Integrated nutrient management by using bioinoculants in seedlings of tea (*Camellia sinensis*) under nursery

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ABSTRACT

A nursery experiment was conducted to standardize the nutrient supply by integrated approach in tea. Five treatments each replicated four times were followed in this trial. Bioinoculants (BF) and/or inorganic fertilisers (IOF) were applied and growth and development of nursery grown BSS (biclonal seed stocks) seedling plants were monitored. During the first six months period of observation, the results indicated that the recommended dosage of inorganic fertilizers with bio inoculants provided significantly higher values of biometric characteristics in terms of stem diameter, number of leaves, biomass production followed by the 100 % recommended practice of IOF and 50% IOF + BF. After 12 months, it was noticed that 100 % IOF had an edge over the other treatments followed by 50 % IOF + BF in most of the biometric parameters.

Key words: Bioinoculants, Integrated nutrient management, tea, etc.,

INTRODUCTION

Tea is the most popular and inexpensive beverage produced from the shoots of commercially cultivated tea plants (*Camellia sinensis* (L.) O. Kuntze). Tea is grown in more than 50 countries, mostly in large plantations. India is the largest producer in the World. Tea nursery is involving the usage of chemical fertilizers especially nitrogenous and phosphatic ones to improve the development of seedlings. Conventional application of inorganic fertilizers is only source of nutrients for the development of seedlings under nursery so far. It may be possible to affect the physical and chemical properties of the soil besides its microbial activities. Hence, [1] stressed the importance of integrated nutrient management in tea to increase its soil health and thus the productivity. Integrated Nutrient Management (INM) involves the use of organic manures, bio fertilizers with a view to reduce the quantity of inorganic sources nutrients.

Coir pith as an industrial waste from coir industries, which is available plenty in the plains of South India when decomposed, served as a good source of organic nutrients to many horticultural crops [2], [3] which will be the best supporting matrix for establishment of beneficial microorganisms. Similarly bio

fertilizers such as *Azospirillum brasilense,* Phosphate solubilising bacteria (*Pseudomonas putida*) and VAM (Vesicular Arbuscular Mycorhizae) are extensively used to make available more quantity of nutrients to the plants so as to increase the productivity with reduction in the use of inorganic fertilizers [4], [5]. With this background, the present study was undertaken on nutrient management by exploitation of bio fertilizers like *Azospirillum brasilense*, Phosphate solubilising bacteria (*Pseudomonas putida*) and VAM (Vesicular Arbuscular Mycorhizae) in combination with inorganic fertilizers in order to standardize the INM in tea under nursery level.

MATERIALS AND METHODS

Morphometric and biometric observation

Bioinculants (BF) and/or inorganic fertilisers (IOF) were applied and growth and development of nursery grown BSS (biclonal seed stocks) seedling plants were monitored regularly. There were 5 treatments namely, 1) 100% Inorganic fertilizers (IOF), 2) Bioinoculants alone (BF - Consortium of *Azospirillum*, phosphate solubilising bacteria and Arbuscular mycorhizae; *Trichoderma* applied separately, 3) Combination of IOF (100%) as recommended in treatment 1 and bioinoculants, 4) 50% reduction in IOF and in combination with bioinoculants and 5) untreated control for comparison. Each treatment, replicated four times with 50 plants (each treatment consisted of 200 plants totally). Fourty plants from each treatment (10 plants from each replicate) were collected randomly and subjected to biometric and biochemical analyses at 6 and 12 months after implementation of treatments. Except the nutrient application, all other nursery practices/plant protection measures were carried out uniformly, irrespective of the treatments. Morphometric parameters such as diameter of stem (mm), number of leaves, height of the shoot (cm), root length (cm), total biomass (fresh and dry weights of shoot and root) and number of secondary roots were documented adopting standard procedures. Dried shoots and roots were used for quantification of nutrient (N, P and K) analyses. Prior to drying, a portion of leaves were sampled and used for estimation of chlorophyll pigments with particular reference to individual treatments. Generated data were statistically analysed in order to interpret the results and to observe the significance of the treatments.

