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**ABSTRACT:** Arbuscular mycorrhizal fungi (AMF) are beneficial symbiotic organisms that play an important role in the water absorption and nutrient assimilation of a wide variety of plants. In this work, the use of polyethylene oxide (PEO) nanofibers was studied as a delivery system for AMF through the coated with nanofibers of common bean seeds (*Phaseolus vulgaris* L var. José Beta). Thus, it was examined the effect of PEO nanofibers on the infective capacity of AMF and the indicator parameters of plant growth promotion (height plant, root length, number of leaves, number of flower buds, number of pods, fresh weight and dry weight). Likewise, it was compared the mycorrhizal colonization rate and the indicator parameters of plant growth promotion between a conventional inoculation strategy and inoculation by nanofiber-coated seeds. The results show that PEO nanofibers did not have any negative effect in the infective capacity of AMF or the indicator parameters of plant growth promotion. On the other hand, the inoculated bean plants showed a significant increase of 200%, 140% and 143% in the number of flower buds, fresh weight and dry weight, respectively. Moreover, no significant differences were observed in the mycorrhizal colonization rate between the two inoculations strategies used, even though the inoculant dose was 97% lower for the nanofiber-coated seeds. Therefore, nanofibers could represent a promising alternative as a seed coating material and the development of easy-to-apply and economically viable technologies for the application of AMF inoculants in the fields of agriculture, horticulture, forestry, ecological remediation and related areas.

**KEYWORDS:** *Seed coating, nanofibers, arbuscular mycorrhizal, agriculture, biofertilizers, nanotechnology*

## INTRODUCTION

Fertilizers are one of the most important requirements for maintaining soil fertility and improving crop productivity, which has promoted economic development over the past few decades.<sup>1,2</sup> However, traditional fertilizers are not fully accessible to plants because they are frequently deposited in poorly soluble forms in the soil, which causes a low nutrient use efficiency and induces a series of negative environmental consequences.<sup>3,4</sup> Furthermore, high costs limit its use, mainly in developing countries, where the need to increase agricultural production is more urgent.<sup>5</sup>

Consequently, it is clear that future transformations in agricultural systems must aim to reduce the use of harmful agricultural inputs without jeopardizing yield.<sup>6</sup> Therefore, the exploration of unconventional technologies is necessary to mitigate the demand for resources and maintain environmental sustainability for future generations.<sup>7</sup>

In this sense, the beneficial soil microorganisms have gained important interest in the last decades as promising tools to fully or partially replace synthetic fertilizers.<sup>8,9</sup> Among these microorganisms are the arbuscular mycorrhizal fungi (AMF), which are a type of mycorrhizal that represent one of the most abundant symbiotic relationships in the biosphere.<sup>10</sup> AMF play an important role in the nutrient uptake and resistance to several biotic and abiotic stress factors from a wide variety of plants.<sup>11</sup> For this reason, AMF have become increasingly important as a tool to reduce the amount of agrochemicals, which are the cause of serious problems in the soil, air, water systems and human health.<sup>12</sup>

However, although AMF inputs represent a promising option for agricultural systems their efficacy may be limited by the unavailability of suitable carriers that allow AMF to easily disperse in the vicinity of the root system and maintain their beneficial activities in the presence of unfavorable biotic and abiotic factors that may interfere with AMF abundance and colonization once applied to the soil.<sup>13-15</sup> In addition, the

application of large-scale inoculant is economically unfeasible, since it would require the use of expensive machines and the undirected propagation of the inoculant to large areas, which represents a high cost per plant.<sup>16,17</sup> Therefore, the development of new inoculation strategies such as seeds coating is essentially necessary for the application of AMF inoculants on a large scale.<sup>13,17</sup>

Recently, due to their unique properties of adaptation, high surface area-to-volume ratio, tunable porosity, absence of residual solvents and high production rate, microfiber/nanofiber mats are presented as an attractive covering material to seeds and the delivery of active ingredients of agricultural interest, such as fertilizers, pesticides, phytohormones and living cells.<sup>18-20</sup>

