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EFFECT OF INOCULATION WITH ARBUSCULAR MYCORRHIZAL FUNGI AND LABELED NITROGEN FERTILIZER ON ROOT COLONIZATION AND SPORE DENSITY OF SOME MEDICINAL PLANTS

Dina I.M. Abd El-Kader[1*], S.H. Salem[2], Fatma I. El-Zamik[2], Howaida M.L. Abd El-Basit[2], M.A. Rizk[1] and O.A. Abd El-Aziz [1]

1. Soil and Water Res. Dept., Nuc. Res. Cen., Atomic Energy Authority, Inshas, Egypt

2. Agric. Microbiol. Dept., Fac. Agric., Zagazig Univ., Egypt

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ABSTRACT: The present study was carried out to study the effect of inoculation with arbuscular mycorrhizal fungi (AMF) and some beneficial microbes under labeled nitrogen (^{15}N) fertilization on AMF root colonization and spore density of some medicinal plants. The occurrences of AMF diversity have been studied in rhizosphere soils of 22 medicinal plants in Sekem Company Farm, and the percentage of mycorrhizal colonization on each plant and the number of spores per 100g rhizosphere soil was calculated. AMF was observed in almost all plants. There were great variations in AMF colonization based on host plant. The highest root infection was observed in black cumin plant, being 77% and the lowest was in parsley plant, being 18.17%. The highest spore population (368/100g rhizosphere soil) was recorded in anise plant and the lowest was observed in lemon grass plant (103/100g rhizosphere soil). The effects of AMF and microbial inoculation under labeled nitrogen fertilizer on root colonization of black cumin and anise plants after 30, 60 and 120 days of cultivation and the rhizosphere spore density at the end of the experiment have been studied. The maximum AMF root colonization in black cumin plant was 83.67% after 120 days in the composite AMF, *Azotobacter chroococcum* and *Bacillus megatherium* in the presence of half nitrogen (^{15}N) fertilizer dose, compared with the maximum AMF root colonization in anise plant that reached 76.67% after 120 days in the same treatment and the same nitrogen dose and this may be due to the difference between root exudates of two plants. The highest spore density in black cumin plant was 377/100g soil with the composite treatment in the presence of half nitrogen (^{15}N) fertilizer dose, while in anise plant it was 385/100g soil in the dual inoculation of AMF and *Bacillus megatherium* using half nitrogen dose.

Key words: Arbuscular mycorrhizal fungi, medicinal plants, mycorrhizal colonization, spore density, black cumin, anise.

INTRODUCTION

Mycorrhiza are a symbiotic relationship between the fungus and plant roots (Frank, 1885). AMF are associated with almost all plants in nature (Hayman, 1982) and are ubiquitous. Symbiosis of Mycorrhizae is one of the best known symbiotic systems in plants which are observable in most ecosystems and most of plants have at least one type of Mycorrhiza (Ardakani *et al.*, 2000). Almost all land plants including cereals, legumes, millets, plantations and horticultural crops ornamental and medicinal plants and forest trees are reported to be host of AMF (Chandra and Kehri, 2006).

The mycorrhizal symbiosis between fungi and plant behaves as a three phase heterogeneous interaction where all components, the soil, the plant and the fungus are in contact. Each component affects mycorrhizae in different ways. But the main drivers of symbiosis are the

*** Corresponding author: Tel. :** +201062600273
E-mail address: dinaibrahim73@yahoo.com

degree of dependence of the plant on the AMF, the fungus efficiency to establish symbiosis and the phosphorus (P) availability in the soil. Also, it was found that mycorrhizae increase nutrients absorption distance around roots which lead to increasing of root colonization and increasing of nutrition uptake (Smith and Read, 2008).

AMF symbiotic association plays a major role in nutrient acquisition (Mosse, 1981). AMF play a key role in soil fertility and plant nutrition. They enhance the uptake and translocation of mineral nutrients – mainly P, N, S, K, Ca, Fe, Cu and Zn from soil of host plants (Giovannetti and Avio, 2002; Smith and Read, 2008). AMF are known to provide phosphorus at very low concentration to the host plants. Also, AMF increase the effective absorbing surface of the host root by as much as ten times (Mehrvarz *et al.,* 2008; Soleimanzadeh, 2010; Alizadeh, 2012).

Mycorrhizae is a multi-functional fungus in agricultural ecosystems which enhances the soil physical quality (with improvement of hyphae), chemical quality (enhanced absorbing nutrients) and biological quality (with development of nutritional channels) (Cardoso and Kuyper, 2006). Among the biotic factors, the inoculation with AMF can influence the production of active ingredients in medicinal and aromatic plants (Kapoor *et al*., 2002; Karagiannidis *et al*., 2011).

Therefore, the present work was aimed to investigate the effect of inoculation of AMF and some beneficial microbes on root colonization of some medicinal plants and spore density in the rhizosphere of these plants under different levels of labeled nitrogen fertilization (^{15}N) in sandy loam soil.

MATERIALS AND METHODS

Occurrence of Amf in the Rhizosphere Samples of Medicinal Plants

Samples collection of plants with roots and soil rhizosphere

Soil samples were collected from the rhizosphere of 22 medicinal plants at Sekem Company Farm, Belbase, Sharkia District, Egypt to study the variations of AMF infection in these plants. Samples were air dried and then preserved in clean plastic bags. Pebbles and other unwanted matters were removed from the spread samples. Large lumps were broken with wooden roller or hand. After grinding soil samples were sieved and fine soils were stored in clean plastic poly propylene bags with labeling tag for mycorrhizal fungal spore estimation and soil physical, chemical, and microbiological analyses at room temperature.

Isolation of AMF by wet-sieving and decanting technique

Quantification and separation of AMF spores from soil sample each medicinal plant rhizosphere was done by using wet sieving and decanting method (Gerdemann and Nicolson, 1963). This technique was used for sieving the coarse particles of the soil and retaining AMF spores and organic particles on sieves of different sizes. For isolation of the spores, 100g of soil were added to 1000 ml of water in a large beaker, then stirred well on magnetic stirred and settled until all the aggregates dispersed to leave a uniform suspension. The suspension was passed through 710µm sieves decanted through 425 µm, 250 µm, 150 µm, 125 µm, 75 µm, 63 µm and 45 µm sieves, consecutively. The contents on the sieves were transferred to clean beaker, suspended in water and adjusted to a known volume 100 ml. One ml was taken from the suspension with Pasteur pipette and transferred into Petri-dish containing a Millipore filter to count AMF spores under a dissecting microscope10-40x. The number of spores was expressed as spores/100g of rhizosphere soil sample.

Identification of AMF spore

AMF spores were identified according to the morphological characteristics described by Gerdemann and Trappe (1974) and Trappe (1982). For identification and nomenclature INVAM's19 World Wide Web site at http:// invam.caf.edu/methods/mycorrhizae was used.

Estimation of root colonization

The root samples were cleaned and stained with trypan blue according to modified method of the Phillips and Hayman (1970) as follows: Roots were carefully washed to remove soil and other particles in running tap water and cut into small (1 cm) pieces. Roots were immersed in 10% KOH solution and heated for 10 min at 80-

90°C in water bath, Then, root pieces were washed several times with distilled water and treated with 1% HCl for 3-4 min. Root pieces were stained with 0.05% trypan blue and left in water bath for 5-10 min at 80-90°C, then placed on clean glass slide with few drops of glycerol. Slides were observed under the microscope to score for any structures associated with mycorrhizal fungi, like hyphae, vesicles or arbuscles in each segment. The percentage of AM fungal colonization was assessed by using the following formula:

$$\text{Percentage of infection} = \frac{\text{No. of root segments infected}}{\text{Total No. of root segments observed}} \times 100$$

AM fungal colonization was visualized in the root tissues of each plant species using frequency distribution method proposed by Biermann and Linderman (1981).

