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Bio-nutrition for improving the vigour of FCV tobacco (*Nicotiana tabacum*) seedlings

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ABSTRACT

The efficacy of different biofertilizers on tobacco (*Nicotiana tabacum* L.) seedbeds was evaluated at the nursery site of ICAR-Central Tobacco Research Institute, Rajahmundry during 2010-11. The combined inoculation of nitrogen fixing (*Azospirillum*/*Azotobacter*), phosphorus solubilizing (*Bacillus subtilis*) and K-mobilizing (*Frateuria aurantia*) bacteria increased the percent germination, seedling height, leaf area, chlorophyll content, dry weight of the seedlings and total number of transplantable seedlings as compared to individual inoculation and uninoculated control after 60 days of sowing. Besides increasing the nitrogen fixing, phosphorus solubilizing and potassium mobilizing abilities, microbial inoculants have played a vital role in suppressing the population of fungal pathogen *Pythium aphanidermatum* causing damping-off in tobacco seedbeds. Improvement in nitrogen, phosphorus and potassium content of the seedlings was observed due to the inoculation of these beneficial microbes individually or in consortia. The results suggest that PGPR based inoculants can be used and should be further evaluated in nursery management practices.

Key words: *Azospirillum, Azotobacter, Bacillus subtilis,* Bio-fertilizer, *Frateuria aurantia,* Tobacco nursery

Flue Cured Virginia (FCV) tobacco (*Nicotiana tabacum* L.) is a high-value commercial crop and an important contributor to the national exchequer as a foreign exchange earning crop. Since tobacco is a quality conscious crop and leaf is the economic product, indiscriminate use of pesticides and fertilizers is not desirable. Utility of microorganisms that improve soil fertility and enhance plant nutrition has continued attraction due to the increase in cost of fertilizers and their negative impact on environment (Asad *et al.* 2008). Hence exploitation of plant growth promoting rhizobacteria (PGPR) is a promising alternative to minimize the use of chemical fertilizers and pesticides (Subhashini 2013). Plant Growth Promoting Rhizobacteria (PGPR) are originally defined as root-colonizing bacteria, i.e *Bacillus subtilis* and *Pseudomonas fluorescens*, that cause either plant growth promotion or biological control of plant diseases (Vassilev *et al.* 2006). The principal mechanisms of growth promotion include production of growth stimulating phytohormones, solubilization and mobilization of phosphate, siderophore production, antibiosis, i.e. production of antibiotics, inhibition of plant ethylene synthesis, and induction of plant systemic resistance to pathogens (Richardson *et al*. 2009).

Leaf being the end product in tobacco, field planting of vigorous and healthy seedlings is the pre-requisite for successful establishment in the field. Nutrition plays an important role in improving the vigour of seedlings. In general, use of chemical fertilizers helps raise healthy seedlings of various agricultural and horticultural crops. There are several reports that PGPR has promoted the growth of reproductive parameters of plants ranging from cereals, pulses, ornamentals, medicinal and aromatic plants, vegetable crops, and even tree species (Cakmakc *et al.* 2007). Treatment with PGPR has increased the germination percentage, seedling vigour, emergence, plant stand, and root growth, shoot growth, total biomass of the plants, seed weight, early flowering, increased grain, fodder, fruit yields etc. (Subhashini and Padmaja 2010). The exact mechanism involved in growth promotion when agronomic crops are inoculated with rhizobacteria include increase in the production of auxin, gibberellins, cytokinin, ethylene, the solubilization of phosphorus and oxidation of sulfur, increase in nitrate availability, the extracellular production of antibiotics, lytic enzymes, hydrocyanic acid, increases root permeability, ACC (1-aminocyclopropane-1-carboxylate) deaminase activity, siderophore production, enhancing biological nitrogen fixation and enhancement in the uptake of essential plant nutrients (Belimov *et al.* 2007) which is extensively studied in numerous species of plant growth promoting bacteria like *Bacillus* (Cazorla *et al.* 2007) and *Pseudomonas* (Subhashini and Padmaja 2010). Furthermore, the plants grow faster and greener with longer roots and shoots than the untreated plants (Subhashini and