Chlorophyll estimation

The leaf samples of known quantity were weighed and ground in a mortar with sufficient quantities of chilled acetone. The finely ground leaves were extracted with acetone, filtered and made up to 50 mL in a volumetric flask using methanol. This solution was diluted five times with acetone and the absorbance

recorded at 645 and 663 nm. The chlorphyll a, b and total were calculated using the given formulae. The values were expressed as mg per gram fresh weight of green leaves.

Chl a = $12.7\ A_{663} - 2.69\ A_{645}$ x V /(1000 x W)

Chl b = $22.9\ A_{645} - 4.68\ A_{663}$ x V /(1000 x W)

Chl total = $20.2\ A_{645} + 8.02\ A_{663}$ x V /(1000 x W)

Sample preparation for nutrient analysis

The uprooted plants were separated into root and stem portion with leaves. After separation the samples were washed with tap water followed by double distilled water. The samples were kept in enamel plates overnight and dried in an oven at 55 to 70° C. The dried samples were homogenized using a mortar and pestle and stored carefully in polythene bags for further analysis.

Estimation of Nitrogen

Total nitrogen in the plant biomass was estimated by kjeldhal method [6]. The acid digested sample was distilled and subjected to quantify the nitrogen through titrimetric method using 0.07N sodium hydroxide. Total Nitrogen was calculated and represented as per centage basis by the formula,

$$\text{Total N \%} = \frac{\text{(Blank value – Titrated value) x Normality of NaOH x 0.014}}{\text{Sample weight taken}} \times 100$$

Extraction and estimation of P & K content

About 0.2 g of homogenized sample from every plant part was taken in 150 mL conical flask to which 10 mL of 9 : 4 mixture of nitric acid and perchloric acid was added and kept overnight for predigesting to avoid the danger of explosion with perchloric acid [7]. The samples were digested on an electric hot plate. The digested samples were transferred to 100 mL standard measuring flasks and made up with distilled water and used as a stock solution. By use of this stock, the phosphorus content was measured spectrophotometrically [8]. The digested samples were directly fed into the flame photometer and the potassium content was estimated using direct reading Flame Photometer (Sherwood 410).

Enumeration of microflora in rhizosphere and non rhizosphere soil

The soil samples were collected and air dried under shade and used for the enumeration of total bacteria, total fungi, Actinomycetes, *Azospirillum*, Phosphate solubilizing bacteria (PSB) and *Trichoderma*.

The media used for enumeration of Bacteria by Nutrient Agar [9]; for Fungi by Rose Bengal Agar [10]; for Actinomycetes by Starch Casein agar medium [11]; for *Azospirillum* by N-free malic acid medium [12]; for PSB by Pikovskaya's medium [13], for *Trichoderma* by *Trichoderma* specific medium and for *Pseudomonas* by King'B medium [14]. Add 10g soil into 100ml of sterile distilled water to make 1:10 dilution (10^{-1}). Vigorously shake the dilution on a vortex for 5 minutes to obtain uniform suspension of microorganisms. The soil samples were serially diluted from 10^{-4} to 10^{-7}. Transfer 1ml aliquots from each dilution to sterile pertidishes. Add approximately 15ml of the cooled medium (45^{o}C) to each Petridish and mix the inoculum by gentle rotation of the Petridish. Upon solidification of the media, incubate all the plates in an inverted position at 30^{o}C for 2-7 days. At the end of incubation, PSB colonies were visually identified from the clear zone around the bacterial colony. The colony were counted and expressed as number of colonies per gram dry weight of soil sample.

Enumeration of *Azospirillum*

For the enumeration of *Azospirillum*, the rhizosphere soil samples were serially diluted upto 10^{6} dilution using sterile distilled water. One milliliter of the soil diluents from each dilution was transferred to the tubes containing 10ml nitrogen free malic acid semi solid medium and kept for incubation for three days at 35±2^{o}C. The presence of *Azospirillum* was indicated by the formation of white characteristic undulating subsurface pellicle with the change of colour of the medium from yellowish green to blue. Enumeration of *Azospirillum* in soil samples were carried out by most probable number method (MPN). One ml of successive dilutions of 10^{-4}, 10^{-5} and 10^{-6} were transferred to test tubes containing nitrogen free malic acid semisolid medium. Five tubes were maintained for each dilution. The tubes were incubated at room temperature for 3 days. The positive tubes were counted and the population was calculated as per MPN tables [15] and expressed as number of *Azospirillum* per gram dry weight of soil samples.