Effect of Inoculation with AMF and some Beneficial Microbes on Root Colonization and Spore Density of Black Cumin and Anise Plants under Different Labeled Nitrogen (^{15}N) Fertilization Doses

Pot experiments were set up from November 2015 to April 2016 to evaluate plant mycorrhization either solely or in combination with a symbiotic N_2 fixer and phosphate dissolving bacteria affecting root colonization and spore density of black cumin and anise plants.

Plastic pots with 30.0 cm height, 30.0 cm diameter were used for cultivation of the above mentioned plants using sandy loam soil. The unsterile soil and mycorrhizal spore density was (77/100g of soil). Each pot was filled up to 8 kg of air-dried soil. The plants were irrigated every two days with 250ml tap water.

Preparation of inocula

Mycorrhizal inoculants

Soil samples containing AMF spores extracted (as previously described) from the rhizosphere of the medicinal plants grown on sandy loam soil of Sekem Company Farm were thoroughly mixed. Soil contained AMF (10 spore/g) was used as standard inoculum in all experiment. The mycorrhizal inoculum was added at rate of 100 g per pot. The major population was 80% *Glomus* spp., 15% *Gigospora* sp., and few from *Aculospora* sp. and *Sctellospora* sp.

Bacterial inoculants

Phosphate dissolving bacteria *(Bacillus megatherium var phosphaticum*) and, a symbiotic N_2 fixers (*Azotobacter chroococcum)* peat based inoculants were provided by Agricultural Microbiology Department, Soil, Water and Environment Research Institute, Agriculture Research Center (ARC), Ministry of Agriculture and Land Reclamation, Giza, Egypt.

Medicinal plants (host genotype)

Seeds of two medicinal and aromatic plants were kindly provided by Sekem company:-

a- Anise (*Pimpinella anisum* L.) belonging to Apiaceae family.

b- Black cumin (*Nigella sativa* L.), plant belonging to Ranunculaceae family.

Soil

Unsterile sandy loam soil was used in this investigation. The soil was collected from the field of Soil and Water Research Department, Nuclear Research Center, Egyptian Atomic Energy Authority, Inshas, Egypt. Physical and chemical characteristics of the experimental soil sample are presented in Table 1. Soil analysis was carried out according to Estefen *et al*. (2013).

Fertilizers

Potassium and phosphorus fertilizers were added at recommended doses in the experiment before planting. Nitrogen fertilizer was applied at recommended dose rates of 400 kg fad.$^{-1}$ ammonium sulfate for black cumin and 200 kg fad.$^{-1}$ ammonium sulfate for anise. It was added after one month from cultivation on three rates zero, 50 and 100% (N_0, N_1 and N_2, respectively) recommended doses. Nitrogen fertilizer was applied in the labeled ammonium sulfate $(^{15}NH_4)_2SO_4$ form with enrichment 2% ^{15}N atom excess (Table 2).

The experiments for nitrogen fertilization (^{15}N) were arranged in randomized complete block design with three replicates and 6 plants pot^{-1} for each treatment. Plants and rhizosphere

Table 1. Physical and chemical properties of the experimental soil sample

Physical properties					
Sand (%)	**Silt (%)**	**Clay (%)**	**Texture class**		
74.0	21.6	4.4	Sand loam		
Chemical properties					
pH	**$CaCO_3$ (%)**	**E.C (dsm^{-1})**	**O.C. (%)**	**Total N (%)**	**Total P (%)**
7.8	0.75	0.22	0.10	0.0028	0.006
Soluble cations and anions meq l^{-1}					
Ca^{++}	**Mg^{++}**	**Na^{+}**	**K^{+}**	**CO_3^{--}**	**HCO_3^{-}**	**Cl^{-}**	**SO_4^{--}**
8.4	4.6	6.0	2.0	0.0	7.3	4.5	9.2

Table 2. Nitrogen-^{15} fertilization (g pot^{-1}) rates applied to the tested plants: dilution ratio between labeled and ordinary forms

Plant	Ammonium sulfate $(NH_4)_2SO_4$ ($^{15}N/^{14}N$) (g pot^{-1})			
	**50%		**100%	
	^{15}N*	**Ordinary**	**^{15}N***	**Ordinary**
Anise	0.136	0.204	0.268	0.402
Black cumin	0.268	0.403	0.536	0.804

* Labeled ^{15}N (2% ^{15}N a.e.), **from the recommended rate.

soil samples were taken at intervals of 30, 60 and 120 days of planting to determine percentage of mycorrhizal infection on black cumin and anise plants. Mycorrhizal spore density was determined at the end of experiment.

The experimental treatments were:

1. M_0: Control (without microbial inoculation)
2. M_1: Inoculated with arbuscular mycorrhizal fungi (AMF)
3. M_2: *Azotobacter chroococcum*
4. M_3: *Bacillus megatherium*
5. M_4: AMF + *Azotobacter chroococcum*
6. M_5: AMF + *Bacillus megatherium*
7. M_6: AMF + *Azotobacter chroococcum* + *Bacillus megatherium*

RESULTS AND DISCUSSION

Occurrence of AMF in the Rhizosphere of some Medicinal Plants

The distribution of AMF in different medicinal plants in Sekem farm soil has been studied in 22 medicinal plants. It is well known that the occurrence of AMF is affected by the climate, soil type and host plant.

Results in Table 3 indicate that all the medicinal plants studied showed AMF associations. The mycorrhizal association changed among various plant species and there was a significant difference in root colonization. AM Fungal colonization and diversity were noted in rhizosphere soil. Mycorrhizae colonization was indicated by the presence of hyphal networks, arbuscules, vesicles and

Table 3. Occurence of AMF in 22 rhizospere samples of the medicinal plants

Name of the plant species	Family name	Type of infection	Infection percentage	Spore population per 100 g soil
Borage (*Borago officinalis* L.)	Boraginaceae	**HV**	38.67 ±1.20	261±2.03
Cornflower (*Centaurea cyamus* L.)	Asteraceae	**HA**	54.00 ±0.58	192±1.73
Pot marigold (*Clandula officinalis* L.)	Asteraceae	**HAV**	58.67 ±0.88	273± 2.03
Cumin (*Cuminum cyminum* L.)	Apiaceae	**HV**	53.00 ±0.58	199±2.31
Lemon grass (*Cymbopgon citratus* L.)	Poaceae	**HV**	58.33 ±0.88	103±2.61
Milk thistles (*Silybum marianum* L.)	Asteraceae	**HA**	19.00 ±1.16	221±3.18
Fennel (*Foeniculum vulgare* L.)	Apiaceae	**HAV**	70.50 ±0.87	261±1.45
Rock-rose (*Helianthemum vulgar* L.)	Cistaceae	**HA**	45.07 ±0.64	262±2.34
Lavender (*Lavandula angustifolia* L.)	Lamiaceae	**HV**	66.80 ±1.17	220±2.03
Chamomile (*Matricaria chamomilla* L.)	Asteraceae	**HV**	60.00 ±1.16	106±3.18
Lemon balm (*Melissa officinalis* L.)	Lamiaceae	**HAV**	54.77 ±0.91	248±1.77
Peppermint (*Mentha piperita* L.)	Lamiaceae	**HV**	46.20 ±0.99	212±2.34
Spearmint (*Mentha viridis* L.)	Lamiaceae	**HV**	56.00 ±1.16	221±2.34
Black cumin (*Nigella sativa* L.)	Ranunculaceae	**HA**	77.00 ±1.16	269±2.65
Sweet Basil (*Ocimum bacillicum* L.)	Lamiaceae	**HV**	26.13 ±0.70	228±1.73
Marjoram (*Originum majorana* L.)	Lamiaceae	**HV**	45.03 ±0.55	282±1.77
Rose Geranium (*Pelargonium graveolus* L.)	Geraniaceae	**HA**	46.00 ±0.58	122±1.77
Parsley (*Petroselinum crispum* L.)	Apiaceae	**HA**	18.17 ±0.60	338±1.16
Anise *(Pimpinella anisum* L.)	Apiaceae	**HV**	72.00 ±1.16	368±1.16
Rosemary (*Rosmarinus officinalis* L.)	Lamiaceae	**HV**	59.40 ±0.87	310±1.77
Sage (*Salvia officinalis* L.)	Lamiaceae	**HA**	47.17 ±1.17	186±1.73
Thyme (*Thymus vulgaris* L.)	Lamiaceae	**HV**	59.13 ±0.47	201±2.34

