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Interaction between *Glomus geosporum, Azotobacter chroococcum,* and *Bacillus coagulans* and their Influence on Growth and Nutrition of Melia azedarach L.

Sevanan RAJESHKUMAR[1], Mathan CHANDRAN NISHA[2], Padanilly CHIDAMBARAM PRABU[1], Lakew WONDIMU[1], Thangavel SELVARAJ[3]

[1]Department of Applied Biology, Faculty of Natural and Computer Sciences, Ambo University College, Post Box. No. 19, Ambo, Western Shoa - ETHIOPIA

[2]Department of Botany, Kongunadu Arts and Science College, Coimbatore, Tamilnadu – INDIA

[3]Department of Plant Sciences, Faculty of Agricultural Sciences, Ambo University College, Post Box. No. 19, Ambo, Western Shoa - ETHIOPIA

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Abstract: A green house nursery study was conducted to assess the interaction between arbuscular mycorrhizal fungus, a nitrogen fixing bacterium, *Glomus geosporum*, *Azotobacter chroococcum,* and a mycorrhiza helper bacterium (MHB), *Bacillus coagulans,* in soil and their consequent effect on growth and nutrition of *Melia azedarach* L. seedlings. Triple inoculation of *G. geosporum*, *A. chroococcum,* and *B. coagulans* resulted in maximum plant biomass, N, P, Zn, and Cu uptake, and biovolume and quality index of *M. azedarach* seedlings. It also increased the mycorrhizal root colonization and spore numbers in the root zone soil of the inoculated plants over uninoculated control plants. The enzyme activity, namely acid phosphatase and dehydrogenase, in the root zone soil was found high in the 3-combination treatments and low in the uninoculated control. Triple inoculation with *G. geosporum* + *A. chroococcum* + *B. coagulans* proved to be the best microbial consortium for inoculating *M. azedarach* at nursery level in order to get healthy and vigorously growing seedlings.

Key Words: *Melia azedarach*, *Glomus geosporum*, *Azotobacter chroococcum*, *Bacillus coagulans*, growth, nutrition

Glomus geosporum, Azotobacter chroococcum ve *Bacillus coagulans* Arasındaki Etkileşim ve Bu Etkileşimin Melia Azedarach L. Büyüme ve Beslenmesine Etkisi

Özet: Arbuskular mycorrhizal mantarları, *Glomus geosporum*, azot fikse eden bakteri, *Azotobacter chroococcum,* ve mycorrhiza yardımcı bakteri *Bacillus coagulans,* arasındaki etkileşimi ve *Melia azedarach* L fidelerinin büyüme ve beslenmesi üzerine etkileri seralarda yapılan çalışma ile araştırılmıştır. *M. azedarach* fidelerine *G. geosporum*, *A. chroococcum,* ve *B. coagulans* mikroorgannizmalarının üçlü inokülasyonları bitki biomasını, N, P, Zn ve Cu alımını, biyohacim indeksini, kalite indeksini maksimum düzeyde artırmıştır. Ayrıca control bitki ile mukayese edildiği zaman bu bitkilerin kök zonlarında mikorhizal kök kolonizasyonu ve spor sayısında artış gözlenmiştir. Mikroorganizmalar ile inoküle edilen bitkiler ile control bitkiler arasında yapılan mukayese sonucu bakteri inoküle edilmiş bitkilerin fosfataz, dehidrojenaz gibi enzim aktviteleri de oldukça yüksek bulunmuştur. *M. azedarach* bitkisinin seralarda sağlıklı ve en güzel şekilde yetiştirilmesinde *G. geosporum* + *A. chroococcum* + *B. coagulans* organizmaların üçlü kombinasyonlarının en uygun mikrobial karışım olduğu sonucuna varılmıştır.

