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Single inoculation with an AM Fungus enhanced growth of *Phyllanthus emblica* compared to its co-inoculation with plant growth promoting rhizomicroorganisms

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Abstract

Sustainable agroforestry recommends use of more biologicals including microorganisms for the cultivation of plants. *Emblica officinalis* is an important forest tree species commonly used in Indian Ayurvedic medicine for curing several human ailments. A glass house study was conducted to evaluate the effect of inoculation with the arbuscular mycorrhizal fungus (AMF) *Claroideoglomus etunicatum* and 5 different plant growth promoting rhizomicroorganisms (PGPR) singly and in combination on the growth of *Phyllanthus emblica* (amla) seedlings. The result brought out that single inoculation with AMF *C. etunicatum* is the best compared to all other treatments in improving the growth of amla seedlings. The performance of this microbial inoculation was later evaluated through large scale forest nursery trials at 3 locations with 500 inoculated and 500 uninoculated amla seedlings at each location. The results of the large scale forest nursery trials validated the results of the glass house study. The increase in biovolume index (BI) of inoculated plants, which indicates the quality of seedlings, was 49% (average of 3 locations) compared to uninoculated plants. The inoculated seedlings under large scale nursery trials were planted in wastelands at 2 locations and their establishment was monitored. The BI of inoculated seedlings was 66% more (average of 2 locations) compared to uninoculated seedlings.

Key words – Biovolume index **–** *C. etunicatum* **–** PGPR

Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous, found in all types of soil, climate and environment all over the world (Garcés-Ruiz et al. 2017). It is important in the field of agriculture, horticulture and forestry, as AMF benefits majority of crops by enhancing nutrition, protection against root pathogens (Sikes et al. 2010) and to withstand abiotic stresses, in exchange for photosynthetic products from plants (Pagano & Cabello 2013). These soil fungi forming a symbiotic association with higher plants, facilitate uptake of diffusion-limited nutrients, particularly phosphorus and increase plant productivity (Wang et al. 2017). Furthermore, AMF colonization stimulates the development of microorganisms in the mycorrhizosphere with antagonistic activity

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towards soil-borne pathogens (Linderman 2000, Battini et al. 2016). Though AMF are not host specific but they exhibit host preference thus suggesting the need for selecting an efficient AMF for a particular host, as evidenced by earlier studies (Bagyaraj 2015, Ashwin et al. 2019).

Several factors influence the rate of success of inoculation like AMF species compatibility with the host, interaction with other soil organisms, environment in the target niche, etc. AMF mutually interact with other beneficial soil microorganisms thereby enhance plant growth (Hashem et al. 2016). Because of the difficulty in mass production of AMF, the best way to utilize AMF for crop production would be to concentrate on crops which are normally grown on nursery beds, root trainers or polybags, where they could easily be inoculated with desired AMF, and then transplanted to the field. Plant growth promoting rhizomicroorganisms (PGPR) are another group of beneficial microorganisms that play an important role in maintaining crop and soil health through nutrient cycling and uptake, suppression of plant pathogens, induction of resistance in plant host and direct stimulation of plant growth (Kloepper et al. 2004, Haas & Defago 2005). AMF interacting synergistically with PGPR in soil and enhancing plant growth has been reported by earlier workers in crop plants and forest tree species (Divyananda et al. 2005, Thilagar et al. 2014)

Phyllanthus emblica L. (syn. *Emblica officinalis* Gaertn.) belongs to the family *Phyllanthaceae*, commonly known as amla, emblic, myrobalan, Indian gooseberry etc. The amla fruit is reported to have antioxidant, (Anila & Vijayalakshmi 2003), antihyperlipidemic, (Anila & Vijayalakshmi 2000) and antidiabetic (Abesundara & Matsui et al. 2000) properties and also acts as an important constituent of many available hepatoprotective formulas (Panda & Kar 2003). It is widely distributed in subtropical and tropical areas of China, India, Indonesia, and Malaysia. It is also recommended for afforesting the degraded wastelands. In an earlier study we have screened and selected the best AMF (*Claroideoglomus etunicatum*) for inoculating amla (Srinivasan et al. 2012).