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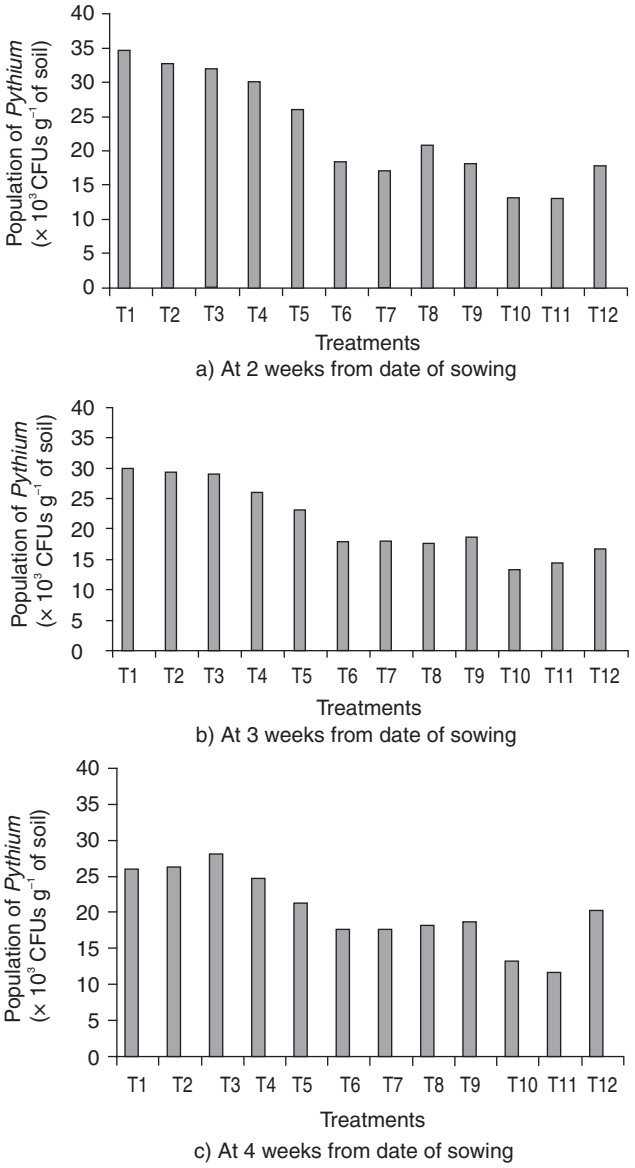

Fig 1 Effect of bio-inoculants on the population of *P. aphanidermatum* (cfu/g) in tobacco seed beds

Padmaja 2009). The objective of this study is to evaluate the effect of different biofertlizers and their combination using free living non symbiotic nitrogen-fixer (*Azospirillum brasiliensis* or *Azotobacter chroococcum*), Phosphorus-solubiliser (*Bacillus subtilis*) and potassium-solubiliser (*Frateuria aurantia*) on growth and nutrient uptake of seedlings in tobacco nursery.

MATERIALS AND METHODS

The experiment was conducted during 2010-11 in the nursery site of ICAR-CTRI, Rajahmundry in a randomized block design with 12 treatments and three replications. The nursery soil was of sandy loam type and poor in nitrogen, phosphorus and organic carbon content. The soil was analysed before starting the experiment for its chemical characteristics such as pH (6.6), electrical conductivity (0.28 dS/m), organic carbon (0.25%), available P (10.1 mg/kg) and available K (130 mg/kg). Nitrogen, phosphorus and potassium (NPK) were applied at the rate of 10: 30: 5 kg/ha as ammonium sulphate, single super phosphate and potassium sulphate, respectively and 4 g of chlorpyrifos dust was also applied to prevent the ant and insect damage to the seed before sowing.