Anahtar Sözcükler: *Melia azedarach*, *Glomus geosporum*, *Azotobacter chroococcum*, *Bacillus coagulans*, büyüme, beslenme

Introduction

Biofertilizers are one of the most important components of the integrated nutrient supply system of sustainable agriculture. Arbuscular mycorrhizal (AM) fungi are a unique group of soil fungi that form symbiotic association with higher plants and facilitate the uptake of diffusion-limited plant nutrients, such as P, Zn, and Cu (1). Interest in these associations are mainly because of the manifold conferred on the host by the fungus. Furthermore, these fungi show a preferential colonization to the hosts, and thereby, the extent to which a host is benefited depends on the fungal species involved in the symbiosis (2). Mycorrhizal fungi interact with a wide range of other soil organisms in the root or in the rhizosphere of the soil. Some form a symbiotic association and in turn modify the host physiology (3, 4). Interaction studies between AM fungi, N_2 fixing bacteria, and mycorrhizal helper bacterium (MHB) has been proved to form a consortium benefiting the growth of a few plant species (5, 6). 'Malai vembu' or 'China berry' *Melia azedarach* L. is a multipurpose indigenous tree of great economic and medicinal importance. The major chemical compound 'Azadirachtin' extracted from seeds and leaves is an insect repellent and ecofriendly in nature. It is extensively used for afforestation in waste lands, drought areas, and polluted lands in Ethiopia. Hence the present investigation was aimed to study the interaction between *G. geosporum* + *A. chroococcum* + *B. coagulans* in the root zone of *M. azedarach* and this may develop a microbial consortium for inoculating this important indigenous tree species in the nursery in Ethiopia.

Materials and Methods

The present investigation was carried out under nursery conditions in a glass house. The potting mix used in the study was a mixture of unsterilized sand:soil:FYM @ 1:1:0.25 by volume. Seeds of *M. azedarach* were sown in polythene bags (300 gauge) of 2.5 kg capacity containing the potting mix. *Glomus geosporum* used in the study was obtained from the culture of AM fungi maintained in the glass house of the Department of Agricultural Microbiology, University of Agricultural Sciences, Bangalore, India, using sand:soil (1:1 v/v) as the substrate, and Rhodes grass as the host. *G. geosporum* inoculum was added to the seedling point at the rate of 12,500 infective propagules per bag, based on the most probable number estimation (7). *A. chroococcum* was grown on Waksmann no.77 medium under shake culture condition for 7 days. *B. coagulans* was multiplied on nutrient broth for 3 days at 30 °C under stationary conditions. The cultures were then centrifuged at 5000 rpm for 7 min. The supernatant was discarded and the pellets containing N-fixing bacterial cells and mycorrhizal helper bacteria were suspended in 250 ml of 0.1 M $MgSo_4$ solution separately. *A. chroococcum* inoculum containing 1.4×10^7 cfu/ml and *B. coagulans* inoculum containing 4.5×10^5 cfu/ml were added at the rate of 5 ml per planting hole in appropriate treatments. The uninoculated control received 2.5 ml of the washings of AM inoculum suspensions passed through 45 µm sieve that contained associated microorganisms but not AM propagules, 2.5 ml of 0.1 M $MgSo_4$ buffer solution, and 5 ml of respective broths with no bacteria. The poly bags with 6 treatments, namely

i. Uninoculated control (without AM fungi, N-fixing bacterium, or MHB),

ii. *Glomus geosporum* (Gg),

iii. *Azotobacter chroococcum* (Ac),

iv. *Bacillus coagulans* (Bc),

v. Gg + Ac,

vi. Gg + Bc, and

vii. Gg + Ac + Bc

were arranged as a completely randomized design. Each treatment was replicated 8 times. The plants were watered regularly.

Plant growth parameters, namely plant height, number of leaves, and stem girth, were recorded on 90, 135, and 180 days after transplanting (DAT). However, only the data on 180 DAT are presented in this paper. Stem girth was measured using a vernier caliper. Biovolume index and quality index were calculated using the formula suggested by Hatchell (8). Plants were harvested 150 DAT. Shoot and root biomass was determined after drying the plant samples to constant weight at 60 °C in a hot air oven. The nitrogen content of shoot and root was determined by microKjeldhal method as outlined by Jackson (9). The per cent protein in the plant samples was calculated by multiplying per cent nitrogen values by the factor 6.25. The phosphorus content of shoot and root was determined by the vanadomolybdate phosphoric yellow color method (9). Micronutrient copper (Cu) was estimated by Atomic

