

Evaluation of the resilience of *Rhizophagus intraradices* inoculated on hybrid *Brachiaria* grass ‘Mavuno’

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Abstract

The technology to produce mycorrhizal biofertilizers has given a remarkable impulse, but it is necessary to know the resilience of mycorrhizal inoculums. The objective was to evaluate the multiplication capacity and persistence of the mycorrhizal species *Rhizophagus intraradices* (*R. intraradices*) on the hybrid *Brachiaria* grass ‘Mavuno’. The number of spores, the spatial distribution of spores in the beds and the percentage and intensity of root colonization in the two propagation beds of *R. intraradices* were determined. Chemical and physical characteristics of the substrate in each bed were analyzed. Student’s *t*-test for two independent samples ($p < 0.05$) and chi-squared test were used for data analysis. *R. intraradices* species reproduced more spores g⁻¹ of substrate in both beds in 2023. In the years 2022 and 2023, the number of spores in bed 1 was 26% and 41% higher than in bed 2, respectively. The spatial distribution of spores in the beds was more heterogeneous in 2023. Nitrogen, phosphorus and organic matter contents differed between the substrates used, which may have influenced the efficacy of *R. intraradices* species. A high potential for multiplication and resilience of *R. intraradices* reflected under the experimental conditions studied was found.

Keywords: arbuscular mycorrhizal fungi; biofertilizers; persistence; replication

Introduction

The use of mycorrhizae in agriculture allows substantial increases in crop yield and quality, improving soil productivity, and has a positive effect on efforts to increase food security sustainably. Different studies have focused on the use of arbuscular mycorrhizal fungi (AMF) to increase yields of different crops, including potato and wheat crops (Elliott *et al.*, 2021), as well as that reported by Rivera-Espinosa *et al.* (2018) in more than 190 crop-soil combinations (20 crops and 12 soil types), such as corn, vegetables, grass species, among others. The benefits of AMF are not limited to crops, but also to the restoration of disturbed ecosystems (Carrillo-Saucedo

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et al., 2022). AMFs are obligated mutualistic symbionts of most plants and in turn, influence the structure of edaphic environments and their multifunctionality (Van der Heijden *et al.*, 2015). Although AMF is not selective to the plant species they colonize, they may show greater compatibility towards some plant species than others (Montesinos-Navarro *et al.*, 2019).

Few studies that determine the resilience and persistence of AMF several years after their application, therefore, there is a lack of sufficient information on the abundance and permanence of AMF propagules to ensure successful root colonization (Islam *et al.*, 2021). Among the AMF genera that have been studied for their persistence and ecological effect, *Rhizophagus* occupies 20% of a total of five genera reported, with *Rhizophagus irregularis* being the species that has shown the greatest persistence, being detected up to three years after application in different cropping systems (Basiru and Hijri, 2022). The persistence of AMF was confirmed by Floc'h *et al.* (2022) after 10 years of studies on canola roots. Their results give rise to new studies on the success of symbiosis establishment and persistence of AMF in soil, as well as their interaction with other soil microorganisms. Nevertheless, the efficacy of AMF inocula must be corroborated by field trials designed to examine the establishment and persistence of mycorrhizal inoculants, employing techniques that facilitate the monitoring of inoculated AMF species (Hart *et al.*, 2018). Based on the above, the objective of the present work was to evaluate the resilience and propagation capacity of the AMF species *R. intraradices* in the hybrid *Brachiaria* grass 'Mavuno'. The above under the hypothesis that some AMF species multiply efficiently under certain experimental conditions in the presence of a host, increasing their resilience and replication capacity.

Materials and Methods

Experimental procedures

The experiment was carried out at the General Terán Experimental Field, National Institute of Forestry, Agriculture and Livestock Research (INIFAP), Mexico, in a greenhouse under semi-controlled conditions. Two beds (1 and 2) were established in 2020 for the propagation of *R. intraradices* in hybrid *Brachiaria* grass 'Mavuno', both beds were constructed of wood with a production capacity of approximately 6.6 m³ of soil and were equipped with a drip irrigation system. The substrate used in each bed was subjected to a physico-chemical analysis at the soil laboratory of the Río Bravo Experimental Field - INIFAP. The methodology described by Durán-Prado *et al.* (2001) for the propagation of *R. intraradices* was used. *Brachiaria* grass was inoculated with INIFAP mycorrhiza (Bajío Experimental Field - INIFAP), prepared with the mycorrhizal species *R. intraradices*. A total of 500 g of inoculated seed was sown in each bed and fertilisation was carried out according to the needs of the crop. A record was made of the activities carried out in both beds until the year 2023. In 2021, the beds were reseeded and the evaluation of the variables studied was carried out in 2022 and 2023.