In previous research, the use of fibrous polymeric matrices has been studied in the coating of seeds and seedlings as an alternative to immobilize beneficial soil microorganisms.<sup>21-24</sup> These formulations are based on the restriction of living cells in spaces confined by a polymer, preventing the free circulation of microorganisms and protecting them from the environment.<sup>25,26</sup>

To our knowledge, the use of biodegradable fiber mats as a delivery system for AMF has not yet been reported. In this research, we studied the use of nanofibers made from polyethylene oxide (PEO) as a delivery system for AMF through the production of bean seeds (*Phaseolus vulgaris* L var. José Beta) coated. We analyze the impact of nanofibers on the infective capacity of AMF and the indicator parameters of plant growth promotion. In addition, we compared the mycorrhizal colonization rate and the indicator parameters of plant growth promotion between a conventional inoculation strategy for AMF and inoculation by coating seeds with nanofibers.

The choice of PEO as a polymer in the present work is due it is an environmentally safe and water soluble coating material.<sup>27</sup> It was hypothesized that the water solubility of PEO would allow its early dissolution near the roots during watering so that AMF inoculant can establish contact with the roots for efficient

Seed coating has the potential to reduce labor and the amount of AMF inoculum required, which results in a cost reduction and a viable strategy for the application of AMF inoculants on a large scale. In this sense, this study is a contribution to our understanding about the type of cell body that can be incorporated in biodegradable nanofiber mats that serve as delivery system for microorganisms in seed coating process for ecological or industrial applications.

## EXPERIMENTAL SECTION

**Materials.** Poly (ethylene oxide) (PEO) ( $M_v$  200,000), acid lactic solution ( $\geq 85\%$ ) and sodium hypochlorite solution (10%) were purchased from Sigma-Aldrich. Hoagland solution was purchased from HiMedia. Hydrochloric acid (99.9%) and potassium hydroxide ( $\geq 85\%$ ) in lentils were purchased from Avantor Macron Fine Chemicals. Trypan blue powder was provided from the Facultad de Agronomía y Veterinaria de la Universidad Autónoma de Santo Domingo (UASD). Powder inoculant composed of expanded clay, kaolinite, natural diatomite and a concentrated mass of 150 spores per gram of arbuscular mycorrhizal fungus *Rhizophagus irregularis* was purchased from Premier Tech Biotechnologies. *Phaseolus vulgaris* L. var. José Beta seeds were purchased from Comercial Agrícola Sanz. AMF inoculant and seeds were stored at 4 °C.

**Preparation of nanofibers.** A 5% (w/v) solution of polyethylene oxide (PEO) was prepared. The polymer was dissolved with distilled water in a shaking thermostatic bath (Boshi Electronic Instrument, China) at 85 °C for 1 hour. The polymeric solution was autoclaved at 121 °C and 15 lb pressure for 15 minutes and stored at 4 °C before use. The elaboration of the nanofibers was realized by the centrifugal spinning technique.<sup>28</sup> Nanofibers were produced using hypodermic needles (Blue Cross, 27G x 1/2 ", United States) until a dense amount of fibers was generated in the vertical collector bars. Centrifugal spinning parameters were set as follows: distance to the collector, 15 cm; relative humidity; 35 %; environment temperature 21 °C.

**Immobilization of AMF inoculant.** Immobilization of the mycorrhizal inoculant was realized according to the methodology described by Campaña and Arias<sup>29</sup>. Fiber sheets were collected and the powder inoculant was added to its surface. Then, three inoculated sheets were overlapped on each other to obtain an inoculated composite sheet. One gram of inoculant was used for each composite sheet. The schematization of the procedure can be found as Scheme S1 of the Supporting Information.