Absorption Spectrophotometer with Cu hollow cathode lamp at a wavelength of 325 nm and similarly Zn was estimated using Zn hallow cathode lamp at a wavelength of 214 nm. Activities of the 2 enzymes, acid phosphatase and dehydrogenase from the root zone soil, were estimated following the method of Tabatabasi (10). The population of *A. chroococcum* and *B. coagulans* in the root zone was estimated by the dilution plate method using Waksmann No. 77 agar medium and nutrient agar, respectively. Soil samples (100 g) were collected from each polythene bag and subjected to wet-sieving and decantation as outlined by Gerdemann and Nicolson (11) to estimate the population of AM fungal spores. Fine terminal feeder roots were stained using 0.05% trypan blue as described by Phillips and Hayman (12) and the per cent root colonization was estimated by adopting the gridline intersect method (13).

Results and Discussion

In general, the response of *M. azedarach* to 5 different treatments for different characters was better compared to control. The performance of the 3-combination treatment was found superior over 2-combination and single treatments (Table 1- 4). As compared to microbial inoculation, mycorrhizal

Table 1. Effect of *Glomus geosporum*, *A. chroococcum* and *B. coagulans* on growth parameters of *Melia azedarach*.

Treatments	Plant height (cm)	Stem girth (mm)	Biomass (g/plant)		
			Shoot	Root	Total
Uninoculated control	16.6^{f}	2.7f	1.4f	0.4e	1.8^{e}
Glomus geosporum (Gg)	23.8^{d}	4.7^{d}	4.4^{d}	4.0^{b}	8.4^{c}
Azotobacter chroococcum (Ac)	19.2^{e}	3.2^{e}	1.5^{e}	0.9^{d}	2.4^{d}
Bacillus coagulans (Bc)	18.5^{e}	3.1^{e}	1.4^{e}	0.6^{d}	2.0^{d}
Gg + Ac	28.5^{b}	6.2^{b}	5.7^{b}	4.3^{b}	10.0^{b}
Gg + Bc	26.4^{c}	5.8^{c}	4.6^{c}	3.9^{c}	8.5^{c}
Gg + Ac + Bc	36.5^{a}	7.6^{a}	6.4^{a}	5.7^{a}	12.1^{a}

Means followed by same letter in each column do not differ significantly at P = 0.05 lead by Duncan's Multiple Range Test.

Table 2. Effect of *G. geosporum, A. chroococcum,* and *B. coagulans* on P, N, and crude protein content of *M. azedarach*.

Treatment	P content mg/plant		N content g/plant		Crude Protein	
	Shoot	Root	Shoot	Root	Shoot	Root
Uninoculated control	159^{f}	1.06^{e}	0.04^{c}	0.01^{c}	0.05^{e}	0.02^{d}
Glomus geosporum (Gg)	15.24^{d}	14.84^{d}	0.14^{b}	0.06^{b}	0.58^{c}	0.33^{c}
Azotobacter chroococcum (Ac)	3.09^{e}	2.06^{e}	0.02^{c}	0.02^{c}	0.21^{d}	0.04^{d}
Bacillus coagulans (Bc)	2.53^{e}	1.60^{e}	0.04^{c}	0.02^{c}	0.06^{e}	0.03^{d}
Gg + Ac	18.24^{b}	16.98^{b}	0.20^{a}	0.08^{b}	1.07^{b}	0.52^{b}
Gg + Bc	16.58^{c}	13.04^{c}	0.12^{b}	0.14^{a}	0.52^{c}	0.41^{c}
Gg + Ac + Bc	27.16^{a}	28.14^{a}	0.20^{a}	0.15^{a}	1.30^{a}	0.85^{a}

Means followed by same letter in each column do not differ significantly at P = 0.05 lead by Duncan's Multiple Range Test.