Preparation of the beds

Prior to planting, the substrate in each bed was leveled. To avoid contamination with other AMF spores, the substrate was sterilized with a formaldehyde solution consisting of 120 ml in 5 L of water per m² of substrate. The beds were covered with black polyethylene for 72 hours, after which they were uncovered and the substrate removed to eliminate any trapped gases. Eight furrows were made in each bed to a depth of 3 cm using a manual furrower. Drip irrigation strips were placed between the furrows and an initial irrigation was applied before planting.

Seed preparation and sowing

The germination percentage of *Brachiaria* 'Mavuno' grass seeds was determined. One hundred seeds were placed in a Petri dish and incubated in humid conditions until germination. The seeds that germinated

were then counted, and the germination percentage was evaluated, which was 92%. Other criteria for good seed quality, such as the absence of phytopathogens and high purity, were also taken into account. Before sowing, *Brachiaria* 'Mavuno' grass seeds were disinfected with a sodium hypochlorite solution (3.5%), followed by thorough washing with sterile distilled water to ensure complete removal of the residual solution. The seeds were then dried in the shade. The sowing procedure consisted of distributing 500 g of seeds along the eight furrows per bed.

Protocol for the sampling of the beds

The first sampling of the beds was carried out in March 2022, with five subsamples of approximately 100 g being extracted from the substrate at various points within each bed, at a depth of 20 cm. The second sampling was conducted in June 2023, involving the division of each bed into three sections and the extraction of five subsamples of approximately 100 g from each section at a depth of 20 cm. Subsequent to both samplings, the subsamples from each bed were placed in a nylon bag, homogenized to form a composite sample, and then sent to the laboratory for analysis. Prior to spore determination, the composite substrate samples from both beds were dried at room temperature.

*Determination of the number of spores of *R. intraradice**

The extraction of AMF spores was performed by the wet sieving and decanting method according to the technique proposed by Gerdemann and Nicholson (1963). Common water was added to 100 g of each homogenized sample, and each sample was washed six times. Each wash was passed through two sieves, 800 μm and 45 μm , respectively. The sample was collected from the 45 μm sieve and centrifuged, first with water and then with sucrose + tween 80, and the spores were then extracted and washed thoroughly with copious amounts of water. The spores were deposited on a counting plate to determine the number of spores per gram of soil. Five replicates were performed for each composite sample from both beds.

*Percentage and intensity of colonization in roots of *Brachiaria* grass*

In 2023 roots were extracted from both beds, and five subsamples of roots were taken from the three sections into which the beds were divided, the subsamples were homogenized for analysis. The finest roots (secondary and tertiary) were selected, clarified with KOH (10%), HCl (1N), and stained with a 2.5% Parker Quink (blue) dye solution, according to the method proposed by Rodríguez-Yon *et al.* (2015). Subsequently, the stained roots were examined under an optical microscope to determine the presence of mycorrhizal structures (hyphae, arbuscules and vesicles). The variables percentage and intensity of colonization were determined according to equations 1 and 2 (Trouvelot *et al.*, 1986).

$$\text{Percentage of colonization} = (\sum(1 - 5) / \sum(0 - 5)) \times 100 \quad (1)$$

$$\text{Colonization intensity} = \sum(A) / (\sum(0 - 5)) \quad (2)$$

Where:

- a) Reading levels are established (0 to 5)
- b) Each reading level is multiplied by a constant (*A*).

Levels	0	1	2	3	4	5
Constant	0	1	2.5	15.5	37.5	47.5

Statistical procedure.

The data were analyzed using a Student's *t*-test for two independent samples ($P < 0.05$) between beds and sampling years (2022 and 2023). Additionally, the Chi-square test of independence was employed, assuming that the variables (spatial distribution of spores and substrate type in the beds) are dependent, i.e.,

that there is an association between them. The number of spores (g^{-1}) was transformed by the square root, while the percentage and intensity of mycorrhizal colonization were calculated using the function $\arcsin\sqrt{x}/100$, as these variables did not present a normal distribution. All statistical analyses were performed with SAS 9.4M4 software for a Windows 10 operating system.

