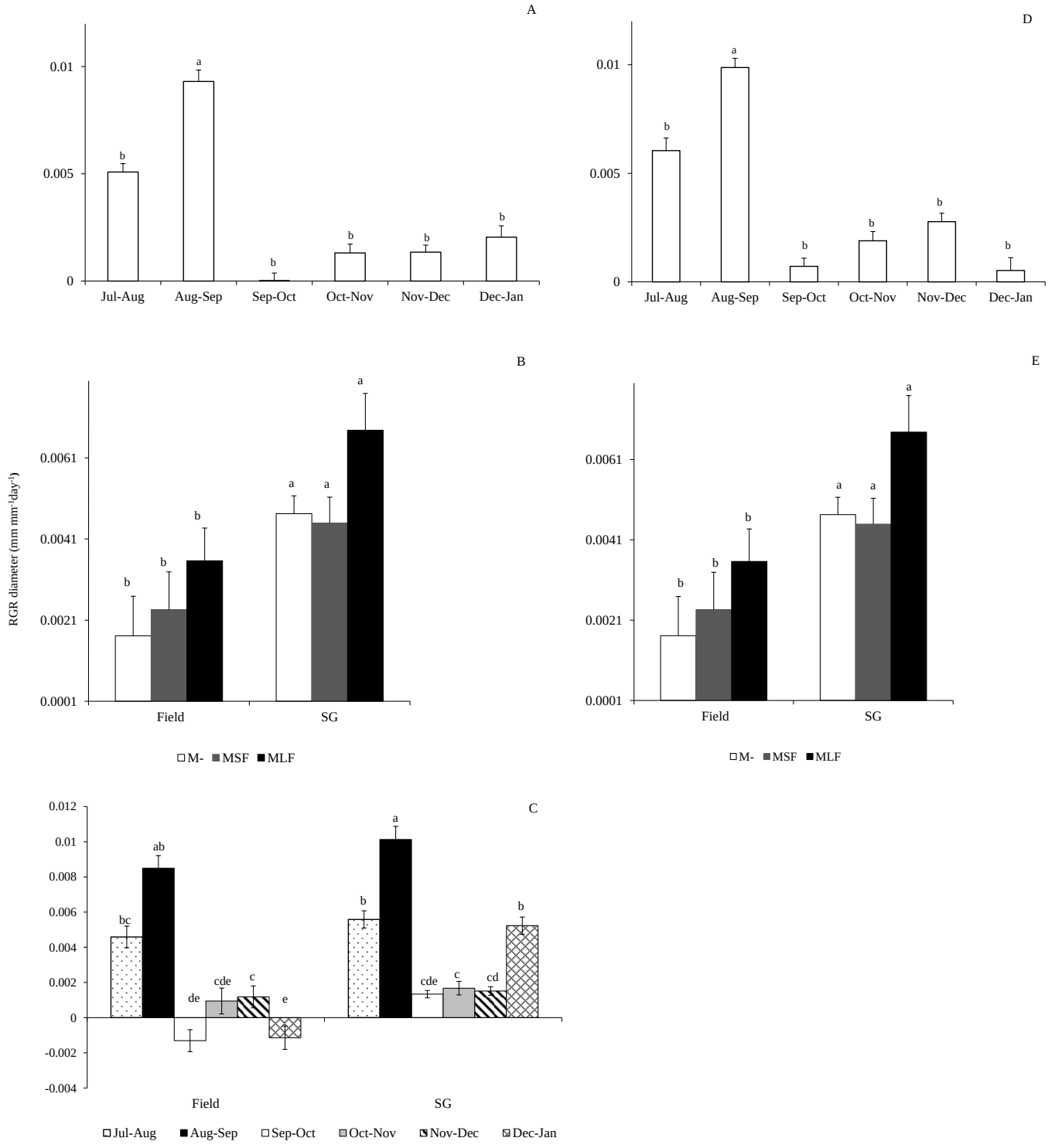

Figure 3. Mean diameter relative growth rates according to time (A, and D), and interactions among factors, inoculum origin (IO)×Zone (B, and E), and Zone×Time (C). A, B, and C correspond to *Pleuranthodendron lindenii* while D, and E correspond to *Pimenta dioica*. Different letters indicate significant differences according to Tukey test ($p < 0.05$). Zone: field and shaded greenhouse (SG).