RESULTS AND DISCUSSION

Effect of Integrated Nutrient Management (INM) on morphometric and biometric parameters of BSS seedlings

Bioinoculants (BF) and/or inorganic fertilisers (IOF) were applied and growth and development of nursery grown BSS (biclonal seed stocks) seedling plants were monitored. During the first six months period of observation, the results indicated that the recommended dosage of inorganic fertilizers with bio inoculants provided significantly higher values of biometric characteristics in terms of stem diameter, number of leaves, biomass production followed by the 100% recommended practice of IOF and 50% IOF + BF (Table

1). After 12 months, it was noticed that 100 % IOF had an edge over the other treatments followed by 50% IOF + BF in most of the biometric parameters. Application of *Pseudomonas aeruginosa LES4* with half dose of fertilizers resulted in growth equivalent to full dose treatment without compromising with the growth and yield of Sesame [16].

The growth promoting effect of Azospirillum in cassava was reported earlier by [17]. Govindan and Purushothaman [18] ascribed the increase in growth characters due to *Azospirillum* to its 'N' fixing role and also the production of phyto-hormones. Ramanandan et al [19] reported that application of recommended NPK fertilizers along with and *Azospirillum* resulted in maximum dry matter production in stem, leaf and tuber at harvest during both the years of investigation as a result of the synergistic interaction between biofertilizers and inorganic fertilizers.

Influence of INM on Chlorophyll content of leaves of BSS seedlings

Chlorophyll was estimated in BSS seedlings which were treated with inorganic and bio fertilisers. Results of chlorophyll content of the leaves, sampled after six months revealed that application of 100% IOF+BF had shown higher amount of total chlorophyll followed by 50% IOF + BF (Table 2) and 100% IOF alone. But bio inoculants alone recorded higher values of chlorophyll content followed by 100% IOF + BF and 100% IOF alone during 12 months period of observation. The nitrogen fixers and Phosphobacteria with phosphate mobilizer present in it might have helped in increasing various growth parameters by exerting its synergistic effect with inorganic and biofertilizers [20]. This could also have accelerated cell division and elongation as well as greater chlorophyll synthesis and higher metabolic activity as suggested by Nazeerahmed and Tanki [21].

Impact of bio inoculants treatment on nutrient status of shoot and root of BSS seedlings

Nutrient status of shoot and root collected from nursery grown BSS seedlings were estimated. At the end of 6 months, it was noticed that the shoot N was significantly higher in 100% IOF + BF followed by 50% IOF + BF (Table 3). No significant variation was observed in terms of P content of shoots of BSS seedlings. Potassium content was significantly higher as it was observed in the case of N. However, 100% IOF + BF and 50% IOF + BF treated roots of BSS seedlings contained significantly higher amount of 'K'. At the end of 12 months, shoots of BSS seedlings followed the identical trend in terms of shoot N. Considering the P content of shoot, 100% IOF alone or in combination with BF and 50% IOF + BF showed significantly higher amount. At the end of 12 months, 100% IOF + BF exerted significant values in terms of NPK content in root. *Casuarina* seedlings inoculated with AM fungi, Frankia, a symbiotic N – fixing actinomycete, associative symbionts of *Azospirillum*, PSB or their mixture showed substantial increase in seedling biomass, nutrient content and use efficiency under nursery conditions [22].

Colonization of microbes in rhizosphere and non – rhizosphere soil

Population dynamics of the microbes in the rhizosphere and non-rhizosphere soils in which BSS seedlings are grown was enumerated and documented in Tables 4 and 5. In rhizosphere soil, application of biofertilizers and the treatment 50% IOF + BF have provided better environment for the soil biota followed by BF alone. In case of non-rhizosphere soil, the number of microbial populations is lesser, in general. However soil application of BF alone followed by 50% IOF + BF offered congenial environment to the bacteria, fungi and actinomycetes. Jayathilake et al reported that highest population of *Azospirillum* x 10^4 CFU/g soil) was recorded in the treatment with *Azospirillum* in combination with VC and FYM (T9) followed by inoculation of Azospirillum with VC and chemical fertilizers. The treatment with RDF (control) recorded the lowest population (1.46 x 10^4 CFU/g soil) and was significantly lower to all the remaining treatments. It sustained the present research findings. It ensures the improvement of soil fertility with higher plant nutrients content and higher population of Azospirillum and phosphobacteria as compared to application of presently recommended dose of chemical fertilizers.