In the present investigation, a glasshouse experiment was undertaken to study the interaction between the selected AMF *C. etunicatum* with 5 different PGPR viz., *Azotobacter chroococcum*, *Pantoea agglomerans*, *Pantoea dispersa*, *Paenibacillus polymyxa* and *Trichoderma harzianum* in order to select the best consortia for inoculating amla. Following the glass house study large scale nursery trials were taken at 3 locations with and without the selected *C. etunicatum* inoculation to validate the results of the glasshouse studies.

Wastelands are unproductive, not fit for cultivation due to rough terrain and eroded soils (Brahmaprakash & Bagyaraj 1998, Maji et al. 2010). Plants in waste lands are subjected to several physiological factors such as hormonal and nutritional imbalance, ion toxicity and susceptibility to diseases (Nadeem et al. 2014). One of the methods recommended for the reclamation of wasteland is the use of suitable microbial inoculants which promote plant growth by regulating nutritional and hormonal balance and by inducing resistance against plant pathogens, thus converting wastelands into cultivable lands. In the present study, it was contemplated to evaluate the growth of amla seedlings raised in large scale nursery trials with the selected AMF and to study their performance when planted in a wasteland for a period of 180 DAS (days after planting).

Materials & Methods

Glass house study

A glass house experiment was conducted to evaluate the response of amla to inoculation with the AMF *C. etunicatum* (selected through an earlier study) and 5 different PGPR, inoculated individually and together to select the best microbial consortium for inoculating amla. The substrate used for growing plants in the study was red sandy loam soil (Alfisol): sand: vermicompost mix in 1:1:0.25 v/v/v ratio. Polybags of the size 30 cm x 20 cm were filled with the substrate to hold 2.5 kg substrate. The substrate mix had a pH of 5.6 and 2.9 ppm available P and an indigenous mycorrhizal population of 18 spores/10 g. AMF *C. etunicatum* maintained in the germplasm bank of CNBRCD, Bangalore using vermiculite: perlite: soil rite 3:1:1 v/v/v ratio as the substrate and

Rhodes grass (*Chloris gayana*) as the host was used in the experiment. The air-dried mix consisting of finely chopped root system plus substrate served as mycorrhizal inoculum.

Three grams of mycorrhizal inoculum having 6,240 infective propagules (IP) were added to the polybag at the seeding point. *Azotobacter chroococcum* was grown for 7 days in Waksman's medium No. 77; *Pantoea agglomerans, Pantoea dispersa and Paenibacillus polymyxa* were grown in nutrient broth for 3 days. The bacterial suspension was then diluted with sterile water to a final concentration to give 10^8 cfu/ ml. *T. harzianum* was grown in *Trichoderma* specific medium for 8 days and macerated in a blender and used as PGPR inoculum having 10^6 cfu/ ml. Five ml of each PGPR suspension was added to the seeding hole of respective polybags depending on the treatment. Each treatment was replicated 12 times. Three seeds were sown in each polybag and watered whenever necessary. Thinning of the seedlings was done to maintain 1 seedling per polybag. The plants were harvested 130 days after sowing (DAS).The plant height was measured from soil surface to the growing tip of the plant. Stem girth was measured 1.0 cm above the soil surface using Vernier calipers. Biovolume index (BI) of the plant was calculated by the formula suggested by Hatchell et al. (1985). Shoot, root and total plant dry weights were determined after drying the samples at 60^{o}C to a constant weight in a hot air oven. The N concentration of the shoot and root was estimated following the micro-Kjeldahl method (Jackson 1973). P concentration was determined by employing the vanado-molybdate phosphoric acid yellow colour method (Jackson 1973). The percent mycorrhizal root colonization was determined by staining fine roots with trypan blue (Philips & Hayman 1970) and estimated adopting the gridline intersect method (Giovannetti & Mosse 1990). The AM fungal spore numbers in the root zone soil was determined by wet sieving and decantation method (Gerdemann & Nicolson 1963).