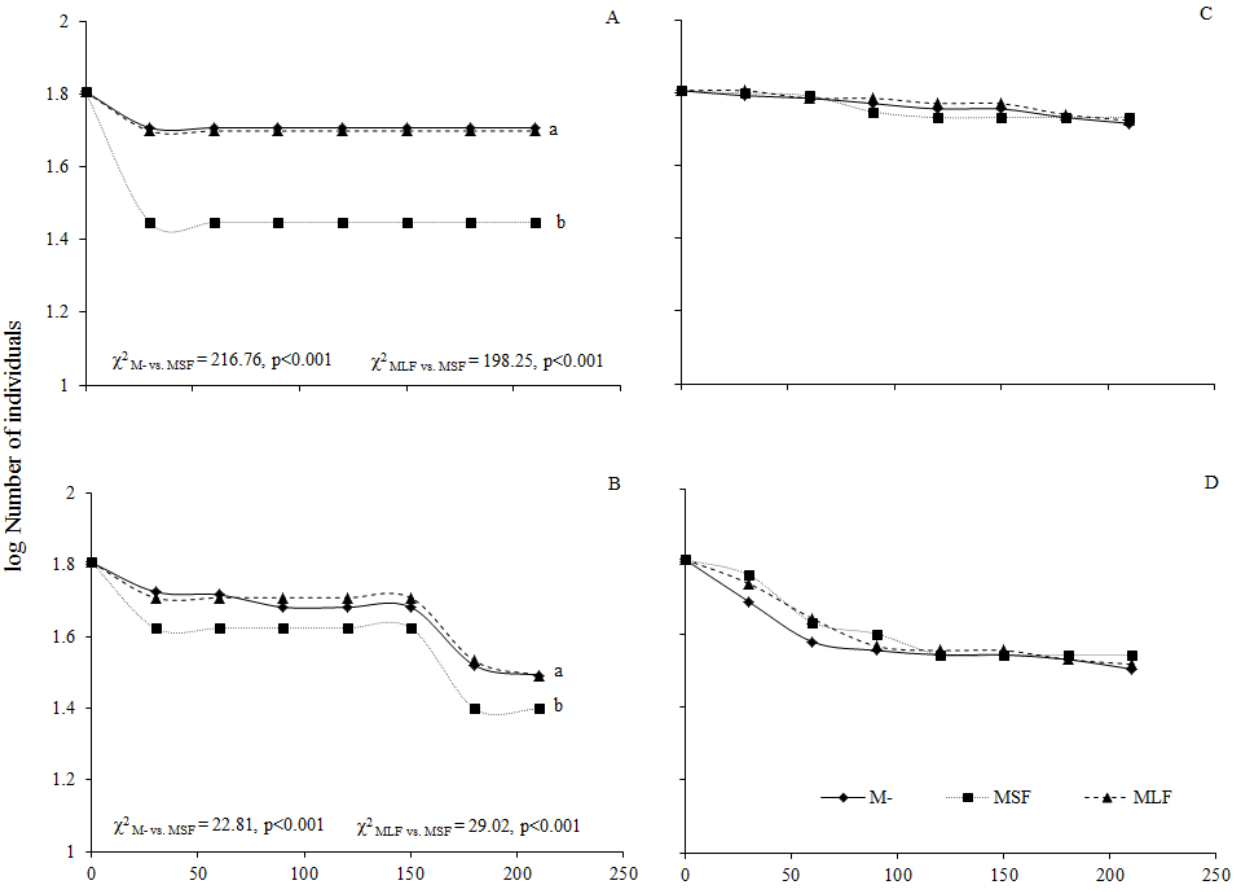

Figure 4. Survivorship curves of both species growing under shaded greenhouse (A and B) and field (C and D) conditions, (A and C, *Pleuranthodendron lindenii*; B and D, *Pimenta dioica*).

If we thoroughly analyze species response to AMF, we found that in general *Pleuranthodendron* in field had the highest mean values growing with MLF treatment while *Pimenta* pattern is not clear, but in general, MLF together with no inoculum had the highest growth and survival responses. The above suggests life history traits (Martínez-Ramos, 1994) are critical for determining plant response to inoculation, predominantly in the case of the former species because as a light demanding, its successful when being transplanted to field beneath tree shade is low, and *Pimenta* as shade tolerant can stand low light field conditions and allocate more resources to keep growing in natural conditions into the forest; this performance has been observed in other shade tolerant species (Álvarez-Sánchez *et al*., 2009).

The AMF did not have a significant effect after transplantation to the field, which indicates that for any of the two life history traits there were not effect of the inoculum for increase survival according to the size of the fragment,. This is contrary to that found by Guadarrama *et al.* (2004); they reported survival differences for the genus *Heliocarpus*, light demanding specie. It is clear that for species used in this study there is not a survival-growth trade off in terms of the benefit by the AMF.

On the other hand, AMF effects on plants can have different quality; some AMF species enhance phosphorus absorption, while other species protect plants from pathogens and parasites (Klironomos *et al*., 2000). This occurs since each AMF species can have a different effect depending on the plant species identity, this is explained by AMF multifunctionality (van der Heijden *et al*., 1998a, 1998b) supported by the fact that there is a great genetic diversity in AMF (Clapp *et al*., 2002). Our inocula shared close to 70% of species, but both inocula (from small and large fragments) had exclusive species (Luna, 2009), that could explain differential plant responses. Whether this can be happening at Los Tuxtlas tropical rain

forest is a question that has to be solved in the short time because it is patent that native forest plants need AMF to grow and survive, now the question is who needs whom, answering it will give major success to restoration actions.

CONCLUSIONS

Despite there was no relationship between plant life history and response to AMF inoculum origin, we found that AMF inoculum factor by itself had a significant and positive effect on relative growth rates of both species, and this result is more evident when inoculum belonged to large fragments.

However, we also found that these positive effects depend on the site conditions where seedlings are growing; AMF inoculum origin and site interaction showed that arbuscular mycorrhiza effects are clearly positive only when seedlings are growing under shaded greenhouse conditions, but when these are transplanted to field, AMF positive effects on seedling height and diameter can not overcome harsh field environmental conditions. And this might be due to the high energetic costs this mutualistic interaction has for seedlings, regardless inoculum origin.

Nevertheless, we widely recommend the use of native AMF to inoculate seedlings before field transplants, but taking care of them, at least the first two years.

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