Results

Physic-chemical characterization of substrates and activities

The substrates of both beds were similar in most of their characteristics, with important differences in the amount of organic matter, nitrogen, phosphorus, copper, and potassium (Supplementary Table 1). The monitoring of activities carried out in both beds is shown in (Table 1). An important low-temperature event occurred in the month of February 2021, and during the months of May 2022 to June 2023 the beds did not have any cultural management activities, only irrigation was applied. On the other hand, in the months of April to June 2023, the beds suffered water stress due to water shortage and high temperatures. Average maximum and minimum temperatures were 32 °C and 21 °C (General Terán Experimental Field, 2023).

Table 1. Management conditions of the beds used for *R. intraradices* spore production

Management of the beds (2020 - 2023)		
Activities	Dates	
Seeding	15/04/2020	
Reseeding	22/04/2021	
Sampling	16/04/2022	12/06/2023
Irrigation	Weekly	
Fertilization	On one occasion in the years 2020 and 2021	

Number of spores

The Student's *t*-test for the number of spores indicates a statistical difference between the soils of the beds studied (Table 2). The number of spores in the year 2022 in bed 1 was 26% higher than in bed 2 (Figure 1A), while in the year 2023, the number of spores in bed 1 was 41% higher than in bed 2 (Figure 1B). The species *R. intraradices* showed a greater resilience by replicating a greater number of individuals during the two years that the study was maintained, and this was reflected in the spore content in the substrates of both beds.

Table 2. Results of the Student's *t*-test for spore production in the years 2022 and 2023

Spore production	2022		2023	
	<i>t</i>	<i>P value</i>	<i>t</i>	<i>P value</i>
Number of spores g^{-1}	6.29	0.003 ***	3.51	0.025 *

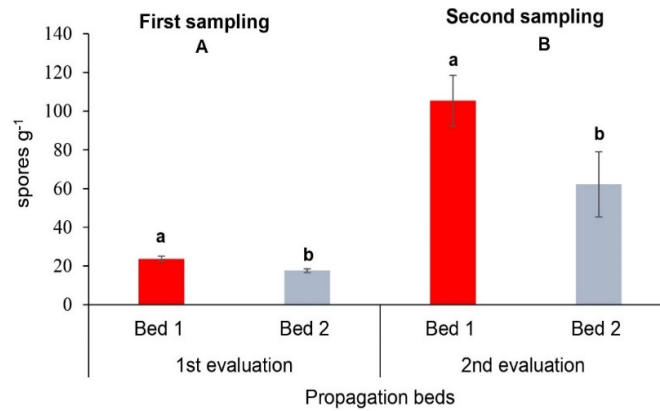


Figure 1. Beds in production of *R. intraradices*

A: 1st evaluation of beds (year 2022), B: 2nd evaluation of beds (year 2023). The bars indicate the standard deviation of the sample.

Spatial distribution of spores

The Chi-square test of independence indicates that there is no significant statistical association between the spatial distribution of spores and the substrate type of the beds. Therefore, both variables are independent (Table 3).

Table 3. Results of the Chi-square test spatial distribution of spores within *R. intraradices* production in the years 2022 and 2023

Chi-square test	Year 2022		Year 2023	
	Chi-square	P value	Chi-square	P value
Pearson	0.037	0.982	2.913	0.233
Likelihood ratio	0.037	0.982	2.930	0.231

Chi-square test ($p < 0.05$) *H₀*: are dependent; *H₁*: are independent

Regarding the spatial distribution of spores within the beds, it was observed that during the year 2022 in both beds the distribution was relatively homogeneous, in contrast to what was observed the following year. In 2023, the spatial distribution of spores was highly variable, with approximately 71% and 77% of *R. intraradices* spores found in the first two sections of beds 1 and 2, respectively (Figure 2).

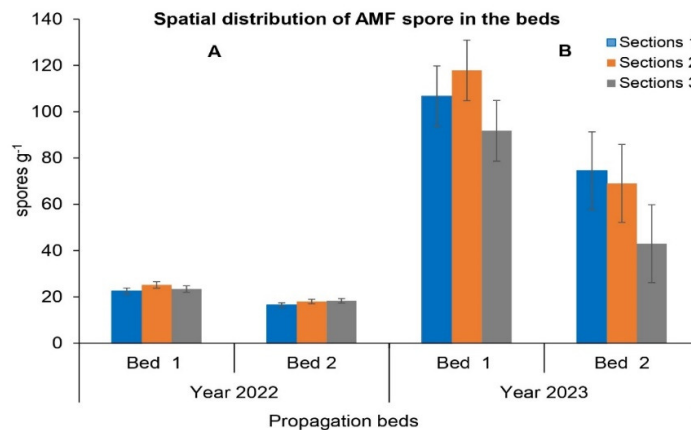


Figure 2. Spatial distribution of spores within *R. intraradices* production beds

A: 1st evaluation of beds (year 2022), B: 2nd evaluation of beds (year 2023). The bars indicate the standard deviation of the sample.

