Plant Archives Vol. 16 No. 2, 2016 pp. 918-924 *ISSN 0972-5210*

DEVELOPMENT OF MICROBIAL CONSORTIA FOR OVERALL IMPROVEMENT OF *AZADIRACHTA INDICA* SEEDLINGS

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Abstract

Azadirachta indica A. Juss (Neem) is one of the important tree of Indian Thar Desert. The growth of neem seedlings under nursery conditions is often limited by damping off, wilts and leaf feeding insects. The uses of chemical fertilizers are not environment friendly, therefore there is a need of biofertilizers that not only increases the physical fitness of plants, but also helps the plants to fight against the pathogens. The present investigation was carried to find out the best combination of biofertilizers that can enhance the growth of neem seedlings by modifying overall growth parameters for healthy nursery practices. Five well known biofertilizers *viz*. *Azospirillum brasilense, Azotobacter beijerinckii, Bacillus thurengensis* (PSB)*, Trichoderma harzianum*, consortia of AMF (Arbuscular mycorrhizal fungi consisting of species of *Glomus*, *Gigaspora*, *sclerocystis and scutellospora*) were tested individually and in consortia for neem seedlings and observed that the consortia regulates the growth more positively as compare to individual. Overall maximum growth was observed in the treatment in which combination of *Azotobacter+Azospirillum+Trichoderma* followed PSB + *Azotobacter, Azospirillum* + AMF and PSB + *Azotobacter* + *Azospirillum*.

Key words : *Azadirachta indica,* biofertilizers, consortia.

Introduction

The land in general regarded as natural reservoir that contains plenty of benefits and habitat for living organism (Pierra, 1991). The increasing human needs can only be met by enhancing the agricultural productivity but on the other hand due to the shortage of land people has stopped using bio-fertilizer and going with the chemical fertilizer for gaining more productivity in shorter duration (Chowdhury *et al*., 2014). Indiscriminate use of chemical fertilizers leads to decline in soil productivity and also of salts to the soil (Aggani, 2013). Moreover, these chemical fertilizers due to the process of biomagnifications causes serious issues to human being like, skin cancer, or effect on the growth of a baby (Yong, 1994; Battu *et al*., 2004).

The agricultural practices can affect the soil parameters both in positive and negative ways and by using such chemicals as fertilizers the natural nutrients on the soil surface get degraded (Fred, 1991) because these fertilizers hampers the growth of beneficial microbes (Katsunori, 2003; Preap, 2009; Richard, 2010).

Biofertilizers makes the soil rich of nutrients by using microorganisms that also establishes symbiotic relationships with the plants therefore these are cost effective renewable sources of plant nutrients which are alternative to the chemical fertilizers. These biofertilizers also mobilizes nutritionally important elements from non usable to usable form (Rajendra *et al.,* 1998). Mainly there are two groups of biofertilizers *i.e.* symbiotic and non symbiotic. Symbiotic group comprises Rhizobium, Frankia and Mycorrhizae that covers most of the terrestrial and aquatic plant community. While non-symbiotic group includes *Azotobactor*, *Azospirillum*, *Pseudomonas* etc. living in free environment.

Azospirillum belongs to family *Spirilaceae* which not only fixes the environmental nitrogen (about 20-40 kg/ha) (Okon *et al*., 1983), but also produces many compound which regulates the plant growth positively. Therefore there are two main characteristics of genus; it fixes atmospheric nitrogen and produces phytohormones (Tien *et al*., 1979). The major affects which inoculation of *Azospirillum* exert on the plants are root elongation (Dobbelaere *et al*., 1999), development of lateral and adventitious roots (Creus *et al*., 2005; Fallik *et al*., 1994; Molina-Favero *et al*., 2008), root hairs (Hadas and Okon, 1987) and branching of root hairs (Jain and Patriquin, 1985). The species also known to produce plant hormones,

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as well as polyamines and amino acids in culture media (Thuler *et al*., 2003). These phytohormones alter metabolism and morphology of plants, leading to better absorption of minerals and water, consequently larger and healthier plants.