Large scale nursery trials

Large scale nursery trials were conducted in Mandya district of Karnataka state, India. It was conducted in 3 different locations viz., Indvalu nursery, Mandya; K. Shettihalli nursery, Srirangapatna, and Eliyur nursery, Pandavapura. It is a semi-arid tract where temperature ranges from 16-35^{o}C. The normal annual rainfall is around 700 mm. Polybags of the size of the size 30 cm x 20 cm holding 2.5 kg were filled with soil : sand : compost in the proportion of 1:1:0.25 by v/v/v. The potting mix had a pH of 5.5-5.7. Five hundred amla seedlings were raised in polybags inoculated with selected AMF (*C. etunicatum*) and 500 seedlings were raised as uninoculated seedlings in forest nurseries at 3 locations mentioned above. Thus at each location there were 1000 seedlings, totaling to maintenance of 3000 seedlings at 3 locations. The method of multiplication, and inoculation of AMF was similar to that described earlier under glass house trial. Using random table 40 seedlings from each treatment at each location were labeled for taking observations on plant height, stem girth and to calculate BI. The methods used for determining plant height, stem girth and biovolume index 180 DAS were also similar to that described earlier under glass house study.

Establishment of inoculated seedlings planted in wasteland

40 inoculated and 40 uninoculated seedlings from each of the large scale nursery trials were planted in wasteland at 2 locations in Mandya district of Karnataka state, India. They are Bilidegilu of Mandya range and Melkote of Pandavapura range. This area comes under the dry zone of Karnataka. The soils are highly leached and poor in bases. The normal annual rainfall is around 710 mm. Observations were recorded 8 months after planting, like plant height and stem girth and biovolume index. The methods used for determining plant height, stem girth and biovolume index were similar to that described earlier under glass house study.

Results

Glass house study

The present glass house investigation on amla was carried out to evaluate the effect of the

selected AMF *C. etunicatum* (*Ce*) and 5 different PGPR viz. *A. chroococcum (Ac), P. dispersa (Pd), P. agglomerans (Pa), P. polymyxa (Pp) and T. harzianum (Th)* individually and together on the growth of amla. Among the treatments, single inoculation with the AMF *C. etunicatum* resulted in maximum plant height but statistically on par with the treatments *Pa, Ce+Pd, Ce+Pa* and differing significantly from all other treatments. Regarding stem girth single inoculation with *Ce* increased significantly compared to all the other inoculated and uninoculated treatments. BI followed a similar trend (Table 1). The dry weight of the shoot, root and total plant dry weight was also significantly higher in *C. etunicatum* treatment except the total plant dry weight in the treatment *Ce+Pd.* However the least total plant dry weight was encountered in the uninoculated treatment, bringing out that single inoculation with *C. etunicatum* is the best for inoculating amla (Table 1). The shoot N concentration was significantly higher in *C. etunicatum* alone and *Ce+Pa* inoculated plants compared to other inoculated and uninoculated treatments. Root N concentration was also highest in the treatment *C. etunicatum* alone differing significantly from all other treatments (Table 2). The shoot P concentration was significantly more in the *C. etunicatum* alone treatment differing from all other inoculated and uninoculated treatments. The root P concentration was also significantly higher in *C. etunicatum* alone treatment but on par with the treatment *Ce+Pa* (Table 2). Mycorrhizal root colonization was significantly more in plants inoculated with *Ce* but was statistically on par with the treatments *Ac, Ce+Ac, Ce+Pd* and *Ce+Th* (Table 2). Mycorrhizal spore numbers in the root zone soil was significantly more in *Ce* and *Pd* treatments compared to other treatments and uninoculated control. The results brought out clearly that inoculation with the AMF *Ce* alone is the best in improving the growth of amla seedlings compared to either single inoculation with PGPR or together with the AMF *Ce.*