**Nanofiber characterization.** Nanofibers were assessed using scanning electron microscopy (SEM). (Quanta FEG 250, United States). Samples were taken from uninoculated and inoculated nanofiber mats, which were affixed to sample stubs to analyze the uniformity of the fibers, the disposition of the AMF inoculant in the fibrous polymer matrix and the diameter of the fibers using the ImageJ 1.52 software.

**Seed coating with nanofiber.** Bean seeds were surface-disinfected by immersion in a 10% aqueous solution of sodium hypochlorite for 5 minutes and finally by five successive rinses in distilled water. The seeds were dried in a Petri dish at room temperature for 30 minutes. Disinfected seeds were manually covered with five layers of fiber-composite sheets. This resulted in 17 spores per seed and a buildup of 25% of seed weight for

seeds were transferred to hermetically sealed bags and stored at 4 °C before use.

**Experimental design.** An independent experiment was conducted using a completely randomized block design of ten blocks and ten replicates. The treatments are described in Table 1. The experiment was conducted in a nursery with a temperature and relative humidity ranging from 23 to 32 °C and from 84 to 85%, respectively, and with an average photoperiod

of 12 hours. In order to minimize differences due to blocks location in the nursery, their positions were weekly swapped.

**Table 1. Description of the treatments for the experimental design**

treatments	abbreviation
untreated seeds	NC
seeds inoculated in the soil	PC
seeds coated with PEO nanofibers	SN
seeds coated with AMF incorporated in PEO nanofibers	SNM

For pot experiment, a commercial substrate mixture composed of Canadian *Sphagnum* peat, perlite, dolomite and gypsum was used. The substrate was subjected to a heat treatment as described by Baker<sup>30</sup> with modifications. The substrate used for this study had the following properties: pH 5-6, 0.7-1.7 dS m<sup>-1</sup> electrical conductivity, 0-100 ppm total nitrogen (N), 0-67 ppm, 3-43 ppm phosphorus ( P), 17-138 ppm potassium (K), 24-160 ppm calcium (Ca), 15-75 ppm magnesium (Mg) and 70-225 ppm sulfur (S), 0-1.5 ppm manganese (Mn), 0-0.5 ppm iron (Fe), 0-0.05 ppm copper (Cu), 0-0.19 ppm boron (B), 0-0.15 ppm zinc (Zn) and 0-0.07 ppm molybdenum (Mo).

Bean seeds were sown in 1-gallon plastic pots. One seed was sown per pot at 3 cm deep and at a distance of 10 cm between pots. Plants that were treated with a conventional inoculation strategy received 4 g of the inoculant 2 cm below an uncoated seed which results in 600 spores per seed. The schematization of conventional inoculation strategy can be found as Scheme S2 of the Supporting Information.

As a source of nutrients, the Hoagland nutrient solution was used as described by Rocha and colleagues<sup>31</sup> with modifications. Each plant received 15 ml of Hoagland solution once a week from week 2 to week 6, likewise, the plants were watered with 1200 mL of running water each week.

All plants were harvested at the end of 50 days to record biometric observations and determine the mycorrhizal colonization percentage.

**Biometric analysis.** Plant height, root length, number of trifoliate leaves, number of flower buds, number of pods, fresh weight and dry weight were evaluated to determine the effects of treatments on the indicator parameters of plant growth promotion.

The height of the plant was determined as described by Pérez and colleagues<sup>32</sup> and the fresh and dry weight according to the methodology described by Chekanai and collages<sup>33</sup> with modifications. The root system was separated from the germination sprout (stem and leaves) and stored in containers

**AMF colonization analysis.** The staining of the roots and the determination of colonization rate of AMF was performed as described by Posada and colleagues<sup>34</sup> with modifications. The roots were discolored with 10% potassium hydroxide (w/v), acidified with 10% hydrochloric acid (v/v), stained with trypan blue 0.05% (w/v) in lactoglycerol and preserved in lactoglycerol (v/v).