Azotobacter belongs to family *Azotobacteriaceae* and are present found in neutral or alkaline soils. The number of these bacteria is checked by organic matter and presence of antagonistic microorganisms in soil. The bacterium produces anti fungal antibiotics which inhibits the growth of several pathogenic fungi in the root region thereby preventing seedling mortality to a certain extent (Chowdhury *et al*., 2014). *Azotobacter* has beneficial effects on plant growth and yield through, biosynthesis of biologically active substances, stimulation of rhizospheric microbes, producing phyopathogenic inhibitors (Chen, 2006), modifies the mechanism of nutrient uptake and ultimately enhancing the biological nitrogen fixation (Somers *et al*., 2008). The *Azotobacter* in addition to the nitrogen fixation produces Riboflavin, Nicotine, Indol Acetic Acid (Brakel & Hilger, 1965) and Gibberellins. Therefore, *Azotobacter* spp. is often regarded as a member of "Plant Growth Promoting Rhizobacteria (PGPR)". It was also concluded that *Azotobacter* inoculants have a significant promoting effect on growth parameters like root, shoot length and dry mass of bamboo and maize seedlings *in vitro* and in pot experiments (Salhia, 2013).

***Trichoderma* :** Plant growth promotion is one of the indirect mechanisms employed by *Trichoderma* spp. which plays a role in the biocontrol of various plant pathogens and in improvement of plant health. Treatment with *Trichoderma* generally increases root and shoot growth, reduces the activity of deleterious microorganisms in the rhizosphere of plants and improves the nutrient status of the plant. *Trichoderma harzianum* is good solubilizer of phosphorus but different strains show wide variation in their ability to solubilize phosphorus. *T. harzianum* was also reported to solubilize MnO_2, metallic zinc and rock phosphate (mostly calcium phosphate) in a liquid sucrose-yeast extract medium. It has observed that seed treatment of corn with *T. harzianum*, planted in low nitrogen soil resulted in plants that were greener and larger in the early part of the growing season.

Phosphate solubilizer : To restore and maintain the effective microbial populations for solubilization of chemically fixed phosphorus and availability of other macro and micronutrients the inoculations with PSB and other useful microbial inoculants in these soils become mandatory to restore to harvest good sustainable yield of various crops. (Role of Bio Fertilizer in Organic Agriculture: A Review by Mishra, Singh, Mishra and Kumar, 2013). Phosphate solubilizing bacteria (PSB) have great prospects to improve plant growth under given conditions such as in P deficient soils when used in conjunction with AM fungi (Gryndler, 2000).

AMF

Mycorrhizal fungi helps plants in adaptation of plants to unfavourable Conditions (Penninsi, 2004). The AMF fungi not only enhances the nutrient absorption capability of their host plant, but also helps the plants to interact with other soil microorganisms that have an effect on root development and performance (Johanssson *et al*., 2004). The most prominent effect of this group of fungi is improved phosphorus nutrition of the host plant in soil (Koide, 1991). The Mycorrhiza modifies the plants root exudates that affects the rhizosphere microbial community called as mycorrhizosphere effect (Johanssson *et al*., 2004). Mycorrhizal fungi as biocontrol agent has been studied by Siddiqui and Mahmood (1995). Interests in AM fungal symbiosis observed in agriculture and forestry (Friberg, 2001). AM fungi help the plants either by increasing the uptake of elements which are immobile or by accumulation of these elements to prevent their concentrations to reach toxic levels (Pawlowska and Charvat, 2004).

The consortia of these biofertilizer can be used as better inoculants as compare to individuals, as dual inoculation of AM fungi and PSB stimulate plant growth better than inoculation with either organism alone (Kim *et al*., 1997). Similar observation has also been made for the consortia of AM-*Azotobacter* (Lakshman, 1996). Although, differences in growth response of a number of crop and tree species have been demonstrated (Guissou *et al.,* 1998), nothing is known about the interaction between AM fungi, PSB and *Azotobacter* on tree seedling growth (Belimov *et al.,* 1999). Furthermore, very little is known about the microbial status of neem and its response to microbial inoculations (Mamta *et al.,* 2003). The present study was undertaken to evaluate the synergistic effect of indigenous AM fungi, PSB, *Azotobacter, Azospirillum* and *Trichoderma* on different growth parameters of neem.

Materials and Methods

Preparation of Potting Mixture

The potting mixture was prepared by using Soil, Sand and Farm Yard Manure in the ratio of 4:4:5 in the month of July. The mixture were then filled in poly-bags and kept in the shade house of nursery (Arid Forest Research

Institute, Jodhpur).

Production of Neem seedlings

The seeds of Neem were selected on the basis of phenotypic superiority, these seeds were then sown in beds and after germination the small seedlings were transplanted in to the polythene bags. One month old poly-bag seedlings were used for the present study. The seedling production was done in green net house of nursery.