Large scale nursery trial

In the large scale nursery trial, it was observed that plants inoculated with *Ce* showed a significant increase in all the plant growth parameters studied at all the three locations (Table 3). Plant height increased by 35%, 30%, and 39% significantly in inoculated plants compared to uninoculated plants at Indvalu, K. Shettihalli, and Eliyur forest nurseries. The stem girth was more in inoculated plants than the uninoculated control plants but difference was statistically significant only in Eliyur forest nursery. The BI of inoculated plants was 42%, 35% and 71% more in Indvalu, K. Shettihalli, and Eliyur forest nurseries respectively compared to uninoculated plants. Hence inoculation with only AMF *Ce* was taken up for further studies.

Table 1 Influence of *Claroideoglomus etunicatum* and PGPR on plant height, stem girth, biovolume index and dry weight of amla (130 DAP) under glasshouse conditions.

Treatments	Plant height (cm/plant)	Stem girth (mm/plant)	Bio-volume index	Dry weight (g/plant)		
				Shoot	Root	Total
Uninoculated (U)	14.4^{e}	5.8^{bc}	83.5^{def}	2.1^{cd}	4.4^{e}	6.5^{e}
Claroideoglomus etunicatum (Ce)	24.8^{a}	7.7^{a}	190.9^{a}	4.8^{a}	13.5^{a}	18.3^{a}
Azotobacter chroococcum (Ac)	15.9^{de}	5.6^{bc}	89.0^{cdef}	2.7^{bcd}	5.8^{de}	$8.5d^{e}$
Pantoea dispersa (Pd)	14.4^{e}	5.6^{bc}	80.0^{ef}	2.1^{cd}	10.1^{abc}	12.2^{cd}
Pantoea agglomerans (Pa)	21.4^{abc}	5.5^{bc}	117.7^{bcd}	1.7^{d}	5.6^{de}	7.3^{e}
Paenibacillus (P)	13.2^{e}	5.5^{bc}	72.6^{f}	2.4^{bcd}	7.5^{cde}	9.9^{cde}
Trichoderma harzianum (Th)	17.1^{cde}	6.0^{bc}	102.6^{cdef}	2.7^{bcd}	6.6^{cde}	9.3^{de}
Ce+Ac	20.8^{e}	5.9^{bc}	112.7^{bc}	3.1^{bc}	8.9^{bcd}	11.9^{cd}
Ce+Pd	22.6^{ab}	6.5^{b}	146.9^{b}	3.3^{b}	12.1^{a}	15.4^{ab}
Ce+Pa	24.4^{ab}	6.1^{bc}	148.8^{b}	2.3^{bcd}	11.5^{ab}	13.8^{bc}
Ce+P	19.9^{bcd}	5.4^{bc}	107.4^{cdef}	2.0^{cd}	7.5^{cde}	9.5^{de}
Ce+Th	13.6^{e}	5.2^{c}	70.7^{f}	2.1^{cd}	6.4^{cde}	8.4^{de}

*Mean values for inoculated plants differ significantly at $P \leq 0.05$ level by Student's t-Test

Table 2 Influence of *Claroideoglomus etunicatum* and PGPR on plant N and P concentration, mycorrhizal root colonization and spore numbers in the root zone soil of amla (130 DAP) under glasshouse conditions.