The treatments were T1-Control; T2 - 100 % recommended dose of fertilizer (RDF); T3 - 75% recommended dose of fertiliser (RDF); T4 - 75% RDF+ *Azospirillum braziliensis* @ 1x 10^8; T5-75% RDF+ *Bacillus subtilis* @ 1x 10^9 / g; T6 - 75% RDF+ *Frateuria aurantia* @ 1x 10^9 CFU; T7- 75% RDF (*Azospirillum* + *B. subtilis*); T8 - 75% RDF (*Azospirillum* + *F. aurantia*) ; T9 - 75% RDF (*B. subtilis* + *F. aurantia*) ; T10-75% RDF (*Azospirillum* + *B. subtilis* + *F. aurantia*); T11 - 75%RDF + (*Azotobacter chroococcum* + *B.subtilis* + *F. aurantia*); T12 - 75%RDF + (*Azotobacter* + *F. aurantia*).

The biofertlisers *Azotobacter, Azospirillum* and *Bacillus subtilis* were obtained from TNAU, Coimbatore and *Frateuria aurantia* was obtained from Gokulum Biotech, Pondicherry. The bacterial cultures were mixed with FYM and applied to the seedbeds before sowing. Effective microbial counts were enumerated before applying to the field. For inoculation with biofertilizers tobacco seed (var. Siri (@ 0.5g of seed/m^2bed) mixed with sand and biofertiliser were sown immediately on the seed beds. Germination counts were taken on 7th day after sowing by using petridishes of 10cm diameter. Petridish was placed on seedbed and count of germinated seed was taken from the petridish covered area. Germination count was calculated using m^2.

For PGPR population measurement, 1.0 g of dry soil was added to 9ml sterilized water to prepare a soil solution. PGPR count in the rhizosphere soil was recorded using dilution plate method 3 days after incubation at 30°C (Subhashini 2013).

The population of *Pythium* in the tobacco seedbeds was estimated at 2, 3 and 4 weeks from the date of sowing following the method of Subhashini and Padmaja (2009). The soil samples collected from different treatments were sieved and 1 g of this sample was suspended in 10 ml of sterile water and shaken well by means of sterile pipette. A dilution of 10^{-2} was prepared. One ml of this dilution was dispersed as small drops in the surface of three-day-old 2 per cent plain agar in Petri plates. These plates were incubated at 24 ± 2ºC and observed after 24 hr. The number of hyphal strands emerging from the perimeter of each drop was counted under low power (10x of the Carl Zeiss microscope). Hyphae of *Pythium* can be easily distinguished from other fungal hyphae by their rapid growth and tendency to grow in a straight line away from the drop. The same method was used to estimate population of *Pythium* in various treatments. Data was subjected to analysis of variance.

The N fixing, P-solubilzing bacteria(PSB) and K-mobilizing bacteria (KMB) from fresh soil sample was estimated using serial dilution techniques on agar plates with specific media at 45 days after sowing. The seedlings

Table 1 Survival and establishment of bioinoculants in the rhizosphere of tobacco seedlings

Treatment	Microbial Population (1×10^5CFUs/g)			
	Bacillus subtlis	*Frateuria aurantia*	*Azospirillum*	*Azotobacter*
Control	34	27	16	9
100 % recommended dose of fertiliser (RDF)	32	26	19	11
75% recommended dose of fertilizer (RDF)	34	32	18	9
75% RDF + *Azospirillum* @ 1×10^8 CFU / g	24	23	51	14
75% RDF + *B. subtilis* @ 1×10^9 CFU / g	44	27	28	5
75% RDF + *F. aurantia* @ 1×10^9 CFU / g	29	105	28	8
75% RDF + *Azospirillum* + *B. subtilis*	46	36	38	10
75% RDF + *Azospirillum* + *F. aurantia*	25	65	31	10
75% RDF + *B. subtilis* + *F. aurantia*	48	87	16	11
75% RDF + *Azospirillum* +*B. subtilis* + *F. aurantia*	87	68	29	7
75% RDF + *Azotobacter* + *B.subtilis* + *F. aurantia*	56	63	19	4
75% RDF + *Azotobacter* + *F. aurantia*	35	54	19	9
SEm±	1.39	2.77	1.96	0.97
CD (P=0.05)	4.09	8.15	5.76	2.85
CV %	5.85	9.37	12.98	18.24

