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Comparison of the Effects of Arbuscular Mycorrhizal Fungal (AMF) Spore Inoculation on The Growth of Mexican Marigold (*Tagetes erecta* L.) Plants Grown with Two Different Techniques

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ABSTRACT: *In recent years, the importance and utilization of plant-root-fungal mutualistic symbiosis in crop production have been increasing. In this system, arbuscular mycorrhizal fungi (AMF) spores are mostly used as the production material. The abundant production of healthy spores is an influential factor in the widespread use of this microbial fertilizer. Various cultivation and inoculation techniques have been developed for AMF spore production. Soil and substrate-based production techniques are widely used for the large-scale production of AMF spores. However, beyond the commercial scale, it is important to obtain reliable results in plant nutrition and growth by AMF spore inoculation without contamination for scientific studies. Therefore, in this study, the growth status of Mexican marigold (Tagetes erecta L.) grown using the Nutrient Film Technique (NFT) and the Hydroponic Technique (HT) by AMF spore inoculation was monitored. For this purpose, a pure mixture of AMF spores (Rhizopaghus irregularis) and a control treatment were applied to the media (500 spores were given to perlite media as plant inoculation material for both systems). Some yield parameters and nutrient contents were determined. At the end of the 3-month study, the differences between the yield and nutrient values of the treatments were found statistically significant ($p<0.05$). The highest plant height, fresh and dry plant weight, root length, and fresh root weight values (12.93cm-3.65g-1.87g-7.32cm-1.18g, respectively) were obtained in the plants inoculated with mix spores in the HT system. The highest values of some macro and micronutrients in the plants were generally obtained from the plants inoculated in the HT system (R. irregularis and mix mycorrhizal spores). The lowest values of the measured parameters were generally obtained from the control treatments in both systems, but the highest values of B, Zn, and Mn (469.44-371.42-1501.05 mg kg-1) were obtained in the non-inoculated plants (NFT system).*

KEYWORDS -*AMF Spores, Substrate, Hydroponic System, Nutrient Film Technique, Tagetes erecta* L.

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I. INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are soil fungi that have formed symbioses with many plant families and are distributed in many regions of the world. Their importance is widely recognized in natural and semi-natural ecosystems. This symbiosis has been shown to be important in terms of increased plant resistance to abiotic and biotic stresses [1]. AMF spore inoculations, originally used as phosphate solvents, have partially reduced the use of agrochemicals for environmentalist concerns. Moreover, since mycorrhizal life increases crop health and yield, its importance has increased in agriculture, horticulture, and forestry [2]. In some countries, AMF spores are even used in large areas such as golf-football fields and parks-landscapes, which are open to the public but are operated by private users. Due to this wide range of uses, many methods have been developed for the large-scale utilization of AMF. Many research centers are working on producing AMF spores using different methods because their production is necessary for both scientific and agricultural applications. Indeed, AMF spores can only be produced in very costly production systems in the presence of a suitable host, even if all growth conditions are met, indicating that there is certain sine qua non for AMF spore production. Considering adaptation to environmental conditions in mycorrhizal inoculations, using "multiple" or "diverse" species is important. Furthermore, to achieve the best results in the shortest time, it is necessary to use many spores as a starting material to colonize the largest root volume possible [3].

Similarly, the growth rate of the root must be high for mycorrhizal spore propagation in NFT and HT systems. For this purpose, using multiple types and high numbers of AMF spores is necessary because using a single species and a low number of AMF spores delays the proliferation of healthy spores. When multiple spore varieties and large numbers of spores are used, it is not easy to keep track of which spore type performs better and reproduces more effectively [4].

Consequently, it becomes difficult to distinguish their effects between plant roots and AMF spores. Additionally, as with other microorganisms, the quality control of AMF spore production is another problem. For this reason, the risks associated with the worldwide spread of numerous commercial AMF spores need to be identified, elaborating on the problems that can arise from the introduction of exotic materials with origins in tropical climates. Furthermore, unwanted microorganisms may be present during the inoculation of AMF spores. Therefore, studies on the production of AMF in plants under in vitro conditions have been proposed [5, 6]. For example, Declerck et al. and Voets et al. [7, 8] produced large amounts of propagules by pre-inoculating a single spore in the roots of a suitable host plant followed by transferring the plants to hydroponic and NFT systems.