Preparation of bio-fertilizers

The cultures of PSB, *Azotobacter* and *Azospirillum* were procured from MTCC. These liophilized cultures were then activated by using particular growth media which was nutrient broth for PSB, *Azotobacter* defined media for *Azotobacter* and *Luria bertani* for *Azospirillum*. The inoculated plates were then kept at 28°C temperature in an incubator for 2 to 3 days for growth. These plates were used as master for the preparation of broth cultures of the microorganism in the above defined media that were kept at 200rpm at 28°C temperature in an incubator shaker for 4 to 5 days. The CFU for each of the liquid culture has been recorded. *Trichoderma viride* was isolated from rhizosphere soil as described by Sundar *et al*. (1995). Colonies of *Trichoderma* were identified following a standard key (Srilakshmi *et al*., 2001). AMF was isolated and mass multiplied as per methods described by Seiverding (1991).

Experimental Design

Randomized block design was adopted.

In case-I, different biofertilizers were given individually *viz*; PSB ($\mathbf{T_1}$), *Azotobacter* ($\mathbf{T_2}$), *Azospirillum* ($\mathbf{T_3}$), *Trichoderma* ($\mathbf{T_4}$) and AMF ($\mathbf{T_5}$), (table 3). In case-II, different biofertilizers were used in group of two *viz*; PSB + *Azotobacter* ($\mathbf{T_6}$), PSB + *Azospirillum* ($\mathbf{T_7}$), PSB + *Trichoderma* ($\mathbf{T_8}$), AMF + PSB ($\mathbf{T_9}$), *Azospirillum* + *Azotobacter* ($\mathbf{T_{10}}$), *Azotobacter* + Trchoderma ($\mathbf{T_{11}}$), AMF + *Azotobacter* ($\mathbf{T_{12}}$), *Azospirillum* + *Trichoderma* ($\mathbf{T_{13}}$), AMF + *Azospirillum* ($\mathbf{T_{14}}$), AMF + *Trichoderma* ($\mathbf{T_{15}}$), in case-III these microorganism were given in group of three *viz*; *Azospirillum* + PSB + *Azotobacter* ($\mathbf{T_{16}}$), *Trichoderma* + PSB + *Azotobacter* ($\mathbf{T_{17}}$), AMF + PSB + *Azotobacter* ($\mathbf{T_{18}}$), PSB + *Azospirillum* + *Trichoderma* ($\mathbf{T_{19}}$), PSB + *Azospirillum* + AMF ($\mathbf{T_{20}}$), PSB + *Trichoderma* + AMF ($\mathbf{T_{21}}$), *Azospirillum* + *Azotobacter* + *Trichoderma* ($\mathbf{T_{22}}$), *Azospirillum* + *Azotobacter* + AMF ($\mathbf{T_{23}}$), *Azospirillum* + *Trichoderma* + AMF ($\mathbf{T_{24}}$), in case-IV biofertilizers were used in the consortia of four *viz*; *Azospirillum* + *Azotobacter* + *Trichoderma* + PSB ($\mathbf{T_{25}}$), *Azospirillum* + *Azotobacter* + AMF + PSB ($\mathbf{T_{26}}$), AMF + *Azotobacter* + *Trichoderma* + PSB ($\mathbf{T_{27}}$), *Azospirillum* + AMF + *Trichoderma* + PSB ($\mathbf{T_{28}}$), *Azospirillum* + *Azotobacter* + *Trichoderma* ($\mathbf{T_{29}}$) and in case-V combination of all biofertilizers were used AMF + PSB + *Azotobacter* + *Trichoderma* + *Azospirillum* ($\mathbf{T_{30}}$), The $\mathbf{T_{31}}$ were kept as control. In each treatment, 20 seedlings were used (table 1).

Maintenance of treated seedlings

The neem seedlings were kept under the shade house conditions of nursery with proper watering and weeding, Slight digging were also done routinely to maintain the proper aeration in the soil. Now-a-days the problems of snail and leech is very prominent in nurseries of western Rajasthan that could be reason for the destruction of plants at seedling stage, for this we first sprayed the bed with solution of brine which seems to be very effective.

Observations

After 3 months of bio-fertilizer inoculations, following observations were taken to assess the response of seedlings inoculated with the bio-fertilizers:

(a) Growth performance (Height and Girth).

(b) Plant dry matter (Bio-mass).

Measurement of growth and biomass

The representative sample of 3 plants from each treatment was selected randomly for growth performance. These samples were excavated completely along with intact root system and brought to the laboratory conditions where following parameters were recorded

a) Shoot weight
b) Root weight
c) Number of leaves
d) Collar diameter

Note:- The height and girth of seedlings were measured with help of thread and scale while to measure the collar diameter digital vernier caliper were used.