The colonization rate of AMF was realized by root segment estimation method.<sup>35</sup> The 4X, 10X and 40X objectives of a compound light microscope (AmScope, United States) were used to examine the samples. The images are viewed using the AmScope 3.7.13522 software corresponding to the AmScope MD130 camera (United Scope LLC, United States).

The following formula was used to calculate colonization rate:

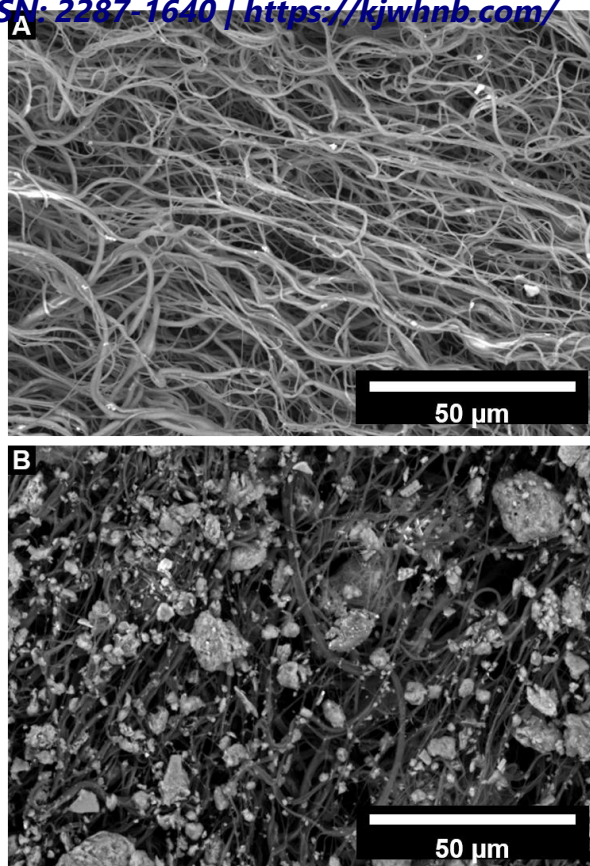
$$(1) \text{ Colonization rate} = \frac{\text{Number of root segments infected by AMF}}{\text{Total number of root segments examined}} \times 100$$

**Statistical analysis.** A bidirectional analysis of variance (ANOVA) and an analysis of multiple comparisons were applied using Fisher's Least Significant Difference (LSD) test to determine the effects of the different experimental groups in each of the following measurements: plant height, length of the root system, number of leaves, number of flower buds, number of pods, total biomass and mycorrhizal colonization rate. In each analysis, significant differences of ( $P < 0.05$ ) were used. GraphPAD 8.0.1 statistical software was used to perform statistical analyzes.

## RESULTS AND DISCUSSION

**Nanofiber characterization.** SEM micrographs show the formation of continuous fibers with little bead formation along them and an average diameter of  $566.75 \pm 145$  nm (Figure 1A). The average diameter of the nanofibers obtained corresponds to that described by Zhang and Lu<sup>36</sup> for 5% PEO nanofibers produced by the centrifugal spinning technique. Likewise, the micrographic images of the SEM show that mycorrhizal powder inoculant was successfully incorporated in the PEO polymer matrices so that it was adhered on the matrix surface and between nanofibers networks (Figure 1B).

The immobilization of the inoculant in the polymeric matrix is based on the absorption immobilization process in which the inoculant remains attached to the surface of the matrix due to physical interactions (hydrogen bridge, ionic bonds, hydrophobic bonds and Van der Waals forces) and the immobilization process known as entrapment, in which the inoculant is captured around the polymer matrix and between sheets of nanofibers as described by Aiswarya and colleagues<sup>25</sup>.



**Figure 1.** SEM micrographs of nanofibers with and without the mycorrhizal inoculant. (A) SEM micrograph with a 2000x magnification of PEO nanofibers. (B) SEM micrograph with a magnification of 2500x of PEO nanofibers with the incorporated mycorrhizal inoculant.