The obligate biotrophic nature of AMF has made it difficult to produce low-cost, high-quality inoculums with large-scale production methods. This is one of the reasons why the commercial use of AMF spores is still under development [9]. The inconsistent performance of AMF spores in production systems depends on the plant, soil, climate, farming systems, and various other factors, and a lack of competent users is a further reason why this microbial fertilizer is still in the development phase [10].

This study was conducted using single and multiple AMF spore cultures to compare NFT and HT plant production systems, which are not yet widely used for AMF inoculum production, in terms of increasing spore numbers. It is hoped that the results of this study will contribute to the production of microbial fertilizers, firstly in the laboratory and for scientific studies and later on a commercial scale.

II. METHODOLOGY

2.1 AMF spores

The first spore material was prepared in the project titled "Molecular Identification of Arbuscular Mycorrhizal Fungi Spores Isolated from the Rhizosphere of *Puccinellia distans* (L.) Parl. in Salt Lake (Konya/Türkiye) Conditions and Use of Spores as Microbial Fertilizer" completed by the Scientific and Technological Research Council of Turkey (TUBITAK). These spores were identified by DNA extraction and using the Nested PCR method [11, 12]. According to the identification results, the spore species was *Rhizopaghus irregularis* (syn. *Glomus intraradices*). Other spores used in this study were the spores belonging to the "Determination of Soil Quality Indices of Three Important and Common Soil Series in Çumra Plain (Konya/Türkiye) " project, which was also completed as a TUBITAK project. Healthy ones among these spores were selected and used as the mycorrhizal material in this study. Since these spores have not been identified yet, they are referred to as "mix" in the study.

2.2 Substrate media

Many of the substrates used as soilless media in mycorrhizal inoculation studies (e.g., perlite, vermiculite, tuff, pumice) can be sterilized and used for mycorrhizal spore inoculation in plant roots [13]. This method is known as spore propagation in soilless media. This study used fine perlite (0-5 cm) as the substrate.

2.3 Host plant

The Mexican marigold (*Tagetes erecta* L.) was used due to its favorable characteristics, including a short vegetation period, adequate root system development, good colonization with different AMF spore varieties, and tolerance to low phosphorus levels (P). The *Tagetes erecta* L. seeds used in the study were obtained from the market under the trademark Miracle.

2.4 Pot

A clear plastic pot with the dimensions of 36*27*8 cm was used in the study. 18 g of perlite was placed inside the pot.

2.5 Nutrient solutions

Since a phosphorus concentration in the range of 1-50 µM in natural soil solutions is sufficient for the growth of AMF spores in substrate-free cultivations, a modified Hoagland solution containing 50 µM phosphorus [14, 15, 16] was used for the study.

2.6 NFT and HT systems

Mexican marigold plants inoculated with AMF spores and grown in quartz sand were transferred to NFT and HT systems for the continued growth and proliferation of AMF spores in the roots. NFT is a system in which a nutrient solution flows through sloping channels [17] to reach plant roots for 15 min every 4 h (about six times a day). HT, which refers to the cultivation of plants in a static nutrient solution, is a system that covers the plant roots, allowing them to grow rapidly and thus increasing the surface area in a short time [18, 19]. After the fifth week of applying the AMF spores (Figure 1) to Mexican marigold plant seeds in the potting medium containing perlite, the plants were transferred to NFT and HT growth media (Figure 2). In the NFT and HT growth media, the plants were grown with a modified Hoagland solution (Figure 3). After two months, the plants were harvested, and their root-top fresh weight, root-top dry weight, plant height, root length, nutrient contents, and mycorrhizal infection statuses were determined.

As for the stages of the study: Mexican marigold seeds were sown in pots containing perlite. *Rhizopaghus irregularis* and mixed mycorrhizal spores were placed just below the seeds. The plants were left to grow (Figure 1). The same protocol was followed without inoculation as a control.