Values are mean of three replicates ± SE. H: Hyphae; A: Arbuscules; V: Vesicles

endospores (Fig. 1). The maximum root infection was observed in black cumin being 77% which belongs to the family Ranunculaceae and lowest root infection was recorded as 18.17% in parsley which belongs to the family Apiaceae. The obtained results agree with Warner and Mosse (1980) who found that the AMF associations depends on root morphology, metabolism and rate of plant growth as well as specific soil plant system in term of chemical nature of root exudates.

The present result also showed that all medicinal plants have rhizosphere soil spore density and root colonized. The highest spore population was recorded as 368/100g soil in anise plant, which belongs to the family Apiaceae. The lowest spore population showing 103/100g soil in lemon grass plant which belongs to the family Poaceae. The major population was represented as *Glomus* species followed by *Gigospora, Aculospora*, and *Sctellospora*. The obtained results are in agreement with the findings mentioned by Koul *et al*. (2012) who found that the AMF endophytes are widespread in all the soils investigated but varied in both number and the type of spores and

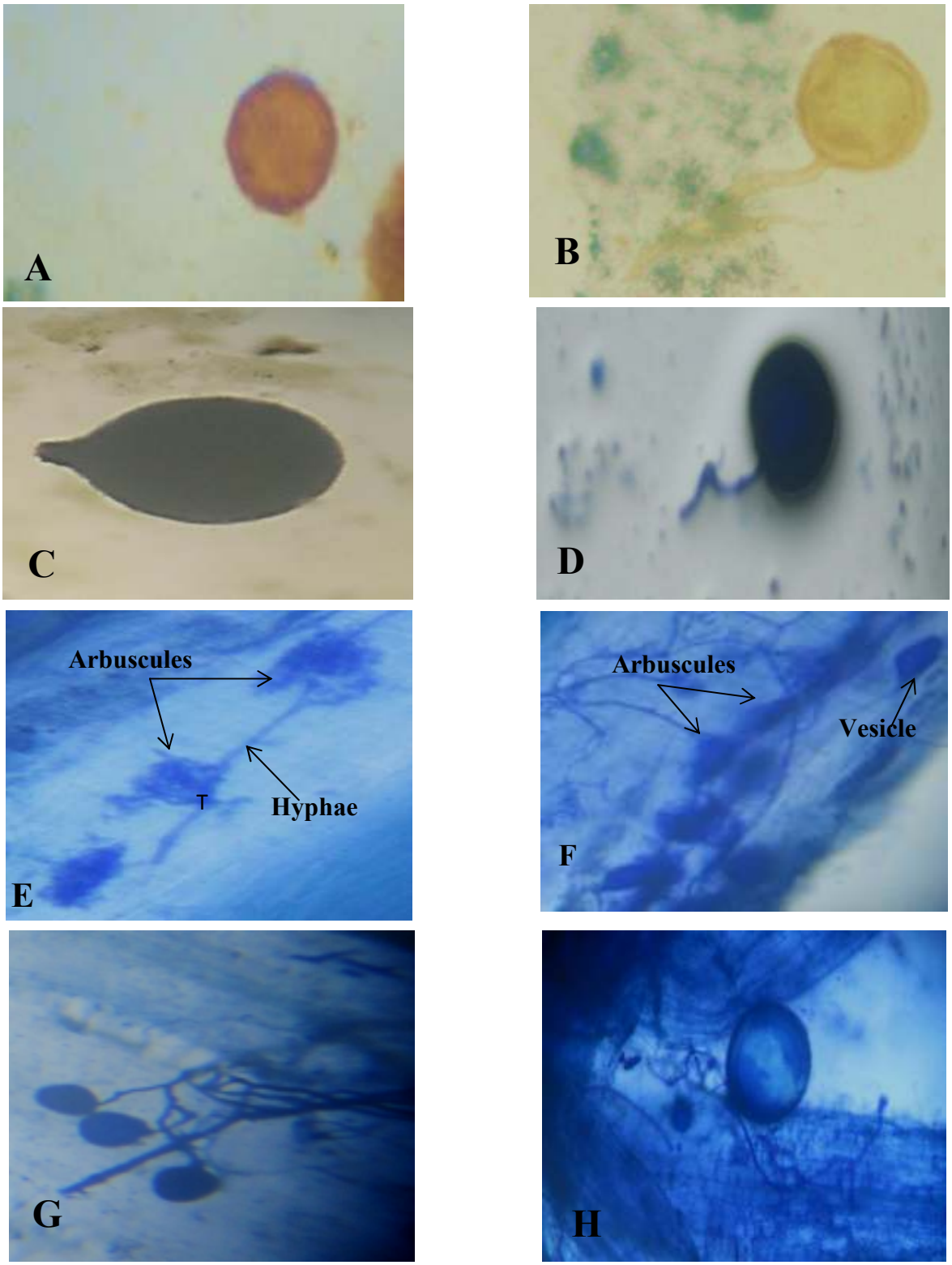

Fig. 1. Light microscopic photograph of AMF extracted from different medicinal plants (40X)

A: *Glomus mosseae* spore B: *Glomus clarum* spore C: *Glomus constrictum* spore
D: *Glomus* spp. with hyphal branching E: Arbuscular and intraradical hyphae
F: Arbuscular, vesicle and intraradical hyphae G & H: Intraradical hyphae and vesicles

sporocarps. They also found that AMF spore count showed no significant or positive correlation with the root colonization percentage. According to Mehrota (2005), AMF in the Fabaceas and Poaceas have great ability to make associations in normal crop conditions. AMF association's records high occurrence also with medicinal and aromatic plants (Urcoviche *et al*., 2014).

Detailed photographic presented in Fig. 1 illustrated the AMF spores and their associations with root system. Table 4 show the difference between *Glomus* sp. isolates. Different varieties of spores were isolated from different medicinal plants in this study. This study showed that biodiversity of arbuscular mycorrhizal fungi differed in different plants. Presence of *Glomus* in different types can lead to an assumption that various species of *Glomus* might have developed a good adaptive mechanism of symbiosis with different host medicinal plants according to Heijden *et al*. (1998).

The obtained results also agree with Sarwade *et al*. (2011) who found that *Glomus* species as major impact on the host plants even under any environmental conditions as presented climatic and chemical factors in their study area. The predominance of various *Glomus* spp. appeared to be general observation reported under curtained ecosystem by others also including medicinal plants (Ram and Bhadauria, 2009; Koul *et al.,* 2012; Muthuraj *et al.,* 2014). *Glomus* indicated high adaptive mechanism for associations with various medicinal plants.