**Biometric analysis.** The indicator parameters of plant growth promotion were determined to analyze the effects of the treatments on the seeds (Figure 2).



**Figure 2.** Treatment of *Phaseolus vulgaris* L var. José Beta seeds. (A) Seeds of *Phaseolus vulgaris* L var. José Beta uncoated. (B) Seeds of *Phaseolus vulgaris* L var. José Beta coated with PEO nanofibers. (C) Seeds of *Phaseolus vulgaris* L var. José Beta coated with PEO nanofibers that have the AMF inoculant incorporated.

Table 2 shows the results on the indicator parameters of plant growth promotion evaluated.

Table 2. Effects of different seed treatments on height, root length, number of leaves, number of flower buds and number of pods.

growth parameters	treatments			
	NC <sup>a</sup>	PC <sup>b</sup>	SN <sup>c</sup>	SNM <sup>d</sup>
height (cm)	52.4 ± 3.4 a	50.4 ± 6.2 a	51.3 ± 5.7 a	53.8 ± 6.1 a
root length(cm)	27.6 ± 4.6 a	31.0 ± 9.5 ab	24.2 ± 4.0 a	29.5 ± 6.0 ab
number of trifoliolate leaves	6.80 ± 1.3 a	8.50 ± 1.6 ab	7.60 ± 1.0 a	8.20 ± 2.7 a
number of floral buds	2.50 ± 2.6 a	6.00 ± 1.9 b	2.80 ± 1.9 a	5.90 ± 3.6 b
number of pods	3.40 ± 1.0 a	4.60 ± 3.6 a	3.60 ± 2.0 a	4.10 ± 2.0 a

<sup>a</sup>NC: untreated seeds

<sup>b</sup>PC: seeds inoculated in the soil

<sup>c</sup>SN: seeds coated with PEO nanofibers

<sup>d</sup>SNM: seeds coated with AMF incorporated in PEO nanofibers

The observations showed plant height, number of trifoliolate leaves and number of pods of the bean plants were not significantly affected by PEO nanofibers or by inoculation with AMF, which corresponds to that reported in previous research for common bean plants previously inoculated with AMF.<sup>37,38</sup>

Similarly, no significant statistical differences were observed in the root length by any experimental groups, which corresponds to that observed by Ganjeali and colleagues<sup>39</sup>, where found no differences between the roots of *Phaseolus vulgaris* L var. Brilliant Red mycorrhizal and non-mycorrhizal. These observations suggested that PEO nanofibers had no effect on root length and that AMF do not necessarily cause significant physiological changes in the root system of plants as described by Dreyer and colleagues<sup>40</sup>.

On the other hand, the number of flower buds in bean plants inoculated with AMF increased by 200% compared to non-inoculated plants. In addition, no significant differences was observed between the treatments inoculated (PC and SNM) and between treatments non-inoculated (NC and SN). The increase flower development is an indicative of the increased vigor of the bean plants as a result of inoculation with AMF as has been described in previous researches.<sup>41,42</sup>

When the total biomass is analyzed, an increase of approximately 140% and 143% is evidenced for fresh and dry matter, respectively, for the experimental groups inoculated compared to the experimental groups not inoculated. In both cases, no significant statistical differences were between the inoculated experimental groups (PC and SNM), see Figure 3.

The increase in total biomass in the bean plant is possibly due to the effects of the fungus on increasing water absorption, improving the structure of the substrate and the absorption of various nutrients as reported for various types of legumes including *Phaseolus vulgaris* L as described by Youssef and colleagues<sup>43</sup>.

**Colonization of mycorrhizal fungi.** The root system of the plants was examined to identify the typical structures of AMF (Figure 4) and determine the effect of the infective capacity of AMF according to the treatment of the seeds.