The dry biomass was estimated after keeping plant material in oven at 70°C for 3 days. The biomass yield improvement percentage was calculated on the basis of total biomass as per the formula given below:

$$\text{Improvement percent} = \frac{\text{Biomass of treated plants}}{\text{Biomass of Control plants}} \times 100$$

Results and Discussion

Growth parameters

The deferent growth parameters of *Azadirachta indica* (Neem) under different biofertilizers (both

Table 1 : Different growth parameters of neem seedlings.

T	Diameter Collar	Root weight		Shoot weight		Net biomass	Leaves No.	Root length	Shoot length	Improvement %
		Wet	Dry	Wet	Dry					
T_1	2.10	0.79	0.40	135	0.48	0.88	5.33	17.3	11.00	1.14
T_2	2.73	0.82	0.39	1.46	0.50	0.89	7.33	16.67	11.33	2.29
T_3	2.70	0.65	0.30	1.65	0.61	0.91	7.00	16.83	13.50	4.59
T_4	2.52	0.68	0.35	1.54	0.57	0.93	9.00	15.53	11.07	6.89
T_5	2.79	0.68	0.39	1.31	0.55	0.94	6.00	13.23	14.20	8.04
T_6	3.30	0.74	0.40	1.42	0.50	0.91	6.33	19.02	11.83	4.59
T_7	2.49	1.00	0.44	1.80	0.66	1.10	7.00	19.00	13.17	26.43
T_8	2.74	0.64	0.33	1.69	0.59	0.92	8.00	13.80	12.17	5.74
T_9	2.42	0.72	0.37	1.45	0.58	0.95	6.67	18.43	12.13	9.19
T_{10}	3.09	1.11	0.46	1.76	0.61	1.07	9.00	19.00	12.33	22.98
T_{11}	2.75	1.03	0.48	1.74	0.64	1.12	9.33	20.02	09.83	28.73
T_{12}	3.00	0.83	0.39	1.51	0.52	0.91	6.33	18.50	14.50	4.59
T_{13}	3.02	0.70	0.36	1.85	0.68	1.04	8.00	15.87	13.67	19.54
T_{14}	2.12	0.68	0.35	1.50	0.56	0.91	5.00	13.47	12.00	4.59
T_{15}	2.95	0.61	0.25	1.76	0.64	0.89	6.67	11.67	13.67	2.29
T_{16}	3.05	0.97	0.40	2.18	0.82	1.22	10.00	25.67	13.37	40.22
T_{17}	2.89	1.04	0.42	2.08	0.72	1.14	9.33	16.33	11.83	31.03
T_{18}	2.83	0.76	0.33	1.45	0.56	0.89	6.33	20.90	12.00	2.29
T_{19}	2.85	0.83	0.38	1.77	0.59	0.97	8.33	19.20	13.90	11.49
T_{20}	2.97	1.19	0.51	1.45	0.50	1.01	6.67	20.00	11.83	16.09
T_{21}	3.02	0.95	0.39	1.90	0.68	1.07	8.33	17.67	12.67	22.98
T_{22}	3.28	1.00	0.42	2.32	0.90	1.32	10.67	22.00	14.90	51.72
T_{23}	2.25	0.94	0.45	1.89	0.66	1.11	9.00	19.70	11.37	27.58
T_{24}	3.04	0.70	0.31	1.92	0.62	0.93	8.33	10.27	11.13	6.89
T_{25}	2.84	1.12	0.48	2.18	0.77	1.25	9.00	17.17	09.83	43.67
T_{26}	3.10	1.15	0.50	2.19	0.85	1.35	10.33	19.43	14.20	55.17
T_{27}	2.58	0.82	0.34	1.78	0.62	0.96	8.50	15.50	11.00	10.34
T_{28}	3.05	0.80	0.35	1.04	0.60	0.95	7.67	11.00	14.83	9.19
T_{29}	3.05	0.80	0.35	1.04	0.60	0.95	7.67	11.00	14.83	9.19
T_{30}	3.01	0.84	0.38	1.49	0.50	0.88	9.00	16.00	14.42	1.14
T_{31}	2.75	0.79	0.34	1.47	0.53	0.87	7.10	15.50	10.67	0.00

Note:- Shoot and root weights were calculated in grams while height is in centimeters and the collar diameter is in MM.