Effect of Inoculation with AMF and some Beneficial Microbes on Root Colonization And Spore Density of Black Cumin and Anise Plants Under Different Labeled Nitrogen (^{15}N) Doses

Mycorrhizal root infection

Black cumin plants

The effect of inoculation with AMF individually or combined with *Azotobacter chroococcum* or *Bacillus megatherium* and mixture of them on Mycorrhizal colonization in *Nigella sativa* roots under different labeled nitrogen doses was evaluated. Mycorrhizae root infection of black cumin was examined after 30, 60 and 120 days of cultivation, as seen in Table 5 and Fig. 2.

Results presented in Table 5 clearly show that AMF root colonization increased significantly with AMF inoculation in all inoculated treatments. The percentage of root colonization after 30 days, in the absence of nitrogen fertilizer ($^{15}N_0$) reached their maximal levels being 42.00 % when inoculated with mixture of AMF, *Az.chroococcum* and *B.megatherium* (M_6) followed by 40.67% for each of M_4 and M_5 treatments. It was also found that inoculated plants with AMF individually recorded 38.33% compared with the control (6%) in the same nitrogen dose. Also, with applying half the recommended nitrogen dose ($^{15}N_1$), the maximum level being 50.33% was obtained in the combination treatment (M_6) followed by 47.33, 44.67 and 43.67% expressed on M_4, M_5 and M_1, respectively compared with the control (8%) in the same ^{15}N-fertilizer dose. In the case of full recommended nitrogen dose ($^{15}N_2$), inoculation with AMF alone or in combination increased AMF root colonization and the maximum was 50% with (M_6) followed by 47.00, 44.67 and 44.67% expressed on M_4, M_5 and M_1, respectively compared with the control (10%) in the same ^{15}N-fertilizer dose. Commonly, the highest infection level being 50.33% at combination (M_6N_1) while control (M_0N_0) showed the lowest infection level being (6.0%).

On the other hand, the percentage of root colonization after 60 days, in the absence of nitrogen fertilizer dose (N_0) reached their maximal levels, being 59.67%, when inoculated with mixture of AMF, *Az.chroococcum* and *B.megatherium* (M_6) followed by 57.33, 49.0 and 48.67% for the M_4, M_1 and M_5 treatments, respectively compared with the un inoculated control giving 9.67% in the same ^{15}N dose. On using half recommended nitrogen dose (N_1) the maximum level, 80.67%, was obtained in the combination treatments (M_6) followed by 74.0, 71.0 and 67.0% expressed on M_4, M_5 and M_1, respectively compared with control 13.67% in the same N-fertilizer dose. In case of full recommended nitrogen dose (N_2) inoculation with AMF alone or in combination significantly increased AMF root colonization and the maximum was 82.33% with (M_6) followed by 73.0, 71.0 and 67.0% with M_4, M_5 and M_1, respectively compared with the control 16.0% in the same ^{15}N-fertilizer dose. The highest AMF root infection percentage after 60 days being 82.33% was recorded in the treatment where combination inoculated with AMF, *Az. chroococcum* and *B.megatherium* was used with full recommended nitrogen dose compared with the control which was 9.67% without ^{15}N-fertilizer.

Table 4. Microscopic characters of AMF spores associated with medicinal plants

AMF	Spore size (µm)	Spore layer	Colour	Other descriptions
***Glomus* spp.**				Sporocarp containing chlamydospores
G. mosseae	200	3	Brown to orange brown	Hyphae are double layered. Spore globose to sub-globose
G. constrictum	110-130 x 150-160	2	Brownish orange to dark brown	Subtending hyphae straight or curved, usually markedly constricted at the spore base. Globose to subglobose, sometimes ovoid
G. clarum	80–150 µm	3	Cream to pale yellow	Forms hypogeous sporocarps, spores in sporocarps mostly

Table 5. Effect of inoculation with AMF and/or some beneficial microbes under labeled nitrogen (^{15}N) fertilization on mycorrhizal root infection after 30, 60 and 120 days from cultivation of black cumin plants

	Infection percentage								
Treatment	**After 30 days**			**After 60 days**			**After 120 days**		
	Nitrogen (^{15}N)			**Nitrogen (^{15}N)**			**Nitrogen (^{15}N)**		
	N_0	**N_1**	**N_2**	**N_0**	**N_1**	**N_2**	**N_0**	**N_1**	**N_2**
M_0	6.00	8.00	10.00	9.67	13.67	16.00	15.67	22.00	24.67
M_1	38.33	43.67	44.67	49.00	67.00	67.00	58.00	74.00	73.33
M_2	9.00	12.00	15.00	18.67	23.33	24.67	27.33	31.00	33.33
M_3	9.00	12.33	15.00	15.67	21.00	25.00	25.33	28.00	28.33
M_4	40.67	47.33	47.00	57.33	74.00	73.00	65.33	78.33	75.67
M_5	40.67	44.67	44.67	48.67	71.00	71.00	61.33	73.67	74.00
M_6	42.00	50.33	50.00	59.67	80.67	82.33	71.67	83.67	82.33
Mean	26.52	31.19	32.33	36.95	50.10	51.29	46.38	55.81	55.95
LSD 0.05	M:1.363; N: 892; NM: 2.361			M:2.41; N: 1.58; NM: 4.17			M:1.72 ; N: 1.13; NM:2.98		

Note: N_0, $N_1$and N_2 are zero%, 50% and 100% Nitrogen (N^{15}), respectively of recommended dose.

M_0: control, M_1: AMF, M_2: *Azotobacter chroococcum*, M_3: *Bacillus megatherium*, M_4: AMF+ *Az.chroococcum*, M_5: AMF+ *B.megatherium* and M_6: AMF+ *Az.chroococcum*+ *B.megatherium*