Table 3. Effect of *G. geosporum*, *A. chroococcum*, and *B. coagulans* on Zn and Cu concentration of *M. azedarach* and acid phosphatase and dehydrogenase activity in root zone soil of *M. azedarach.*

Treatment	Zn concentration µg/g		Cu concentration µg/g		Acid phosphatase activity*	Dehydrogenase activity*
	Shoot	Root	Shoot	Root		
Uninoculated control	38.7^{g}	26.0^{g}	18.6^{e}	16.8^{e}	5.06^{f}	57.4^{f}
Glomus geosporum (Gg)	153.0^{d}	95.2^{d}	37.0^{c}	33.2^{d}	14.4^{c}	342.2^{d}
Azotobacter chroococcum (Ac)	56.2^{f}	41.8^{f}	22.2^{d}	21.4^{e}	6.2^{e}	117.8^{e}
Bacillus coagulans (Bc)	62.0^{e}	53.2^{e}	26.6^{d}	32.4^{d}	6.8^{e}	135.2^{e}
Gg + Ac	389.4^{b}	189.2^{b}	60.4^{b}	56.2^{c}	23.3^{b}	384.0^{c}
Gg + Bc	251.8^{c}	166.8^{c}	54.9^{b}	64.2^{b}	13.3^{d}	412.8^{b}
Gg + Ac + Bc	507.8^{a}	232.8^{a}	89.2^{a}	77.2^{a}	32.3^{a}	498.2^{a}

Means followed by same letter in each column do not differ significantly at P = 0.05 lead by Duncan's Multiple Range Test.
* Enzyme activity expressed in µg/g soil/h.

Table 4. Effect of *G. geosporum*, *A. chroococcum,* and *B. coagulans* on biovolume index, quality index, mycorrhizal root colonization, spore numbers, and the population of *Azotobacter* and *Bacillus* in the root zone soil of *M. azedarach*.

Treatments	Biovolume Index (BI)	Quality Index (QI)	Root colonization (%)*	Spore numbers/ 100g soil	Azotobacter (× 10^3/g soil)	Bacillus (× 10^4/g soil)
Uninoculated control	119	0.22	26.4^{f}	185.0^{e}	1.5^{f}	0.3^{f}
Glomus geosporum (Gg)	519	0.89	72.5^{d}	832.8^{c}	3.4^{d}	2.8^{e}
Azotobacter chroococcum (Ac)	202	0.31	31.3^{e}	320.4^{d}	4.7^{c}	2.1^{e}
Bacillus coagulans (Bc)	171	0.26	30.5^{e}	348.6^{d}	2.5^{e}	5.0^{d}
Gg + Ac	1036	0.95	77.6^{c}	942.6^{b}	6.5^{b}	8.5^{c}
Gg + Bc	876	0.91	82.4^{b}	965.6^{b}	3.5^{d}	10.5^{b}
Gg + Ac + Bc	2076	1.06	92.4^{a}	1024.6^{a}	8.7^{a}	12.5^{a}

Means followed by same letter in each column do not differ significantly at P = 0.05 lead by Duncan's Multiple Range Test.
* Values are angular transformed

inoculation showed significant increase in plant height, stem girth, plant biomass, and plant N, P, Cu, and Zn contents of *M. azedarach.* Similar results were obtained in onion when inoculated with the AM fungi, *Beijecrinckia mobilis* and *Aspergillus niger* (14), and also in neem (*Azadirachta indica*) when inoculated with *G. mosseae* + *A. chroococcum* + *B. coagulans* (6). The possible outcome of improved plant growth indicates the improvement in biomass accumulation and this in turn improves P uptake. The total biomass of *M. azedarach* was found to be highest in the plants treated with *G. geosporum* + *A. chroococcum* + *B. coagulans* followed by *G. geosporum* + *A. chroococcum*, both being significantly different from each other compared to uninoculated plants, which were statistically on par with *B. coagulans* alone (Table 1). Similar results were obtained in chickpea by Poi et al. (15), and in neem by Sumana et al. (6). The total biomass increased by 87% over uninoculated plants, by 18% over *G. geosporum* + *A. chroococcum,* and by 30% over *G. geosporum* alone. The P, N, and crude protein contents of the plants showed the same trend, which resembled the earlier works performed on other crops (6,16). These observations uphold the synergistic interaction between AM fungi, N-fixing bacteria, and MHB. The increase in the P content of shoot and root was almost equal and significantly higher in plants with triple inoculation of *G.*

geosporum + *A. chroococcum* + *B. coagulans* followed by dual inoculation of *G. geosporum* + *A. chroococcum* over uninoculated control plants (Table 2). The increase in total P content due to the effect of triple inoculation was high (99%) over control, and low (33%) over dual inoculation treatment, and medium (51%) over *G. geosporum* alone.