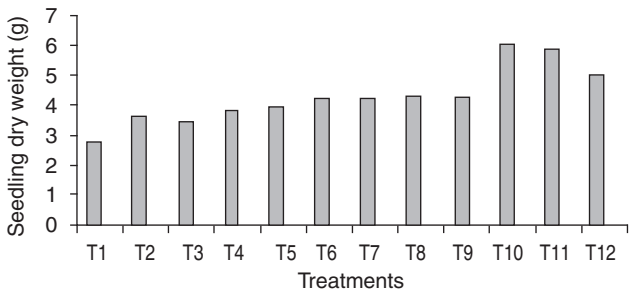

Fig 2 Effect of bio-inoculants on dry weight (g) of seedlings in tobacco nurseries

were pulled and leaf area was measured using Leaf area meter (Model CI 202) and total chlorophyll content was determined following the method suggested by Hiscox and Israelstam (1979).

At 60 days after sowing, tobacco seedlings were pulled, washed with deionized water, dried at 70°C and the dry weight was recorded. Seedlings were ground into fine powder and analyzed for total N, P and K (Jackson 1973).

The data were subjected to analysis of variance appropriate to RBD, treatment means where significant were compared using critical difference method.

RESULTS AND DISCUSSION

The results on the effect of seed inoculation with single, double or triple combination of bioinoculants on the population of bioinoculants in the rhizosphere of the respective treatments, germination, seedling height,

Table 2 Effect of biofertilisers on chlorophyll content and growth of FCV tobacco seedlings

Treatment	Seedling height (cm)	Germination count	Leaf area (cm^2/plant)	Total chlorophyll (mg/g fresh weight)
Control	13.33	48	157	0.70
100 % recommended dose of fertiliser (RDF)	14.70	59	195	0.87
75% recommended dose of fertilizer (RDF)	13.43	54	185	0.78
75% RDF + *Azospirillum* @ 1×10^8 CFU / g	14.56	52	240	0.93
75% RDF + *B. subtilis* @ 1×10^9 CFU / g	14.73	58	261	0.96
75% RDF + *F. aurantia* @ 1×10^9 CFU / g	14.54	59	253	0.95
75% RDF + *Azospirillum* + *B. subtilis*	15.27	58	280	1.03
75% RDF + *Azospirillum* + *F. aurantia*	14.93	56	292	1.09
75% RDF + *B. subtilis* + *F. aurantia*	16.20	63	291	1.10
75% RDF + *Azospirillum* +*B. subtilis* + *F. aurantia*	17.01	75	336	1.21
75% RDF + *Azotobacter* + *B.subtilis* + *F. aurantia*	17.13	72	350	1.22
75% RDF + *Azotobacter* + *F. aurantia*	15.33	68	293	1.12
SEm ±	0.21	1.94	6.29	0.02
CD (P=0.05)	0.63	5.69	18.4	0.05
CV%	2.38	6.35	4.18	2.80

chlorophyll content, leaf area, incidence of root rot, dry weight, number of healthy transplants and NPK content is discussed below.

Enumeration of population of P. aphanidermatum

The present study revealed that the population of *P. aphanidermatum* was gradually reduced in all the treatments up to 30 days after sowing (Fig 1). Population of *P.aphanidermatum* was recorded highest at 2 weeks after sowing in all the treatments. The maximum reduction in the pathogen population was noticed in treatments 75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia* and 75% RDF + *Azotobacter* + *B.subtilis* + *F. aurantia*. These findings are in confirmation with the report of Subhashini and Padmaja (2009) for tobacco damping off disease. The reduction in population of *Pythium* was due to competition of antagonist, in addition to the antibiotics. Subhashini and Padmaja (2009) reported that *Pythium* spp are poor competitors and thus their population may be replaced due to the competitive effect of antagonist and found a correlation between the ability of various bacterial strains to compete for nutrients with germinating oospores of *P. aphanidermatum* and their efficiency as biocontrol agents against damping off caused by *P.aphanidermatum*. Osburn *et al.* (1989) implicated, competition for infection sites as a mechanism for biological control of sugar beet infected by *Pythium ultimum* and further documented that *Pseudomonas putida* strain colonized pericarp, seed and roots of sugar beets.