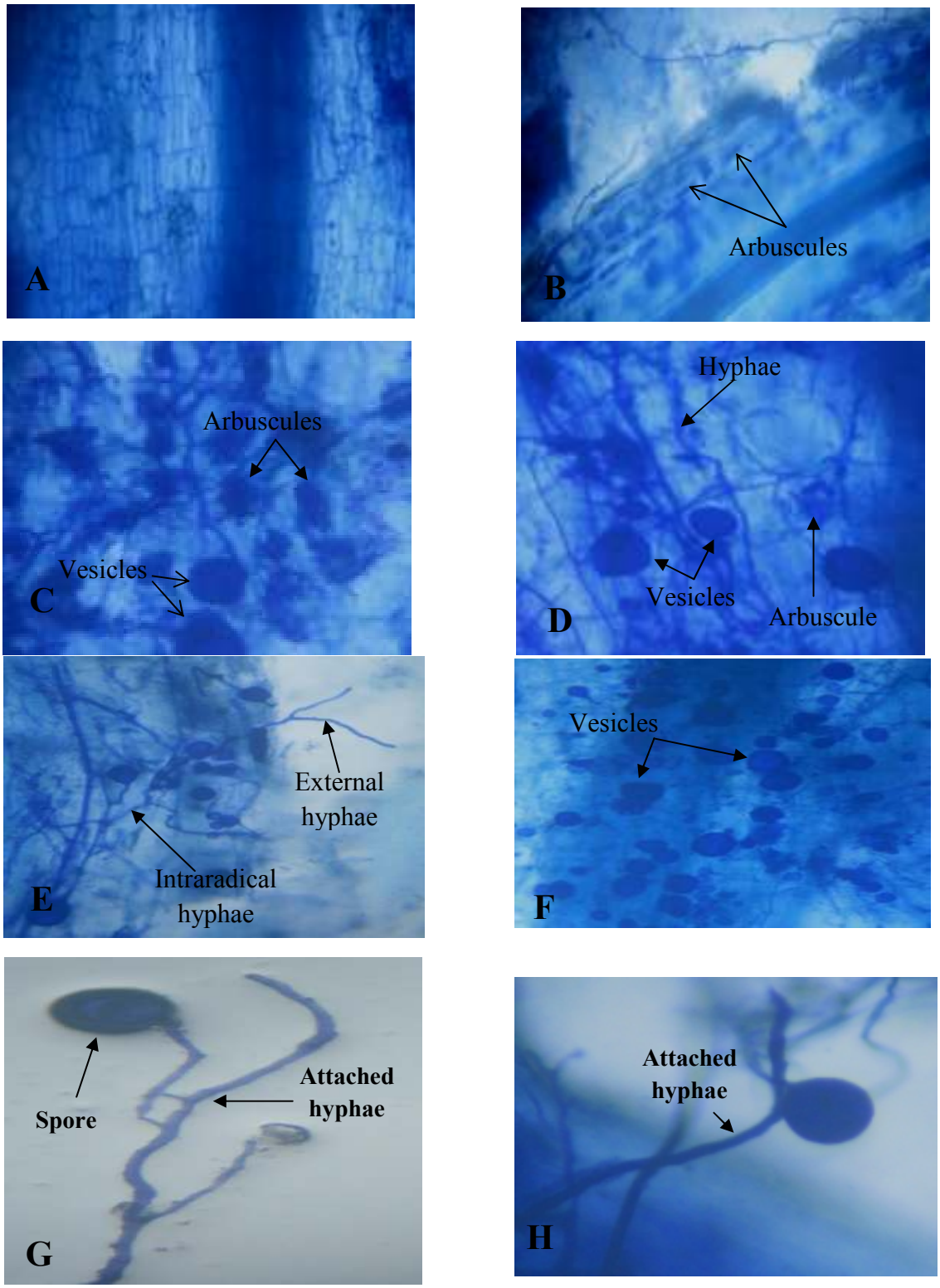

Fig. 2. Microscopic photographs showing root colonization in black cumin stained with trypan blue (40X)

A: Non mycorrhizal colonization B: Infection after 30 days C&D: Infection after 60 days
E & F: Infection after 120 days G&H: Vesicle and intraradical hyphae

AMF root infection percentage after 120 days in the absence of nitrogen fertilizer dose ($^{15}N_0$) reached their maximal levels being 71.67% when inoculated with mixture of AMF, *Az.chroococcum* and *B.megatherium* (M_6) followed by 65.33and 61.33% for the M_4 and M_5 treatments, respectively compared with the un-inoculated control which was 15.67% in the same ^{15}N dose. It was also found that inoculated plants with AMF alone without nitrogen giving 58.0% compared with the control (15.67%) in the same dose. Also, with half recommended nitrogen dose ($^{15}N_1$) the maximum level being 83.67% in the combination treatment (M_6) followed by 78.33, 74.00 and 73.67% expressed on M_4, M_1 and M_5, respectively compared with the control (22.00%) in the same N-fertilizer dose. In the case of full recommended nitrogen dose ($^{15}N_2$) inoculation with AMF alone or in combination gave significant increases percentage for AMF root colonization and the maximum reached 82.33% with (M_6) followed by 75.67, 74.00 and 73.33% expressed on M_4, M_5 and M_1, respectively compared with the control (24.67%) in the same N-fertilizer dose. The maximal levels being 83.67% was obtained in treatment inoculated with AMF, *Az.chroococcum* and *B.megatherium* (M_6) when nitrogen was half of the recommended dose compared to the other treatments. It was also noticed that the percentage of infection in *Nigella sativa* plants increased significantly by increasing nitrogen fertilization (^{15}N) levels compared with control.

Results showed also that the combined of AMF with bacterial inoculation treatments resulted in a higher AMF colonization in comparison with AMF as single inoculants ones. These results agree with Fitter and Garbaye (1994) who found that rhizobacteria increased the capacity of AMF to colonize the roots of plants. However, according to Gianinazzi-Pearson (1982), free-living bacteria such *Azotobacter* spp. and *Azospirillum* spp. can increase microbial populations in the rhizosphere of mycorrhizal plants. Production of mycorrhizal propagules (spores, hyphae and colonized roots) permits the inoculation of these organisms in plants growing in soils where AMF inoculum levels are reduced, and this is an important part of the process of soil microbiota recuperation (Smith and Read 1997). These results differ from those of Constantino *et al*. (2008) who reported that the combined inoculation of AMF and rhizobacteria resulted in a lower colonization in comparison with the treatments with AMF as a single inoculant.

Anise plants

The obtained results presented in Table 6 and Fig. 3 clearly show that mycorrhizae root

Table 6. Effect of inoculation with AMF and/or some beneficial microbes under labeled nitrogen (^{15}N) fertilization on mycorrhizal root infection after 30, 60 and 120 days from cultivation of anise plants

Treatment	Infection percentage								
	After 30 days			After 60 days			After 120 days		
	Nitrogen (^{15}N)			Nitrogen (^{15}N)			Nitrogen (^{15}N)		
	N_0	N_1	N_2	N_0	N_1	N_2	N_0	N_1	N_2
M_0	5.00	6.66	7.33	9.00	13.33	13.33	10.67	15.33	14.67
M_1	27.00	34.66	36.66	38.67	58.33	58.00	40.33	68.00	71.00
M_2	9.33	12.33	12.00	15.67	21.33	22.33	16.67	24.00	24.33
M_3	8.66	14.00	13.00	16.00	21.67	22.67	16.33	24.00	23.33
M_4	30.33	34.33	38.00	43.33	65.67	65.00	46.00	71.33	72.33
M_5	31.33	34.00	37.00	44.33	63.67	64.00	46.67	70.00	70.67
M_6	32.66	36.66	38.66	50.33	70.33	66.67	52.33	76.67	76.33
Mean	20.62	24.66	26.09	31.05	44.90	44.57	32.71	49.90	50.38
LSD 0.05	M:1.19; N:1.56; NM: 2.06			M:3.03 ; N:1.99; NM: 5.26			M:3.14 ; N: 2.05 ; NM:5.44		

Note: N_0, $N_1$and N_2 are zero%, 50% and 100% Nitrogen (N^{15}) respectively from recommended dose.
M_0: control, M_1: AMF, M_2: *Azotobacter chroococcum*, M_3: *Bacillus megatherium*, M_4: AMF+ *Az.chroococcum*, M_5: AMF+ *B.megatherium* and M_6: AMF+ *Az.chroococcum*+ *B.megatherium*