Treatments	N concentration (%)		P concentration (%)		Myc. Root colonization (%)	Myc. Spores no./10g
	Shoot	Root	Shoot	Root		
Uninoculated (U)	0.664^{f}	0.240^{k}	0.487^{c}	0.370^{d}	$2\ (4.9)^{f}$	1.6^{g}
Claroideoglomus etunicatum (Ce)	5.350^{a}	2.490^{a}	0.519^{a}	0.511^{a}	$43\ (41.1)^{a}$	15.0^{a}
Azotobacter chroococcum (Ac)	1.191^{ef}	0.409^{j}	0.459^{c}	0.413^{bcd}	$35\ (36.2)^{ab}$	9.3^{cde}
Pantoea dispersa (Pd)	1.760^{cde}	0.467^{i}	0.376^{h}	0.458^{ab}	$11\ (19.8)^{de}$	13.6^{ab}
Pantoea agglomerans (Pa)	1.838^{cd}	0.466^{i}	0.422^{f}	0.389^{cd}	$23\ (28.6)^{bc}$	8.6^{cdef}
Paenibacillus (P)	0.616^{f}	0.883^{d}	0.363^{i}	0.387^{cd}	$35\ (13.2)^{ab}$	0.6^{g}
Trichoderma harzianum (Th)	1.725^{cde}	0.562^{h}	0.489^{c}	0.364^{d}	$20\ (26.4)^{cd}$	11.0^{bc}
Ce+Ac	3.595^{b}	2.242^{b}	0.496^{bc}	0.439^{bc}	$35\ (36.2)^{ab}$	6.3^{ef}
Ce+Pd	4.008^{b}	1.205^{c}	0.409^{g}	0.436^{bc}	$30\ (33.1)^{ab}$	11.0^{bc}
Ce+Pa	3.603^{b}	2.243^{b}	0.452^{e}	0.388^{cd}	$23\ (28.6)^{bc}$	9.6^{cd}
Ce+P	5.163^{a}	0.826^{e}	0.506^{b}	0.510^{a}	$18\ (25.0)^{cde}$	6.6^{def}
Ce+Th	2.307^{c}	0.588^{g}	0.457^{e}	0.382^{cd}	$35\ (36.2)^{ab}$	7.3^{def}

* Arc sine transformed values are given in parenthesis; Legend as in Table 1

Table 3 Influence of *Claroideoglomus etunicatum* inoculation on amla in large scale nursery trials at three locations (180 DAS)

Treatments	Plant height (cm/plant)	Stem girth (mm/plant)	Biovolume index
Indvalu nursery, Mandya			
Uninoculated	34.00	4.60	160.93
Inoculated	46.16	4.85	229.44
t-test value	4.22**	NS	3.46**
K.Shettihalli nursery, Srirangapatna			
Uninoculated	30.07	4.52	136.35
Inoculated	39.13	4.71	185.21
t-test value	5.49**	NS	4.56**
Eliyur nursery, Pandavapura			
Uninoculated	46.08	10.37	484.53
Inoculated	64.36	12.79	829.17
t-test value	5.15**	5.46**	6.25**

NS = Not significant; * Significant at $P \leq 0.05$

Establishment of inoculated seedlings planted in wasteland

Amla seedlings inoculated with *Ce* in forest nurseries were planted in wasteland at two locations at Bilidegilu of Mandya district and Melkote of Pandavapura district and monitored for 8 MAP. The plant height in inoculated plants was 45% and 21% more than the uninoculated plants at Bilidegilu, and Melkote respectively (Table 4). Stem girth in inoculated plants was significantly more in both the locations than the uninoculated plants. The increase being 21.5% and 27% respectively at Bilidegilu and Melkote. The BI which indicates the quality of the plants was significantly more in inoculated plants in both the locations, the increase being 77% and 55% compared to uninoculated plants planted in wasteland at Bilidegilu and Melkote respectively (Table 4). The results of the present study bring out the benefit of inoculating amla with selected AMF *C. etunicatum*.