Percentage and intensity of colonization in roots

In the case of the percentage and intensity of colonization in the year 2023, the Student's *t*-test ($p < 0.05$) did not show statistical differences between the beds (Table 4). This shows that colonization with *R. intraradices* was efficient in both beds; however, a greater presence of mycorrhizal structures (arbuscules, hyphae, vesicles) was found in the roots of bed 1 compared to those found in the roots of bed 2 (Figure 3B).

Table 5. Results of the Student's *t*-test for percentage and intensity of colonization in the year 2023

Year 2023	<i>t</i>	<i>P</i> value
Percentage of colonization	-0.85	0.442 <i>ns</i>
Intensity of colonization	2.37	0.077 <i>ns</i>

Student's *t*-test ($p < 0.05$) *significant; **highly significant; ***highly significant and *ns* not significant.

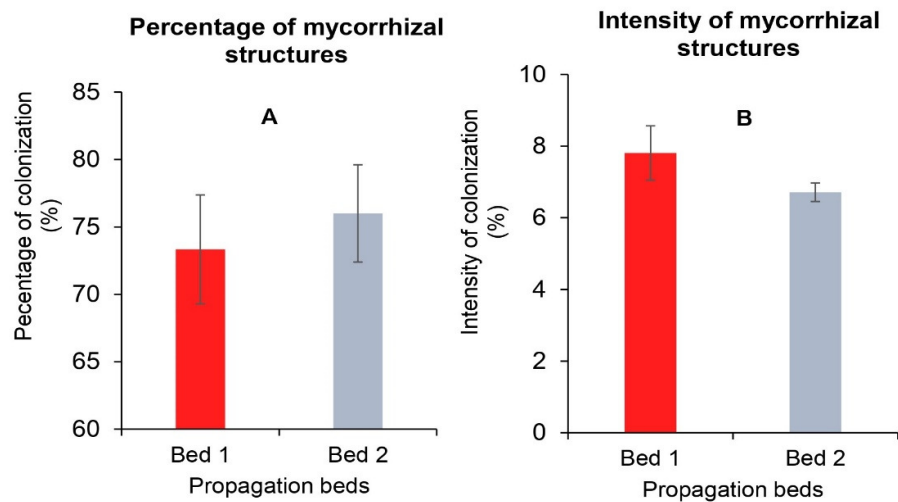


Figure 3. Beds in production of biofertilizer year 2023

A: percentage of colonization, B: intensity of colonization). The bars indicate the standard deviation of the sample.

Discussion

Number of spores

The results demonstrate that the chemical composition of the substrate in bed 1 had a positive impact on the response of the *R. intraradices* species. By 2023, the number of spores g^{-1} of substrate in bed 1 had increased fourfold in comparison to the 2022 results. In bed 2, the number of spores g^{-1} of substrate was three times higher in 2023 than in 2022. The higher number of spores obtained in bed 1 in 2023, compared to bed 2, may be attributed to the difference in phosphorus and nitrogen levels between the substrates. Previous studies have indicated that high phosphorus availability can negatively impact mycorrhizal symbiosis. However, when nitrogen levels are low, excess phosphorus can reverse this effect, promoting mycorrhizal colonization (Nouri *et al.*, 2015). The nitrogen-phosphorus ratio in soil plays a significant role in mycorrhizal symbiotic function (Li *et al.*, 2019). Research indicates that both phosphorus and nitrogen regulate mycorrhizal symbiosis (Wang *et al.*, 2017).

The genus *Rhizophagus* is one of the most studied as a biofertilizer, and some of the species in this genus have demonstrated a great capacity for replication in different soil environments and crops. Under field conditions, *R. intraradices* has been reported to persist for one year and two months after inoculation (Basiru and Hijri, 2022), but its efficiency in replication and infectivity after this period has not been documented.

Similarly, persistence of *Rhizopogon irregularis* was reported for three years after application as a commercial inoculant at four agricultural sites in semi-arid to sub-humid environments in Canada, suggesting that survival of *R. irregularis* was mainly dependent on soil type (Salomon *et al.*, 2022).