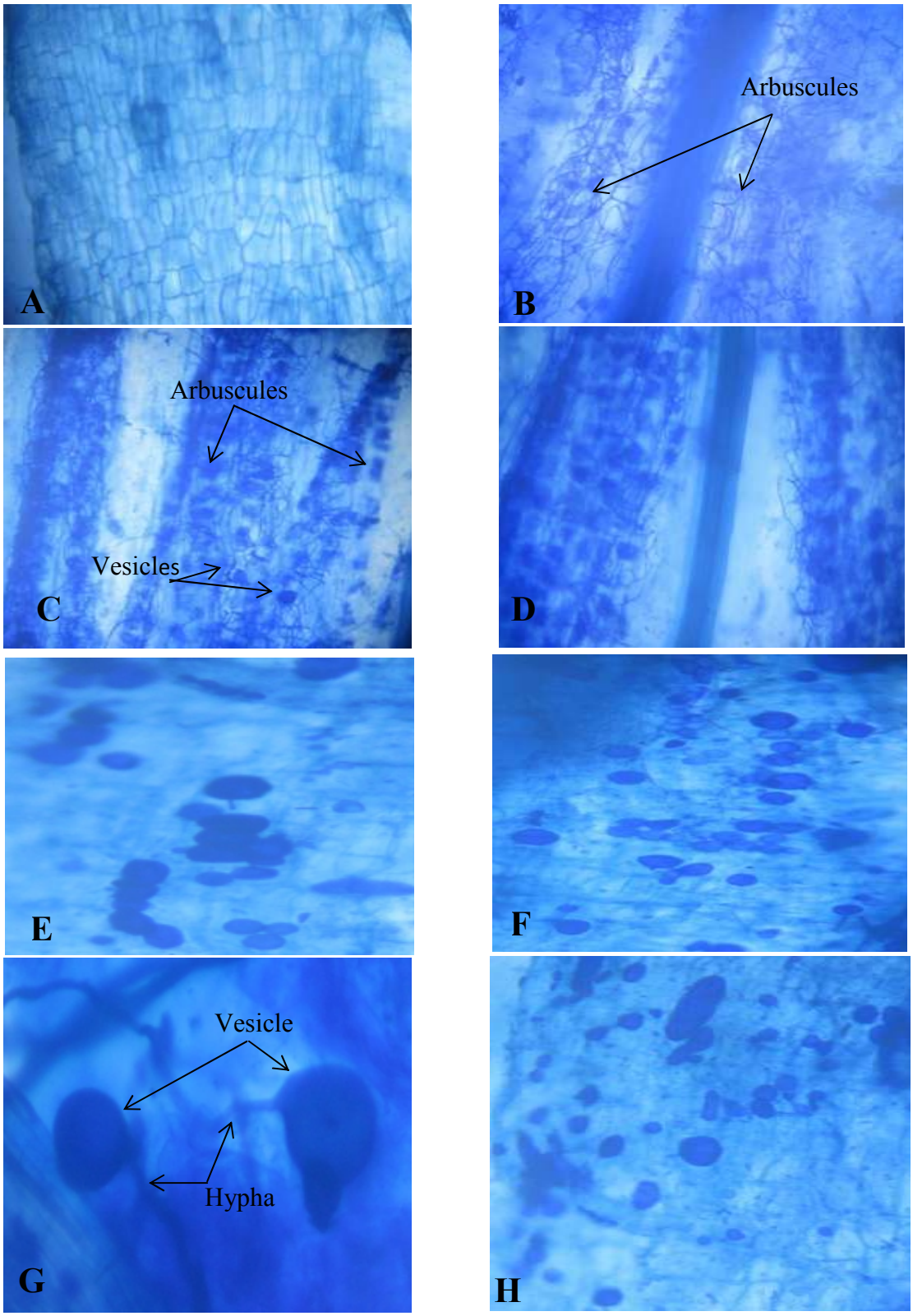

Fig. 3. Microscopic photographs showing root colonization in anise stained with trypan blue (40X)

A: Non mycorrhizal colonization
B: Infection after 30 days
C&D: Infection after 60 days
E to H: Infection after 120 days

colonization of anise behaved the same general trend of black cumin. The percentage of root colonization after 30 days, in the absence of nitrogen fertilizer dose (N_0), the maximal levels was 32.66% when inoculated with AMF, *Az.chroococcum* and *B.megatherium* (M_6) compared with the un-inoculated control which was 5.00% in the same ^{15}N dose. Also, with half recommended nitrogen dose (N_1) the maximum level being 36.66% reached in the combination treatment (M_6) compared with the control which was 6.66% in the same ^{15}N -fertilizer dose. In case of full recommended nitrogen dose (N_2) inoculated with AMF alone or in combination significantly increased AMF root colonization and the maximum was 38.66% with (M_6), while AMF individual being 36.66% compared with control 7.33% in the same ^{15}N -fertilizer dose.

As well, after 60 days, when zero nitrogen fertilizer dose (N_0) was applied, the percentage of root colonization reached their maximal levels being 50.33% when inoculated with AMF, *Az.chroococcum* and *B.megatherium* (M_6) compared with control which was 9.0% in the same ^{15}N dose. With half recommended nitrogen dose ($^{15}N_1$) the maximum level reaching 70.33% was obtained in the combination treatment (M_6) compared with the control which was 13.33% in the same ^{15}N -fertilizer dose. In the case of full recommended nitrogen dose ($^{15}N_2$) the maximum was 66.67% with (M_6) compared with control (M_0) which valued 13.33% in the same N-fertilizer dose. AMF root infection percentage after 120 days at the absence of nitrogen fertilizer dose (N_0) reached their maximal levels being 52.33% when inoculated with AMF, *Az.chroococcum* and *B.megatherium* (M_6) compared with the control which was 10.67% in the same ^{15}N dose. With half recommended nitrogen dose ($^{15}N_1$) the maximum level being 76.67% in the combination treatment (M_6) compared with control 15.33% in the same N-fertilizer dose. When added full recommended nitrogen dose ($^{15}N_2$) the maximum percent was 76.33% with AMF, *Az.chroococcum* and *B.megatherium* (M_6) compared with control (14.67%) in the same N-fertilizer dose. The obtained photographic present in Fig. 3 illustrated that AMF stages in the anise plant from cultivation.

Generally, the maximal infection levels being 76.67% in treatment inoculated with AMF, *Az. chroococcum* and *B.megatherium* when nitrogen $^{15}N_1$ was used in a half of the recommended dose after 120 days from cultivation compared to the other treatments. It was also noticed that the percentage of infection in anise plants increased significantly by inoculation with AMF individual or dual with *Az.chroococcum* or *B.megatherium*. Results revealed a significant interaction effect between nitrogen fertilization and mycorrhizae colonization on percentage infection in anise plant. According to Smith and Read (2008), the availability of P in soils is the most important edaphic factor for the operation of the Mycorrhizal symbiosis. Moreover, this effect is of great value on the expected productivity of anise plants since percentage of infection plays an important role in the photosynthetic potentialities of anise plants, according to Corkid *et al.* (2002), Chen *et al.* (2005), Lestingi *et al.* (2007) and Ali *et al.* (2009).

Mycorrhizae spore density in rhizosphere soil

The density of mycorrhiza spores in the rhizosphere soil of black cumin and anise plants was determined at the end of the experiment. Obtained results presented in Table 7 clearly show that AMF spore density increased significantly with AMF and microbial inoculation in all treatments. The density of the rhizosphere mycorrhiza spores in the absence of nitrogen fertilizer dose ($^{15}N_0$) reached their maximal levels being 346 spores/100g soil when inoculated with AMF, *Az.chroococcum* and *B.megatherium* (M_6) compared with the un-inoculated control which gave 23 spores/100 g soil in the same ^{15}N dose. Using half recommended nitrogen dose ($^{15}N_1$) the maximum level obtained was 377 spores/100 g soil in the combination treatments (M_6) followed by 344 and 334 spores/100g soil expressed on M_1 and M_5, respectively compared with the control 23 spores/100 g soil in the same ^{15}N -fertilizer dose. In case of full recommended nitrogen dose ($^{15}N_2$) inoculation with AMF alone or with microbial combination increased significantly AMF spore density and the maximum reached 305 spores/100g soil with (M_1) followed by 288, 276 and 269% expressed on M_5, M_4 and M_6, respectively compared with control 19 spores/ 100 g soil in the same ^{15}N -fertilizer dose.