The rhizosphere is the zone of soil surrounding the root which is affected by it. The significance of the rhizosphere arises from the release of organic material from the root and the subsequent effect of increased microbial activity on nutrient cycling and plant growth. In the rhizosphere the quantities and the types of substrates are different from those in the bulk soil and this leads to colonization by different populations of bacteria, fungi, and microfauna.

The association between organisms and roots can be beneficial (water uptake, soil stabilization, growth promotion, N2 fixation, biocontrol, antibiosis, symbiosis), harmful (infection, phytoxicity) or neutral (nutrient flux, free enzyme release, attachment, alle opathy, competition) — these effects often depend on soil conditions and therefore must be regarded as variable. Interactions that are beneficial to agriculture include mycorrhizae, legume nodulation and production of antimicrobial compounds that inhibit the growth of pathogens. Clearly the goal in manipulating the rhizosphere must be to increase the balance of beneficial effects as the rhizosphere is deeply affected by fertilization.

CONCLUSION

The seedling nursery experiment was concluded and the results indicated that application of BF with 50% reduction in inorganic fertilizers registered desired growth parameters. This can be adopted in nursery conditions instead of using 100% of IOF, which is practiced all three years. Results also indicated that integration of BF along with IOF where further cost reduction from 100% IOF application is expected. According to the soil biota, incorporation of bioinoculants enhanced the soil microflora thereby the soil

health has been improved. Application of bioinoculants alone may found on par with growth characteristics of nursery grown control plants, but considering soil health, bioinoculants alone keep the soils "FERTILE" in terms of soil microflora.

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Biotechnology, Govt of India for financial support for carry out this research work and Dr.P.Mohan kumar, Director and Dr.B.Radha Krishnan, Joint Director, UPASI Tea research Institute, Valparai, for his encouragement and Dr.R. Rajkumar,Assistant Director for critical suggestions.

REFERENCES:

[1] P. Swaminathan, "Integrated nutrient management in tea", *UPASI scientific conference*, Coonoor, India. 14 Sep. Bulletin No. 45, 13-28, 1992.

[2] D. Selvi Ranganathan and D. Augustine Selvaseelan, "Effect of mushroom spent rice straw compost on soil physical and physic chemical properties of alluvial and laterite soils". *Madras Agric. J*., 84 (1): 15-19, 1997.

[3] M. Baskar, "Quality appraisal of coir pith and its utilisation in tomato and vegetable cowpea cultivation under greenhouse condition", *M.Sc. (Ag.) Thesis*. Tamil Nadu Agricultural University, Coimbatore – 3, 1996.

[4] A. Morita and S. Konishi, "Relationship between VAM infection and soil P concentration in tea fields", *Soil Sci. Plant Nutr.* 35: 139-143, 1989.

[5] S. Merina Premkumari, "Response of certain horticultural crops to inoculation of VAM fungi, *Azospirillum* and phosphobacteria". *M.Sc. (Ag.) Thesis*. Tamil Nadu Agricultural University,Coimbatore–3, 1991.

[6] AOAC, In: *Official methods of analysis*, 15th edition (eds.) S. Williams and D.C. Washington. Pp: 105 – 107, 1996.

[7] B.A. Haunter,M.S. Johnson, and D.J.Thompson, "Ecotoxicology of Cu and Cd in contaminated grassland ecosystem", *J. Appl. Ecol.*, 24: 573 -586, 1987.

[8] H. Bray and L. T.Kurtz, "Determination of total organic and available forms of phosphorus in soils", *Soil Sci.*, 59: 39-45, 1945.

[9] R.E. Gordon, W.C. Haynes and C.H.N. Pang, In: The genus *Bacillus. Hand book No.427,* Washington D.C. U.S. Department of Agriculture, pp: 50-60, 1973.