Nitrogen content was highest (94%) in plants treated with *G. geosporum* + *A. chroococcum* + *B. coagulans*, and followed by *G. geosporum* + *A. chroococcum* treated plants with 92% increase compared to control. Crude protein content of these 2 best treatments also followed the similar trend (Table 2), which suggests the favorable role of AM fungi and MHB in enhancing N content of the plants.

The Zn and Cu concentrations in both shoot and root samples were highest in the plants with triple inoculation followed by dual inoculation *G. geosporum* + *A. chroococcum* and *G. geosporum* + *B. coagulans* compared to control (Table 3). This is supported by previous studies performed in sorghum (17), neem(6), and fodder crops(4).

As phosphorus is essential for the process of nitrogen fixation (18), triple inoculation might have influenced the plants with both P and N nutrition. This may be the cause for enhanced biovolume and quality index of *M. azedarach* seedlings inoculated with *G. geosporum* + *A. chroococcum* and *B. coagulans* (Table 4). Higher values of both indices indicate higher quantity of the seedlings and hence better establishment at the field site.

Mycorrhizal root colonization and spore numbers in the root zone soil were significantly more in plants inoculated with *G. geosporum* + *A. chroococcum* + *B. coagulans* compared to the plants treated with only *G. geosporum* (Table 4). There was a significant correlation between percent root colonization and mycorrhizal spore numbers in the root zone soil supporting the observation made by Meyer and Linderman (19) and Uma Maheshwari, (20).

The root-zone soil of plants inoculated with *G. geosporum* + *A. chroococcum* + *B. coagulans* had higher *Azotobacter* population followed by samples treated with *G. geosporum* + *A. chroococcum* and *A. chroococcum* alone (Table 4). Thus suggesting the stimulatory effect of AM symbiont on *A. chroococcum* supports earlier works of Bagyaraj and Menge (21) and Sumana et al., (6). *B. coagulans* (MHB) numbers were highest in the root zone soils of plants inoculated with *G. geosporum* + *A. chroococcum* + *B. coagulans* followed by treatments *G. geosporum* + *B. coagulans* and *B. coagulans* alone compared to control plants (Table 4). This suggests a synergistic activity in MHB, which enhances the activity of *G. geosporum* by producing organic acids, which serve as a carbon source to the fungus or by producing hydrolytic enzymes thus enabling the AM fungus to penetrate and ramify in the root system of the host (6, 22).

The soil metabolic activity and soil health is measured by the activity of soil enzymes like acid phosphatase and dehydrogenase. High enzyme activity was recorded in triple inoculated treatments, i.e. *G. geosporum* + *A. chroococcum* + *B. coagulans* compared to all other treatments and low enzyme activity was noticed in the uninoculated treatment (Table 3). This suggests that there exists a synergistic interaction among these organisms in stimulating the growth of plants upholding the observations made by Sekar et al. (23) and Lakshmipathy et al. (24).

Based on the response of different characters like plant biomass, P and N content, Zn, and Cu concentrations, and soil enzyme activity, it can be concluded that the 3-combination treatment, *G. geosporum* + *A. chroococcum* + *B. coagulans,* is the best consortium of microorganisms for inoculating *M. azedarach* seedlings in the nursery. Inoculation with such a microbial consortium may result in healthy, vigorously growing *M. azedarach* seedlings. Therefore, this technology, being simple and ecofriendly, can be adopted easily by forest nurserymen for inoculating *M. azedarach* seedlings in the nursery.

Corresponding Author:

Sevanan RAJESHKUMAR

Department of Applied Biology,

Faculty of Natural and Computer Sciences,

Ambo University College, Post Box. No. 19,

Ambo, Western Shoa - ETHIOPIA

E-mail: dhiksha_rajesh@yahoo.co.in

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