Spatial distribution of spores

The spatial distribution of spores within the beds demonstrated notable heterogeneity over the two-year study period. A notable disparity was evident in 2023 (Figure 2). The heterogeneous spatial distribution of spores observed in 2023 may be attributed to inadequate maintenance and structural damage to the greenhouse roof. Some areas of the beds were devoid of vegetation, and the substrate where the *R. intraradices* species was propagated was exposed to solar radiation. This direct exposure of the soil to the sun may have had a negative impact on spore replication, particularly given that solarization is a widely used method for soil sterilization (Katan and DeVay, 1991). It is well documented that soil solarization is an effective agroecological technique for eradicating pathogenic microorganisms from soil. However, it should be noted that prolonged exposure of the substrate to solar radiation can also affect the viability of beneficial microorganisms. Several studies have identified a correlation between AMF spore density and soil depth. These studies have reported that a higher concentration of spores is located at an initial depth of 10-20 cm (Liang *et al.*, 2021). It is important to note that soil temperatures during solarization can range from 35-60 °C. This primarily affects the surface layer of the soil; however, the effect of solarization varies due to the influence of depth on temperature (Stapleton, 1990).

Percentage and intensity of colonization in roots

The increased presence of intraradical mycorrhizal structures (arbuscules, hyphae, and vesicles) in the roots of plants grown in the soil of bed 1 suggests that the chemical composition of the soil, particularly its phosphorus and nitrogen content, may facilitate the establishment and growth of this symbiotic relationship, leading to an increase in the observed structures. Similarly, *R. intraradices* exhibited the capacity to establish and persist in the substrates for a period of two years without reinoculation in the beds, indicating a notable resilience to the environmental conditions under which the beds were subjected. In mycorrhizal symbiosis, phosphorus is one of the most extensively studied nutrients. It can be a significant factor in AMF-host interactions, with increases in phosphorus levels capable of completely and temporarily inhibiting the emergence and development of arbuscules, while leaving the rest of the intraradical mycorrhizal structures unaffected (Kobae *et al.*, 2016). In contrast, low levels of nitrogen (nitrate) in the soil can counteract the ineffectiveness of AMF when high levels of phosphorus are present, indicating that mycorrhizal symbiosis is contingent on the availability of either of these two nutrients (Nouri *et al.*, 2015).

There is a documented correlation between the growth of mycorrhizal structures and an adequate supply of nutrients. However, an overabundance of fertilizer can impede this process, as it may lead to a diminished importance assigned by the plant to the mycorrhizal symbiosis (Ramírez *et al.*, 2017). Similarly, when soil phosphorus levels are elevated, the efficiency of AMF is constrained due to the cost of carbohydrates for the host plant exceeding the benefits provided by the mycorrhizal symbiosis (Qin *et al.*, 2020). In grasses, the availability of nutrients in the soil determines the presence of mycorrhizal structures both inside and outside the root. When grasses are adequately fertilized, the number of mycorrhizal structures is reduced (Gustafson and Casper, 2004). Pellegrino *et al.* (2022) reported the presence of AMF species *Funneliformis mosseae* and *R. irregularis* inoculated on *Medicago sativa* L. in roots and soil for at least two years after inoculation. They associated the positive effect on AMF species persistence with an increased abundance of mycorrhizal structures in roots.

The present study is limited to semi-controlled conditions, and leaves us with the question, will *R. intraradices* species show comparable patterns of replication and infectivity under field conditions? Confirmation of inoculum establishment will remain a challenge due to the inherent complexity of AMF

genetic organization and high variability (Hart *et al.*, 2018). There is currently a significant gap in the understanding of mycorrhizal inoculum establishment and persistence. Future AMF inoculation studies could focus on understanding the resilience of these biofertilizers, which will provide information on the longevity of mycorrhizal inoculants in soil and facilitate the development of efficient AMF inoculation strategies.

Conclusions

The species *R. intraradices* replicated more spores g⁻¹ of substrate in both beds in 2023. In 2022 and 2023, the number of spores in bed 1 was 26% and 41% higher than in bed 2, respectively. The spatial distribution of spores in the beds was heterogeneous in 2023. The contents of nitrogen, phosphorus and organic matter were different among the substrates used, which may have influenced the efficacy of the *R. intraradices* species. The *R. intraradices* species reflected a high potential for propagation and persistence, demonstrating a high capacity for resilience.

Authors' Contributions

Conceptualization: AME and CMRC; Software: AME; Validation: RRG and MER; Formal analysis: AME and BESM; Investigation: AME and CMRC; Resources: MER and CMRC; Data curation: AME; Writing—original draft: AME; Visualization: BESM, RRG; Supervision: MER; Project administration: AME and CMRC. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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