[10] J. P. Martin, "Use of acid rose Bengal and streptomycin in the plate method for estimating soil fungi", *Soil Sci*, 69: 215-232, 1950.

[11] E. Kuster and S.T. Williams, "Selection of media for isolation of *Streptomycetes"*, *Nature, Lond*, 202: 928-929, 1964.

[12] J. Dobereiner and J. M. Day, "Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen fixing sites", In: Proceedings of the First International Symposium on Nitrogen Fixation.(eds.) W.E. Newton and C.J. Nyman, Washington State University Press, Pullman, pp. 518-538, 1976.

[13] R.I. Pikovskaya, "Mobilization of phosphorus in soil in connection with vital activity of some microbial species", *Microbiologiya*, 17: 362 - 370, 1948.

[14] E.O.King, M.K. Wart and D.E. Raney, "Two single media for the demonstration of pyocyanine and fluorescein", *J. of laboratory and clinical medicine*, 44: 301-307, 1954.

[15] W.G.Cochran, "Estimation of bacterial density by means of most probable number", *Biometric*, 6: 105-116, 1950.

[16] Sandeep kumar, Piyush Pandey and D.M.Maheswari, "Reduction in dose of chemical fertilizers and growth enhancement of sesame (*Sesamum indicum* L.) with application of rhizospheric competent *Pseudomonas aeruginosa* LES4", Abst: P 2.9. pp:49, 2009.

[17] S. Suchetha, "Effect of application of *Azospirillum*, zinc and iron on growth and development of Cassava", *M.Sc. (Horticulture) Thesis*, Tamil Nadu Agricultural University, Coimbatore. 1989.

[18] M. Govindan and D. Purushothaman, "Production of phytohormones by the nitrogen fixing bacteria, *Azospirillum*". *Agril. Res J. Kerala*, 22 (2):133 –138, 1984.

[19] G. C.Ramanandan, Ravisankar and D. Srihari, " Integrated Nutrient Management for Cassava Under Rainfed Conditions of Andhra Pradesh", *Journal of Root Crops*, Vol. 34 No. 2, pp. 129-136, 2008.

[20] K. Bano, R.D. Kale, and G.G.Gajanan, "Culturing of earthworm Edurillus euginal for cast production and assessment of worm cast as biofertilizers", *J. Soil Biology Ecology*, 7 (2): 98-114,1987.

[21] L. Nazeerahmad. and M.I. Tanki, "Effect of nitrogen and phosphorus on seed production of turnip (*Brassica rapa* L.)". *Veg. Science*, 15 (1):1-7, 1998.

[22] V. Rajesh kannan and T. Muthukumar, "Studies on nutrient use efficiency of bioinoculants and biocontrol agent interactions in *Casuarina equisetifolia* (Forst.) seedlings under tropical nursery condition", First Asian PGPR Congress for sustainable agriculture organized by Acharya N G Ranga Agri University, India and Auburn University, USA. Absrt: P2.1. pp 45, 2009.

	Treatment											
Morphometric parameters	T1(100% IOF) 6 months	12 months	T2(BF) 6 months	12 months	T3(100% IOF+BF) 6 months	12 months	T4(50% IOF+BF) 6 months	12 months	T5(Control) 6 months	12 months	C.D. at P = 0.05: 6 months	12 months
Diameter of Stem(mm)	2.41	2.9 (0.49)	1.38	2.3 (0.92)	2.7	3 (0.3)	1.82	2.6 (0.78)	1.25	1.9 (0.65)	0.52	0.37
No of leaves	12	15.8 (3.8)	8	11.4 (3.4)	13	15.1 (2.1)	10	15.2 (5.2)	7	9.4 (2.4)	1.71	1.03
Shoot height(cm)	15	19.2 (4.2)	14	16.3 (2.3)	18	18.9 (0.9)	14	18.5 (4.5)	13	15 (2.0)	0.8	1.13
Root length (cm)	30	33.6 (3.6)	26	27.5 (1.5)	32	32.1 (0.1)	29	33.1 (4.1)	23	25.7 (2.7)	3.9	2.10
Shoot fresh weight (g)	2.91	4.22 (1.31)	2.4	3.55 (1.15)	3.69	4.12 (0.43)	2.52	3.94 (1.42)	2.28	3.01 (0.73)	0.41	0.49
Root fresh weight (g)	1.34	2.71 (1.37)	1.09	2.16 (1.07)	1.77	3.25 (1.48)	1.21	2.84 (1.63)	1.04	1.91 (0.87)	0.27	0.46
Shoot dry weight (g)	0.21	0.95 (0.74)	0.16	0.71 (0.55)	0.26	0.95 (0.69)	0.19	0.87 (0.68)	0.15	0.55 (0.4)	0.04	0.09
Root dry weight (g)	0.22	0.61 (0.39)	0.17	0.52 (0.35)	0.27	0.78 (0.51)	0.18	0.67 (0.49)	0.17	0.36 (0.19)	0.03	0.11
No of secondary roots	2	3.5 (1.5)	1	2.8 (1.8)	2	3.9 (1.9)	1	4.1 (3.1)	1	2 (1.0)	0.6	1.40