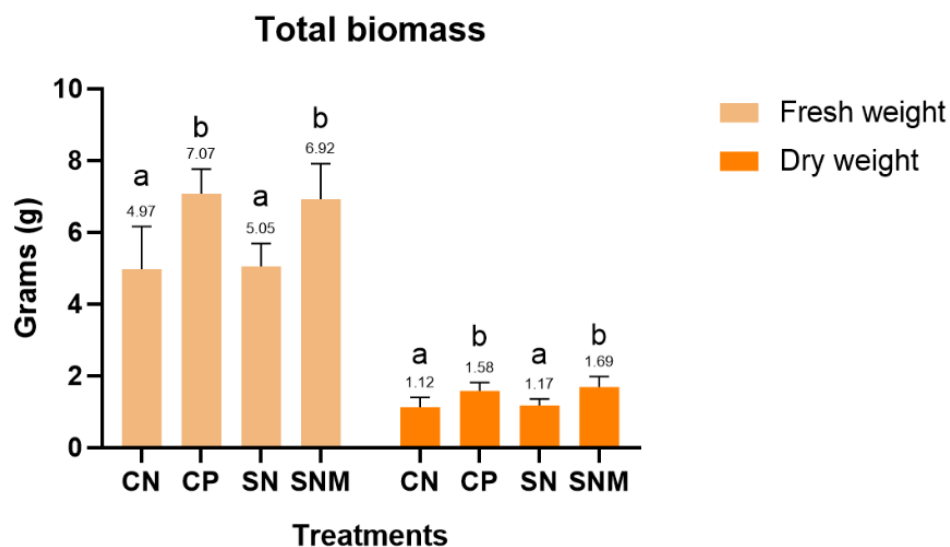
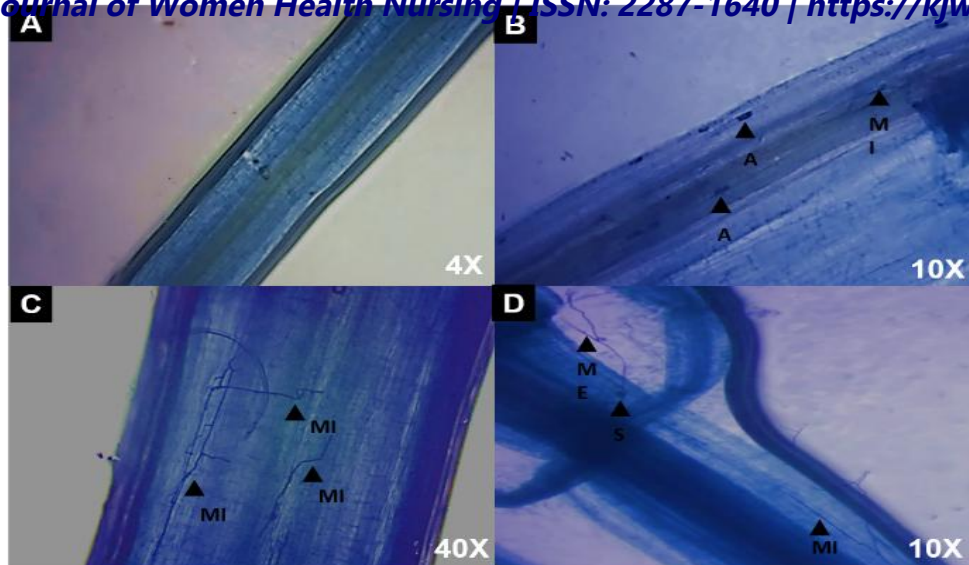


Figure 3. Graph of intervals between fresh weight and dry weight as a function of the different treatments. Different letters on the graph represent specific statistical differences for the multiple comparison test using Fisher's LSD method and 95% confidence. NC (untreated seeds); PC (seeds inoculated in the soil); SN (seeds coated with PEO nanofibers); SNM (Seeds coated with mycorrhizal inoculant immobilized in PEO nanofibers)