Figure 1. *Tagetes erecta* L. seeds and germination in perlite.

Approximately five weeks later, some of the roots of each plant were removed, and mycorrhizal infection rates were determined according to the method reported by Koske and Gemma [20]. The plants with root infection rates of 50% or above were removed from the medium and transferred to NFT and HT media (Figs. 2 and 3). The same treatment was performed for the control plants.

Figure 2. *Tagetes erecta* L. seedlings transferred to NFT and HT systems in the fifth week of growth.

Figure 3. Nutrient film technique (NFT) and hydroponic system (HT).

After about two months, the plants were harvested, and their root and above-root fresh weight (g), above-root dry weight (g), plant height (cm), root length (cm), as well as P, K, Ca, Mg, Na, B, Fe, Cu, Zn, and Mn contents and AMF infection statuses were determined.

Fresh and dry weights were measured on a precision balance (±0.1 g). The dry weights of the above-root parts of the plants were placed in paper bags and kept in an oven at 70°C until they reached constant weight, and dry weights were measured on a precision balance (±0.1 g). Plant heights were determined using a ruler, by measuring the length between the crown and the extreme point of the plant. Plant root length was determined by measuring the lengths of three randomly selected roots.

Roots of three plants from each medium were collected, cleared, and softened with KOH (10%) and HCl (25%) and then stained with Trypan blue (0.05%) [21], by converting lactophenol to lactoglycerol. The dark-pigmented roots were naturally cleared with 10% H_2O_2 for one hour before staining with Trypan blue. The percentage of root colonization was calculated using the gridline intersect method, and when the number of roots was small, using the slide method [22].

After harvesting, 0.3 g of the plant samples was dried in an oven at 65-70°C and dissolved in 5 mL of 65% HNO_3 and 2 mL of 35% H_2O_2 in a microwave device (CEMMARSXpress), and then, the volumes were completed to 25 mL with ultra deionised water. The samples were filtered through blue-banded filter paper. The concentrations of P, K, Ca, Mg, S, Fe, Zn, Mn, Cu, B, P, K, Ca, Mg, S, Fe, Zn, Mn, Cu, and B in the filtrates were determined by ICP-OES (Varian, Vista).

The results obtained in the experiment were subjected to analysis of variance according to the randomized block design. The mean values of the treatments, whose differences were determined by the F-test, were grouped based on the LSD test ($p<0.05$). The deviations between the treatments were determined using the MINITAB Ver. 19.0 program.

III. RESULTS AND DISCUSSION

The results of Duncan's test applied to the values of the measurements and analyses performed on the plant samples are given in Tables 1 and 2. The effects of the systems and mycorrhiza treatments on plant height, plant fresh-dry weight, root length, and root fresh weight values were found significant ($p<0.05$) compared to the control (Tables1 and 2). The highest values of plant height, fresh and dry weight, root length, and root fresh weight (12.93 cm-3.65g-1.87g-7.32cm-1.18g, respectively) were obtained in the plants inoculated with mix spores in the HT system. The lowest values of plant height, fresh and dry weight, root length, and root fresh weight (7.12 cm-0.80g-0.21g-4.21cm-0.40g, respectively) were obtained in the control group of the HT system.

Table 1. Effects of different growth media and AMF spore inoculation methods on some growth parameters of Mexican marigold (*Tagetes erecta* L.).