Table 1. Morphometric and biometric parameters of BSS seedlings observed at 6 and 12 months interval

Values in parentheses indicate the difference between 6 and 12 months of obseration

Table 2: Chlorophyll content of leaves of BSS seedlings – 6 and 12 months

Treatment	6 months (mg. / g fr.wt.)			12 months (mg. / g fr.wt.)		
	Chl. a	Chl. b	Total chl.	Chl. a	Chl.b.	Total chl.
100% IOF	2.92	1.49	4.10	3.13	1.71	4.84
Bio inoculants alone	2.34	1.19	3.55	3.91	2.12	6.02
100%IOF+BF	2.92	1.59	4.64	3.34	1.74	5.08
50% IOF + BF	2.86	1.53	4.13	3.05	1.55	4.60
Control	1.92	0.99	2.84	2.68	1.22	3.90
C.D. at P = 0.05:	**0.06**	**0.09**	**0.80**	**0.68**	**0.58**	**1.21**

Table 3: Effect of bio inoculants treatment on nutrient status of shoot and root of BSS seedlings (12 months)

Treatment	Shoot			Root		
	N(%)	P(%)	K(%)	N(%)	P(%)	K(%)
100% IOF	3.71	0.23	1.47	2.40	0.14	1.21
BF	3.21	0.13	1.25	1.55	0.14	1.21
100%IOF+BF	4.13	0.27	1.74	3.18	0.23	1.78
50% IOF + BF	3.92	0.23	1.56	3.15	0.15	1.26
Control	2.67	0.12	1.18	1.35	0.14	1.05
C.D. at P = 0.05:	**0.92**	**0.05**	**0.70**	**0.92**	**0.05**	**0.33**

Table 4: Microflora in rhizosphere soil - 12 months

Treatment	Colony forming unit / g. dry wt						
	Bacteria ($x10^7$)	*Fungi* ($x10^5$)	*Actinomycetes* ($x10^6$)	*PSB* ($x10^5$)	*Azospirillum* ($x10^6$)	*Trichoderma* ($x10^3$)	*Pseudomonas* ($x10^4$)
100% IOF	2.8	1.0	1.5	3.3	2.4	0	5.0
BF	6.4	10.0	6.5	4.3	4.7	3.5	12.0
100% IOF+BF	3.0	1.3	1.0	7.0	3.6	0	0.5
50% IOF + BF	13.7	4.0	1.5	6.3	7.8	5.5	27.0
Control	2.6	2.5	1.6	3.7	0.08	0	1.0

Table 5: Microflora in non rhizosphere soil - 12 months

Treatment	Colony forming unit / g. dry wt						
	Bacteria ($x10^6$)	Fungi ($x10^4$)	Actinomycetes ($x10^4$)	PSB ($x10^5$)	*Azospirillum* ($x10^6$)	*Trichoderma* (0)	*Pseudomonas* (0)
100% IOF	2.2	1.1	10.0	0.13	0.1	0	0
BF	7.6	6.5	3.2	2.1	0.4	0	0
100%IOF+BF	5.1	2.0	1.0	0	0.4	0	0
50% IOF + BF	6.2	3.1	5.7	0	0.1	0	0
Control	2.1	4.3	1.0	1.0	0.1	0	0