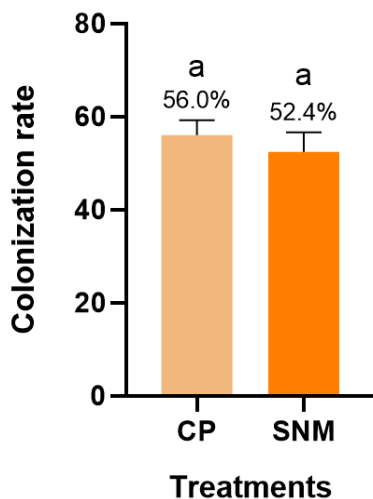


**Figure 4.** Micrographs of the stained roots under a compound light microscope. (A) Non-mycorrhizal root region. (B) Root region with presence of multiple arbusculos (A) and internal mycelium (MI). (C) Root region with branched internal mycelium (MI). (D) Root region with presence of external mycelium (ME), spores (S) and internal mycelium (MI).

Figure 5 shows an interval graph for the mycorrhizal colonization rate as a function of the inoculated treatments.

The analysis of mycorrhizal infectivity did not reveal significant differences in the colonization rate between the traditional inoculation method and the inoculation by coating the seeds with nanofibers that have the AMF inoculant incorporated, even though the dose of inoculum per seed was 97% less than conventional inoculation strategy.

These observations suggest that PEO nanofibers were able to dissolve early, allowing the release of the inoculant without presenting deleterious effects on the mycorrhizal symbiosis. It also shows that even by significantly reducing the amount of inoculant, AMF can colonize the germinating seed by direct contact as described by Adholeya and colleagues<sup>44</sup>.



**Figure 5.** Interval graph of the colonization rate as a function of the inoculated treatments. Different letters on the graph represent significant statistical differences for the multiple comparisons test using Fisher's LSD method and 95% confidence. PC (seeds inoculated in the soil); SNM (seeds coated with mycorrhizal inoculant immobilized in PEO nanofibers).

## CONCLUSION

The centrifugal spinning technique proved to be an effective technique for the elaboration of continuous and uniform nanofibers of polyethylene oxide, which allowed obtaining fibrous mat for the disposition of the mycorrhizal inoculant. Furthermore, nanofibers evidenced to be effective for the immobilization of the inoculant between sheets of fibers and on their surface.

PEO nanofibers demonstrated to be effective for AMF inoculant since it did not affect either the infective capacity or the beneficial properties. Contrary to the non-inoculated treatments, the bean plants inoculated showed an increase in flower development and a greater amount of fresh and dry matter. In addition, both inoculate strategy showed similar colonization rate, despite the amount of spores in seed coated with nanofibers was significantly lower than the traditional inoculation strategy.

Considering that for commercial mycorrhizal inoculants, producers recommend to inoculate 250-300 seedlings with 1 kilogram<sup>45</sup>; according to the results obtained, the amount of inoculated plants could increase up to 4000% per kilogram using seeds coated with inoculated nanofibers, which would represent a significant increase in the yield of the mycorrhizal inoculant.

To our knowledge, this study is the first report where the application of AMF using nanofibers as a delivery system is studied and the results indicate that it could represent a promising approach for plant inoculation, reducing the amount of inoculant needed and facilitating inoculation in large-scale.

In addition, in our knowledge, this study is also the first report where the coating of seeds with biohybrid nanofibers produced without starting from the mixture between the polymeric material and the active biological ingredient, a fact that is of interest to those biological systems that may present a significant reduction in their viability cell if they undergo the spinning process.

Therefore, this study demonstrate that the nanofibers could represent a promising platform as a coating material in seed treatment and as efficient microbiological inoculants delivery system, although the development of technologies for its application on an industrial scale is still necessary.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

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### Notes

The authors declare no competing financial interest.

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