Systems x application	Plant height (cm)	Plant wet weight (g)	Plant dry weight (g)	Root length (cm)	Root wet weight (g)
NFTxControl	7.78B*	2.24BC	0.87AB	6.07AB	0.72AB
NFTx*Rhizopaghus irregularis*	9.95AB	2,14BC	0.78AB	6.13AB	0.72AB
NFTx mix	10.78AB	1.55B	0.78AB	6.18AB	0.73AB
HTxControl	7.12B	0.80C	0.21B	4.21B	0.40B
HTx*Rhizopaghus irregularis*	10.00AB	2.47BC	0.74AB	6.88AB	0.78AB

HTxmix	12.93A	3.65A	1.87A	7.32A	1.18A

***: The difference between numbers with different letters is statistically significant (p<0.05)**

In some macro and micronutrient analyses in the plant, the difference between the treatments was found statistically significant (p<0.05) (Table 2). The highest concentration values of macro elements (P, K, Ca, and Mg) were obtained in the mix spore inoculation treatments in the NFT system (0.60, 5.34, 2.11, and 0.64%, respectively). Additionally, the highest concentration values of Ca and Mg were obtained in the *R. irregularis* inoculation treatments in the NFT system (2.13 and 0.70%, respectively). The highest Ca concentrations were obtained in both the *R. irregularis* (1.97%) and mix spore inoculation (1.97%) methods in the HT system (1.94%).

The highest values for micronutrients B, Fe, Cu, Zn, and Mn (469.44, 222.29, 182.64, 371.42, 371.42, and 1501.05 mg kg^{-1}, respectively) were obtained in the control plants in the NFT system. In contrast, the lowest values (68.31, 62.99, 205.69 and 534.45 mg kg^{-1}, respectively) for Fe, Cu, Zn, and Mn were obtained in the *R. irregularis* inoculated plants in the NFT system, while the lowest value for B (261.42 mg kg^{-1}) was obtained in the mix spore inoculated treatment in the HT system.

Table 2. Effects of different growth media and AMF spore inoculation methods on macronutrient (%) and micronutrient (mg kg^{-1}) contents of Mexican marigold (*Tagetes erecta* L.) plants.

	Macronutrients (%)			
Systems x application	**P**	**K**	**Ca**	**Mg**
NFTxControl	0.52AB	4.87AB	1.23B	0.57ABC
NFTx*R.irregularis*	0.50AB	4.90AB	2.13A	0.70A
NFTx mix	0.60A	5.34A	2.11A	0.64A
HTxControl	0.36C	3.36C	1.43B	0.42C
HTx*R.irregularis*	0.41BC	4.81AB	1.94A	0.58AB
HTxmix	0.44BC	4.38B	1.97A	0.43BC

	Micronutrients (mg kg^{1})				
Systems x application	**B**	**Fe**	**Cu**	**Zn**	**Mn**
NFTxControl	469.44A	229A	182.64A	371.42A	1501.05A
NFTx*R.irregularis*	452.41AB	68.31C	62.99C	205.69C	534.45C
NFTx mix	355.24BC	158.67AB	116.74B	249.30BC	977.38BC
HTxControl	446.93AB	87.16B	74.82BC	246.52BC	887.32BC
HTx*R.irregularis*	380.88AB	181.13AB	167.93AB	243.17BC	815.54BC
HTxmix	261.42C	137.94AB	105.02BC	341.23AB	1007.71B

***: The difference between numbers with different letters is statistically significant (p<0.05)**

As seen in the results, mycorrhizal spore inoculation caused an increase in plant productivity parameters, especially in the HT system. In fact, NFT and HT are not natural media for the survival and reproduction of AMF spores infecting plant roots. A soilless media can be a source of abiotic stress for both plants and AMF spores infecting plant roots. The conditions may limit the growth of plant root development, and this limitation may have the same effect on the development of AMF spores in the root. However, AMF spores proliferated in this study. This indicated that AMF spores proliferate more under stress conditions [1].

In the interactions of AMF spores with plants, there may be some changes in plant form in response to abiotic stress. Although the plants were grown in the same nutrient solution in the experiment, their macro and micronutrient contents were different due to the usage of different AMF spore species. The highest values in the macronutrient contents of the plants were obtained in the mix spore inoculation in the NFT system, which indicated that AMF spores may have mechanisms to adapt to abiotic stress independently of their host plants [23, 24, 25]. Moreover, the fact that the highest concentration values of micronutrients were obtained in the control treatments in the NFT system may be because the exclusion mechanism of AMF spores may have been disrupted. Toxicity caused by heavy metals (e.g., Cu, Pb, Co, Cd, and Zn) in plants can be reduced by AMF through hyphal 'metal binding,' which reduces the bioavailability of elements. Additionally, although the nutrient solutions used in this study were the same, the differences in plant growth and nutrient element contents may have varied depending on the techniques of the growing systems [26].