Table 4 Response of amla seedlings inoculated with *Claroideoglomus etunicatum* and planted in wasteland (8 MAP)

Treatments	Plant Height (cm/plant)	Stem girth (mm/plant)	B.I.
Biledegalu, Mandya			
U	42.05	6.97	293.08
I	61.15	8.47	517.94
t- test value	2.76**	2.01*	34.46**
Melkote, Pandavapura			
U	42.00	11.79	494.97
I	51.40	14.96	768.69
t- test value	6.32**	2.59**	128.91**

U = Uninoculated; I = Inoculated; MAP = Months after planting; ** Significant at P ≤ 0.0; *Significant at P ≤ 0.05

Discussion

Glass house study

Of the various inoculation treatments studied, *Ce* alone inoculated treatment significantly enhanced plant height, stem girth and BI of the amla seedlings compared to all the other dual inoculated treatments with PGPR and the uninoculated control treatment. This is rather strange as most of the publications report the positive influence of AMF + PGPR on the growth of plants (Ramachandran et al. 2016, Finkel et al. 2017). However higher plant growth due to single inoculation with AMF alone as compared to inoculation with AMF+PGPR has been reported earlier by few workers, though it is rare (Tahmatsidou et al. 2006, Ballesteros-Almanza et al. 2010). Higher BI values indicate higher quality seedlings and hence better establishment when planted in the field (Hatchell et al. 1985). Single inoculation with the AMF *Ce* significantly enhanced shoot, root and total plant dry weights compared to uninoculated plants. Increased plant growth because of AMF inoculation is well documented (Thilagar & Bagyaraj 2015, Ashwin et al. 2019). AMF is known to improve uptake of diffusion-limited nutrients and stimulate the development of microorganisms in the mycorrhizosphere with antagonistic activity towards soil-borne pathogens (Linderman 2000). The shoot and root N concentration and P concentration were also highest in plants inoculated with *Ce* alone. This is because AMF produce extraradical mycelium that takes up mainly P, and also Cu and Zn (Bucking & Kafle 2015). Various mechanisms have been suggested for increased P uptake by mycorrhizal plants like external hyphae exploring greater volume of soil for P away from the root, effective P acquisition by external hyphae by production of phosphatases and Pi transporters and smaller radii of absorptive system (Marschner & Dell 1994, Bagyaraj et al. 2015).

Inoculation with *M* alone recorded the highest percent mycorrhizal root colonization and spore number in the root zone soil. Earlier workers have reported that inoculating plants with *C. etunicatum* increases the percent mycorrhizal root colonization and spore numbers in the root zone soil (Hemlata et al. 2012). The results of the present study wherein AMF alone promoted plant growth better compared to AMF + PGPR inoculation brings out the negative impact of the microbial partners on each other leading to reduced effect on plant growth. It also shows the incompatibility between the AMF *C. etunicatum* and the PGPR used in the present study. This supports the view expressed by few works that microbial consortia may lead to increased, reduced or similar effect compared to individual organism used in the consortium (Sarma et al. 2015).

Large scale nursery trials

Large scale nursery trials taken with selected treatment of *Ce* alone at 3 forest nurseries with 1500 inoculated and 1500 uninoculated plants validated the glasshouse study results. The increase in plant height because of inoculation was 34% (average of 3 locations) compared to uninoculated

plants. The BI of plants which indicates the quality of seedlings was 49% (average of 3 locations) compared to uninoculated plants. This result showed a positive influence of the selected AMF on the growth of amla seedlings.

Establishment of inoculated seedlings planted in wasteland

The plant height and BI of inoculated seedlings planted in wastelands at two locations 8 months after planting was 33% and 66% more (average of 2 locations) respectively compared to uninoculated plants. This suggests that inoculated plants establish better even in wastelands. The plants thus established in the wastelands will reduce soil erosion, increase soil fertility and provide a cleaner environment. This simple nursery technology of inoculating amla with AMF *Ce* will help in afforestation programmers especially in wastelands and degraded forests.

Conclusion

The results show the incompatibility between the AMF *C. etunicatum* and the 5 PGPR used in the study. It can be concluded that inoculation with the AMF *C. etunicatum* is the best AMF for improving the growth and nutrition of amla seedlings raised in forest nurseries. Inoculated seedlings planted in wasteland establish better compared to uninoculated seedlings.

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Conflict of Interest

All the authors of the manuscript declare that they do not have any conflict of interest.

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