Results presented in Table 7 also show that AMF spore density in the rhizosphere of anise was determined at the end of the experiment increased significantly by AMF and/or microbial inoculation in all inoculated treatments. Mycorrhizal spore density in the absence of nitrogen fertilizer dose ($^{15}N_0$) showed the highest level being 334 spores/100gsoil when inoculated with AMF individual (M_1) compared with un-inoculated control which was 22 spores/100gsoil in the same N dose. Also, with half recommended nitrogen dose ($^{15}N_1$) the maximum level being 385 spores/100 g soil in the treatments (M_5) compared with control (21 spores/100 g soil) in the same ^{15}N-fertilizer dose. In case of full recommended nitrogen dose ($^{15}N_2$) the maximum was 337 spores/100 g soil with (M_5) compared with the control (18 spores /100 g soil) in the same ^{15}N-fertilizer dose.

Generally, The density of mycorrhizae spores significantly influenced by AMF and/or microbial inoculation. It is also clear from the results recorded that number of mycorrhiza spores in the rhizosphere soil of black cumin plant reached their maximal levels namely 377 spores/100 g soil in treatment inoculated with mixed of AMF, *Az.chroococcum* and *B.megatherium* using half dose of nitrogen ($^{15}N_1$) while the minimal one being 19 spores/100g soil in control with full recommended dose ($^{15}N_2$). The number of mycorrhiza spores in the rhizosphere soil of anise plant reached their maximal levels which gave 385spores/100g soil in treatment inoculated with AMF and *B.megatherium* (M_5) using half dose of nitrogen ($^{15}N_1$) compared with the minimal one which being 18 spores/100 g soil in the control with full recommended dose ($^{15}N_2$).

Table 7. Effect of inoculation with AMF and/or some beneficial microbes under labeled nitrogen (^{15}N) fertilization on mycorrhizae spore density in the rhizosphere of black cumin and anise plants

Treatment	Mycorrhizae spore density spores/100g soil					
	Black cumin			Anise		
	Nitrogen (^{15}N)			Nitrogen (^{15}N)		
	N_0	N_1	N_2	N_0	N_1	N_2
M_0	23	23	19	22	21	18
M_1	344	344	305	334	326	296
M_2	35	68	60	60	64	64
M_3	33	78	65	69	67	79
M_4	293	287	276	297	380	291
M_5	279	334	288	328	385	337
M_6	346	377	269	332	379	324
Mean	**193**	**216**	**183**	**206**	**232**	**201**
LSD0.05	M:27.33 ; N: 17.89 ; NM: 47.33			M:12.32 ; N: 8.062 ; NM: 21.38		

Note: N_0, $N_1$and N_2 are zero%, 50% and 100% Nitrogen (N^{15}), respectively from recommended dose.

M_0: control, M_1: AMF, M_2: *Azotobacter chroococcum*, M_3: *Bacillus megatherium*, M_4: AMF+ *Az.chroococcum*, M_5: AMF+ *B.megatherium* and M_6: AMF+ *Az.chroococcum*+ *B.megatherium*

The obtained results revealed that no correlation can be drawn between number of Mycorrhiza spores in soil and inoculation with AMF and/or *Az.chroococcum* and *B. megatherium* according to Xavier and Germida (2003) since they observed some AMF spore associated bacteria (AMB) such as *Bacillus pabuili* have the ability to enhance AMF root colonization and can also improve plant growth (Artursson *et al*., 2006). Our results were in accordance with Pawaar and Kakde (2012) who found that the maximum spore population was observed during rainy season which coincides with flowering time of the plants. This may be correlated with the fact that during this period most photosynthatase is allocated to roots and rhizomes, which helps fungal symbiont to produce more spores (Wallen, 1980; Gemma and Koske, 1988).

Note

Data revealing the assessment of ^{15}N and it's effect on the growth and productivity of the plants will be presented and discussed in our next work.

REFERENCES

Ali, F.S., G. Zayed, O.A. Saad and E. Abdul-Mohsen (2009). Optimization of nitrogen fertilizer level for maximum colonization of mycorrhizae on root of Coriander plants. Afri. Crop Sci. Soc., 9th Conf., Cape Town, South Africa.

Alizadeh, O. (2012). A critical review on the nutrition role of arbuscular mycorrhizal fungi. Extreme life, Biospeol. and Astrobiol. Int. J. Bioflux Soc., 4 (1): 1-7.

Ardakani, M.R., D. Mazaheri, F. Majd and G.H. Nour-Mohamadi (2000). The study of *Mycorrhiza* and Streptomyces' efficiency and different levels of phosphorus on grain yield and some characters of wheat. Iranian J. Crop Sci., 2(2): 17- 28.

Artursson, V., R.D. Finlay and J.K. Jansson (2006). Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ. Microbiol., 8: 1-10.

Biermann B. and R. Linderman (1981). Quantifying vesicular-arbuscular mycorrhizae: A proposed method towards standardization. New Phytol., 87: 63-67.

Cardoso, I.M. and T.W. Kuyper (2006). Mycorrhizal and tropical soil fertility. Agric., Ecosyst. and Environ., 116: 72-84.

Chandra, S. and H.K. Kehri (2006). Biotechnology of VA Mycorrhiza: Indian Scenario. New India Publishing Agency. Delhi, India, 23-70.

Chen, X., H. Wu Chun, J. Tang and S.J. Hu (2005). Arbuscular mycorrhizae enhance metal lead uptake and growth of host plants under a sand culture experiment. Chem., 60 (5): 665-671.

Constantino, M., R. Gomez-Alvarez, J. Alvarez-Sol´ıs, V. Geissen, E. Huerta and E. Barba (2008). Effect of inoculation with rhizobacteria and arbuscular mycorrhizal fungi on growth and yield of capsicum chinense Jacquin. J. Agric. and Rural Develop. Tropics and Subtropics, 109 (2): 169–180.

Corkid, L., D.L. Rowland, N.C. Johnson and E.B. Allen (2002). Nitrogen fertilization alters the functioning of arbuscular mycorrhizas at two semiarid grasslands. Plant and Soil, 240 (2): 299-310.

Estefen, G., R. Sommer and J. Ryan (2013). Methods of soil, plant and water analysis: a manual for West Asia and North Africa region, Int. Cent. Agric. Res. Dry Areas (ICARDA), 3rd Ed.

Fitter, A. and J. Garbaye (1994). Interactions between mycorrhizal fungi and other soil organisms. Plant and Soil, 159:123–132.

Frank, A.B. (1885). Uber di auf werzel symbiose beruhende Ernahrung gewisser Baume durch unterirdische plize. Ber. Dtsch. Bot. Ges., 3: 128–145.

Gemma, J. and R. Koske (1988). Seasonal variation in spore abundance and dormancy of *Gigaspora gigantea* and in mycorrhizal inoculum potential of dune soil. Mycologia, 80: 211 -216.

Gerdemann J. and T. Nicolson (1963). Spores of mycorrhizalendogone species extracted from soil by wet -sieving and decanting. Trans. Brit. Mycol. Soc., 235-244.

Gerdemann, J. and J. Trappe (1974). The endogonaceae in the Pacific Northwest. Myc. Memoir., 5 : 1-76

Gianinazzi-Pearson, T. (1982). Relation between the critical concentration of nitrogen, phosphorus, and potassium in 17 different vegetable crops and duration of growth. J. Sci. Food Agric., 31:1343–1353.

Giovannetti, M. and L. Avio (2002). Biotechnology of arbuscular mycorrhizas. In: Khachatourians, G.G., Arora Dilip, K. (eds) Appl. Mycol. Biotechnol., Agric. Food Prod. Elsevier, Amsterdam, 2: 275–310

Hayman, D.S. (1982). Practical aspects of vesicular arbuscular mycorrhiza. In: Advances in Agric. Microbiol., (Subba Rao, NS Ed.). New Delhi, Oxford IBA, 325-373.