There are two hypotheses that predict how AMF spores will respond to abiotic stress [27]. The first is the stress exclusion hypothesis, which predicts that the number and diversity of AMF spores will decrease with abiotic stress. On the other hand, the stress adaptation hypothesis predicts that AMF spores will grow more in response to abiotic stress to maintain their activity. It is believed that the plants in the experiment in this study may have shown an adaptation mechanism to stress. However, detailed physiological and molecular studies should be carried out to understand the behaviors of plants and AMF spores against stress [28, 29, 30].

After harvesting the experimental plants, the roots were stained with Trypan blue, and their mycorrhizal infection status was examined. Notably, the infection rate was 100% in all plant roots, but pure *R. irregularis* and mix spores showed different densities in different systems. In an NFT system, oxygen is supplied from the air at the spray intervals, while in hydroponics, oxygen in the water is supplied by a submerged pump. This difference was effective in the proliferation of AMF spores. Pure *R. irregularis* spores were denser in the NFT

system (Figure 4), whereas mix spores were less dense (Figure 5). In other words, impure mix spores preferred the oxygen in the water and multiplied more in the hydroponic system (Figure 6), while pure *R. irregularis* spores multiplied less intensively in the same system (Figure 7).

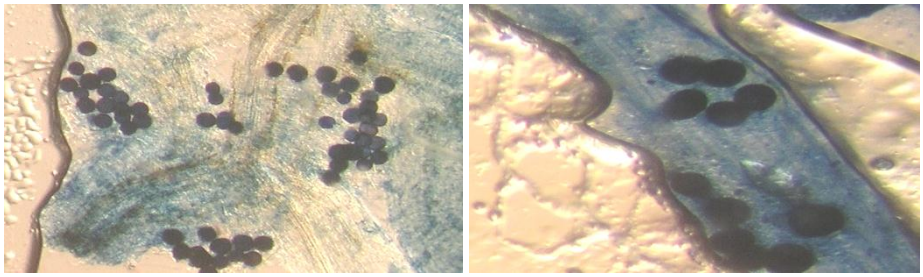

Figure 4. *R. irregularis* spores on roots of Mexican marigold (*Tagetes erecta* L.) grown in the NFT system.

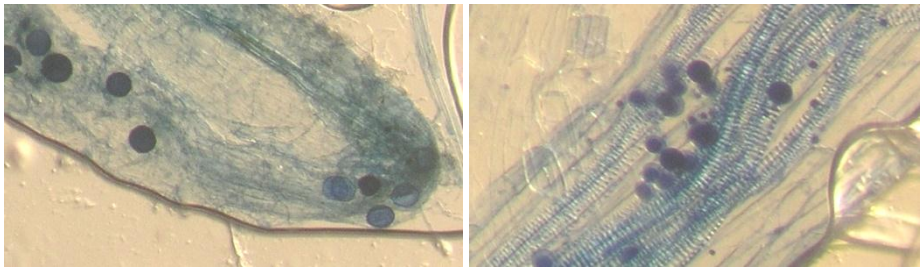

Figure 5. Mix spores on roots of Mexican marigold (*Tagetes erecta* L.) grown in the NFT system.

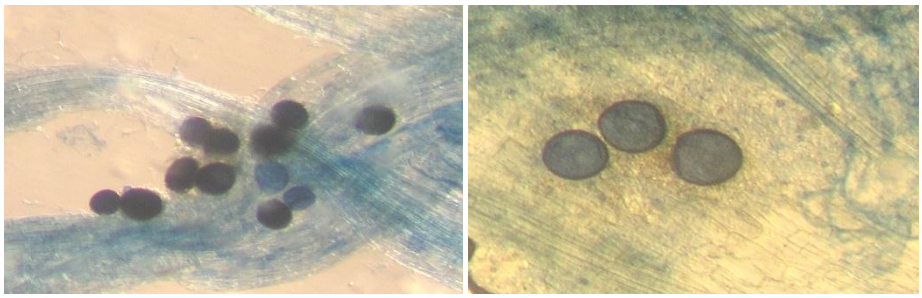

Figure 6. *R. irregularis* spores on roots of Mexican marigold (*Tagetes erecta* L.) grown in the HT system.