individually and in consortia) showed variations to the considerable extent (table 1). The results showed that the consortia of the biofertilizers were more effective as compared to individual microorganisms. However the inoculation of PSB, *Azotobacter*, *Azospirillum* and *Trichoderma* showed improvement in the plant height as compare to un-inoculated seedlings, but the height of neem seedlings both in terms of root and shoot length were found maximum for treatment $\mathbf{T_{16}}$ that includes the consortia of PSB, *Azotobacter* and *Azospirillum* followed by $\mathbf{T_{22}}$ with consortia of *Trichoderma*, *Azotobacter* and *Azospirillum*. It has also been observed in many of the other studies that Phosphate solublizing bacteria along with nitrogen fixing bacteria led to significant increase in radicle and shoot length, shoot weight, coefficient of velocity of germination, seedling vigority index, and significant decrease in mean time of germination (Jahanian *et al*., 2012). The coller diameter was found maximum for $\mathbf{T_{22}}$ (*Trichoderma* + *Azotobacter* + *Azospirillum*) followed by $\mathbf{T_{26}}$ (PSB + *Azotobacter* + *Azospirillum* + AMF) and $\mathbf{T_{16}}$ (PSB + *Azotobacter* + *Azospirillum*). Other studies also suggest that the Inoculation of mycorrhizal fungi with symbiotic nitrogen fixers like *Azospirillum* and *Azotobacter* may improve plant growth and yield due to supplementing the growing plants with fixed nitrogen and growth promoting

substance (Sumner, 1990). Phosphate solubilizing bacteria (PSB) on the other hand solubilize insoluble phosphorus by producing organic acids, which are taken up by the mycorrhizal plants (Rodríguez and Fraga, 1999). AM fungi enhance the uptake and translocation of phosphorus (P) and nitrogen from the soil solution to the root cells (George *et al*., 1995). Similarly, the studies carried out at Hsing University, Taiwan showed 33.2% increase in growth of *Leucaena leucocaphala* seedlings by inoculation with phosphate solubilizing bacteria and synergistic effect on the growth was observed when inoculated with mycorrhizal fungi because the P released upon the activity of PSB does not reaches to the roots due to inadequate diffusion (Barea *et al*., 2005) and the partner AM fungi could improves the uptake of solubilized P (Barea *et al*., 2002). The assessment of growth in terms of number of leaves/plant was found maximum for $\mathbf{T_{22}}$ that was followed by $\mathbf{T_{26}}$ & $\mathbf{T_{16}}$.

Biomass production

Biomass is one of the important criteria for the assessment of overall fitness of a plant. In the present study the biomass were calculated in terms of improvement percentage. Significant difference was observed in biomass of seedlings in relation to different biofertilizers. Again the biomass accumulation was found greater in the seedlings which were treated with consortia instead of individual. Within different combinations of biofertilizers the maximum biomass was recorded for the treatment number 26 with 55.17% in terms of Improvement percent over the control, that contains the multimicrobial interaction of PSB + *Azotobacter* + *Azospirillum* + AMF, which was followed by 51.72% Improvement having the combination of *Trichoderma* + *Azotobacter* + *Azospirillum*. Similar results were also observed by Sharma *et al*. (2015), where the inoculation of AMF, PSB and *Azotobacter* both in isolation and in combination resulted in improvement in growth and biomass of *Tectona grandis* in all the pH and organic matter levels. Some of the previous studies also suggested that AMF enhances the nutrient uptake in seedlings that directly increases the biomass (Marschner and Dell, 1994). Similar affect on biomass accumulation has also been observed by PSB and free living nitrogen fixers (Bongale and Nadiger, 1989). Multimicrobial interactions between AM fungi, PSB and *Azospirillum* have been reported to be synergistic when inoculated together (Muthukumar *et al*., 2001; Belimov *et al*., 1995). Muthukumar *et al*. (2001) confirmed this by inoculating the Neem tree seedlings with *Glomus intraradices*, *Glomus geosporum*, *Azospirillum brasilense* and isolated PSB individually or in various combinations under nursery conditions.

Conclusion

The microorganisms *viz*, *Azospirillum brasilense, Azotobacter beijerinckii, Bacillus thurengensis, Trichoderma harzianum*, consortia of AMF (Arbuscular mycorrhizal fungi consisting of species of *Glomus*, *Gigaspora*, *sclerocystis* and *scutellospora*) individually as well as in different combinations were used on neem seedlings in greenhouse condition, to study their efficacy in promoting growth. The data were recorded on the growth parameters like shoot length, root length, collar diameter, fresh weight and dry weight after 90 days of treatment with these biofertilizers. The results showed that the consortia of the biofertilizers were more effective as compared to individual microorganisms.

Acknowledgment

The authors would like to thank DST for providing the financial support, ICFRE and the Director, Arid Forest Research Institute for providing necessary research facility, encouragement and constant inspiration needed during the course of investigation.

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