Heijden, V.D., J.N. Klironomos, M. Ursic, P. Moutoglis and R. Streitwolf-Engel (1998). Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nat., 396:69-72.

Kapoor, R., B. Giri and K.G. Mukerji (2002). Mycorrhization of coriander (*Coriandrum sativum* L.) to enhance the concentration and quality of essential oil. J. Sci. Food Agric., 82: 339–342.

Karagiannidis, N., T. Thomidis, D. Lazari, E. Panou-Filotheou and C. Karagiannidou (2011). Effect of three Greek arbuscular mycorrhizal fungi in improving the growth nutrient concentration, and production of essential oils of oregano and mint plants. Sci. Hort., 129:329–334.

Koul, K.K., S. Agarwal and L. Rafiq (2012). Diversity of arbuscular mycorrhizal fungi associated with the medicinal plants from gwalior-chambal region of madhya Pradesh-India. Ame.-Euras. J. Agric. Environ. Sci., 12 (8): 1004-1011.

Lestingi, A., D.D. Giorgio, F. Montemurro, G. Convertini and V. Laudadio (2007). Effects of bio-activators on yield and quality composition of triticale forage and food resource. J. Food Agric. Environ., 5 (1): 164-171.

Mehrota, V.S. (2005). Mycorrhizas: Roles and Applications. Allied Publishers, New Delhi.

Mehrvarz, S., M.R. Chaichi and H.A. Alikhani (2008). Effects of phosphate solubilization of microorganisms and phosphorus chemical fertilizer on yield and yield components of barley (*Hordeum vulgare* L.). Ame.– Euras. J. Agric. Environ. Sci., 3(6): 822 - 828.

Mosse, B. (1981). Research Bulletin, Hawaii Institute of Tropical Agric. and Human Res., 194: 5-81.

Muthuraj, K., V.J.P. Joseph and N. Nagarajan (2014). Arbuscular mycorrhizal fungal diversity and root colonization of some medicinal plants rhizospheric soil of Madayipara Hills, Kannur, Western Ghat Kerala, World J. Pharm. and Pharmaceut. Sci., 3 (6): 1114-1122.

Pawaar J.S. and U.B. Kakde (2012). Study of arbuscular mycorrhiza associated with some important medicinal plants in Suburban Area of Mumbai. Online Int. Interdisciplinary Res. J., {Bi-Monthly}, II: 116-127.

Phillips, J. and D. Hayman (1970). Improved procedures for clearing roots and staining parasitic and vesicular-Arbuscular Mycorrhizal fungus for rapid assessment of infection. Trans. Br. Mycol. Soc., 55: 158-161.

Ram, U. and S. Bhadauria (2009). Vesicular arbuscular mycorrhizal association with some medicinal plants growing on alkaline soils of Manipuri District, Uttar Pradesh, Mycorrhiza News, 13: 12-14.

Sarwade P.P., S.S. Chandanshive, M.B. Kanade and U.N. Bhale (2011). Diversity of Arbuscular Mycorrhizal (AM) Fungi in some common plants of Marathwada region. Int. Multidiscipl. Res. J., 1(12): 11-12.

Smith, S.E., and Read, D.J. (1997). Mycorrhizal Symbiosis. San Diego, California: Elsevier Science Ltd.

Smith, S.E. and D.J. Read (2008). Mycorrhizal Symbiosis. 3rd Ed. New York, Acad. Press.

Soleimanzadeh, H. (2010). Effect of VA – Mycorrhiza on growth and yield of Sunflower (*Helianthus annus* L.) at different

phosphorus levels. Int. J. Chem. Biol. Engin., 3 (3): 159-163.

Trappe, J. (1982). Synoptic key to the genera and species of Zygomyceteous mycorrhizal fungi. Phyto. Pathol., 72: 1102-1108.

Urcoviche, R., M. Castelli, R. Gimenes and O. Alberton (2014). Spore density and diversity of arbuscular mycorrhizal fungi in medicinal and seasoning plant. Afr. J. Agric. Res., 9: 1244–1251.

Wallen, B. (1980). Changes in structure and function of Ammophila during primary succession. Oikes, 34: 227 -238

Warner, A. and B. Mosse (1980). Independent spread of Vesicular Arbuscular Mycorrhizal fungi in soil, Trans. British Mycol. Soc., 74: 407 - 410.

Xavier, L.J. and J.J. Germida (2003). Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. Soil Biol. Biochem., 35: 471-478.

تأثير التلقيح بفطريات الميكورهيزا الداخلية والنيتروجين المرقم على استعمار الجذور وكثافة الجراثيم في بعض النباتات الطبية

دينا إبراهيم محمـــد عبدالقادر[١] - سمير حماد ســـالم[٢] - فاطمه إبراهيم الزامـــك[٢]

هويدا محمد لبيب عبدالباسط[٢] - مجدى أحمد رزق[١] - أسامه عبدالباسط عبدالعزيز[١]

١- قسم بحوث الأراضى والمياه - مركز البحوث النووية - هيئة الطاقة الذرية - إنشاص - مصر

٢- قسم الميكروبيولوجيا الزراعية - كلية الزراعة - جامعة الزقازيق - مصر

تم إجراء هذا البحث بهدف دراسة تأثير التلقيح بفطريات الميكورهيزا الداخلية (AMF) والنيتروجين المرقم (^{15}N) على استعمار الجذور وكثافة الجراثيم فى بعض النباتات الطبية، أيضا قد تم دراسة تنوع الميكورهيزا فى ريزوسفير ٢٢ نبات طبى من مزرعة شركه سيكم ببلبيس – شرقية، وتم حساب نسبه استعمار الجذور بالميكورهيزا فى كل نبات وعدد الجراثيم فى ١٠٠جم تربة، لوحظ وجود AMF فى معظم النباتات، وهناك تنوع كبير فى استعمار AMF فى كل نبات بالنسبة لنفس منطقه الدراسة، وكانت أعلى نسبه إصابة فى نبات حبه البركة (٧٧%) وأقلهم فى البقدونس (١٨,١٧%)، وسجلت أعلى كثافة جراثيم في نبات الينسون (١٠٠/٣٦٨جم تربة) وأقل كثافة جراثيم كانت فى حشيشه الليمون (١٠٠/١٠٣جم تربة)، بالإضافة لذلك فقد تم دراسة تأثير التلقيح ب AMF والنيتروجين المرقم على استعمار جذور وكثافة الجراثيم فى نباتى حبة البركة والينسون، وكان أعلى استعمار للجذور AMF فى حبة البركة بنسبة ٨٣,٦٧% بعد ١٢٠ يوم فى المعاملة AMF والازوتوباكتر كروككم والباسلس ميجا ثريم مع نصف الجرعة من السماد النيتروجينى بالمقارنة بأعلى استعمار للجذور AMF ٧٦,٦٧% بعد ١٢٠ يوم فى نفس المعاملة ونفس جرعة النيتروجين، وعلاوة على ذلك كانت أعلى كثافة للجراثيم فى حبة البركة (١٠٠/٣٧٧جم تربة) فى نفس المعاملة بينما فى الينسون (١٠٠/٣٨٥جم تربة) فى المعاملة AMF والباسلس ميجا ثريم ونصف جرعة النيتروجين.

المحكمـــون:

١- أ.د. أحمد عبدالوهاب عبدالحافظ أستاذ الميكروبيولوجيا – كلية الزراعة – جامعة عين شمس.

٢- أ.د. عصام الدين محمـــد جويلي أستاذ الميكروبيولوجيا المتفرغ– كلية الزراعة – جامعة الزقازيق.