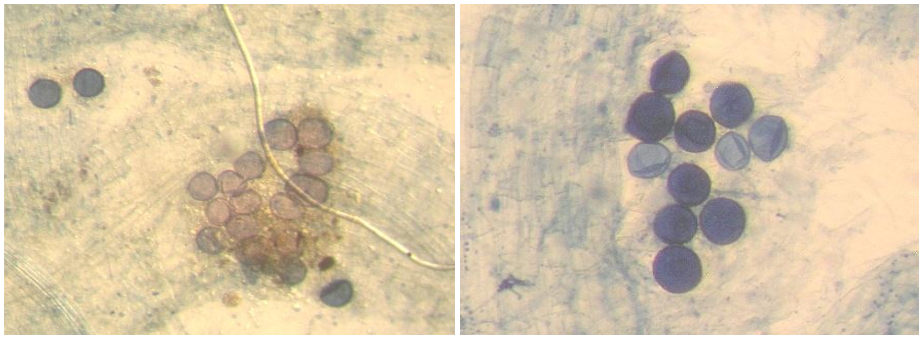

Figure 7. Mixed spores on roots of Mexican marigold (*Tagetes erecta* L.) growing in the HT system.

Since AMF formation is normally a symbiotic relationship between terrestrial plants and spores, both techniques are suitable for the propagation of AMF spores and for studies on plant nutrient content and yield characteristics [31]. Indeed, in a study conducted by Tajini et al. [32] in a hydro aeroponic system, it was observed that *R. intraradices* (basionym *G.intraradices*) [33] successfully multiplied in rhizobium-inoculated bean roots, while *Glomus rosea* did not grow in the same system. The researchers reported that the growth and spread of AMF spores were successful in solution systems where sufficient O_2 was provided, while for those that were not successful, the reason was not clearly understood.

Both the NFT and HT systems used for spore production by mycorrhizal plants provided a controlled flow of nutrients while ensuring that the plant roots were harvested free of soil or any substrate.

Arbuscular fungi, characterized by arbuscules consisting of branched hyphae in cortical cells, normally

extend outward from the root surface, thus extending the reach of the system for nutrient uptake, or in short, its surface area. Therefore, the outer hyphae in the root of a plant infected with AMF spores contribute to plant growth by facilitating access to nutrients [34, 35]. However, in the NFT and HP systems used in this study, the root systems developed weaker than normal because the nutrient solution reached the root surface of the plant.

Similar to substrate-based production systems, the combination of the host plant and the mycorrhizal fungal spores determines the optimal nutrient ratio for AMF spores and plants. However, in hydroponic systems, AMF spores may not be required for plant growth, as the nutrients in the nutrient solution used are directly available to the plant [9, 36). Therefore, the importance of AMF spores in plant growth may not be clear. This clarity can be more accurately determined in stress environments under field conditions.

IV. CONCLUSION

Different solution systems used in the study gave results for a specific purpose in a short time. According to the results that were obtained, the HT system was effective in the infection of the plant with the AMF and the healthy growth of the plant. The prominent spore in the HT system was the mix spore. On the other hand, the media in which the nutrient content of the plant and spores increased more was the NFT system. Mix spores were also effective in this media.

Since no species determination was made, it is not known which AMF spore species increased or decreased more in the mix spores. However, the fact that the highest values in plant growth and nutrient contents were obtained in the mix spore treatments was attributed to the presence of more spore species in mix spores increasing the chance of adaptation in plant development. Therefore, it should be taken into consideration that the agricultural activities carried out during plant production should be selected to involve methods that will not adversely affect the number and variety of natural AMF spores in soils.

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