Inoculum establishment in the tobacco rhizosphere

The treatments 75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia* and 75% RDF + *Azotobacter* + *B.subtilis* + *F. aurantia* significantly increased the extent of population of applied bioinoculants in the root system compared to the uninoculated control (Table 1). The results indicated that the population size of the introduced bioinoculants varied in accordance with the treatment, with chemical fertilizer showing the least population in the rhizosphere. Absence of bioinoculants resulted in a lower level of bacterial population in the rhizosphere, compared to the single, double or triple inoculated treatments with RDF. The colonization of *F. aurantia* showed a strong stimulating effect on the population of KMB (Subhashini 2014). It has already been noted that PSB can act as helper bacteria complementing the ability of nitrogen fixers and K-mobilizers to colonize plant roots (Riedel *et al.* 2008). Specialized bacterial activities such as the production of vitamins, amino acids, and hormones may be involved in these interactions and the presence of rhizobacterial inoculation might have assisted in the multiplication of beneficial bacteria (Sala *et al.* 2007).

Seedling productivity

The first sign of adaptability of PGPR strains to the root system of tobacco was the proliferation and clumping of bacteria around the root tips followed by entry into cortical cells of the root (Subhashini and Padmaja 2010). In case of *B. subtilis* it was able to enter into vascular tissues of the root system, indicating its endophytic nature (Compant *et al.* 2005). Seed inoculation with single, double or triple combination of bioinoculants enhanced the population of bioinoculants in the rhizosphere of the respective treatments (Table 1), germination, seedling height, chlorophyll content, leaf area (Table 2), incidence of root rot (Fig 1), dry weight (Fig 2), number of healthy transplants (stem girth of pencil thickness; Fig 3) and NPK content (Table 3). Higher seed germination was observed with treatment 75% RDF + *Azospirillum* + *B.subtilis* + *F. aurantia* and lowest germination was observed in uninoculated control. Durgannavar *et al*. (2004) reported improved seed germination in different crops upon inoculation with different nitrogen fixing bacteria and AM fungi.

Sabannavar and Lakshman (2011) stated that the introduced populations of beneficial soil microorganisms like *Azotobacter* and phosphate-solubilizing bacteria exerted a synergistic effect on seedling growth. Significant increase in the seedling height was observed upon inoculation of tobacco nursery seed beds with various beneficial microorganisms (Table 2). The present study revealed that combined application of consortia of all the three microorganisms (*Azospirillum/Azotobacter, B. subtilis and F. aurantia*) significantly improved number of healthy

Table 3 Effect of bio-inoculants on nutrient content (NPK) in tobacco seedlings

Treatment	Nutrient content (%)		
	N	P	K
Control	2.690	0.180	1.330
100 % recommended dose of fertiliser (RDF)	2.823	0.217	1.723
75% recommended dose of fertilizer (RDF)	2.710	0.233	1.750
75% RDF+ *Azospirillum* @ 1 × 10^8 CFU / g	2.837	0.237	1.760
75% RDF + *B. subtilis* @ 1 × 10^9 CFU / g	2.700	0.273	1.777
75% RDF + *F. aurantia* @ 1 × 10^9 CFU / g	2.710	0.250	1.963
75% RDF + *Azospirillum* + *B. subtilis*	2.727	0.277	1.830
75% RDF + *Azospirillum* + *F. aurantia*	2.790	0.250	1.900
75% RDF + *B. subtilis* + *F. aurantia*	2.787	0.290	1.913
75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia*	4.110	0.373	2.160
75% RDF + *Azotobacter* + *B.subtilis* + *F. aurantia*	4.120	0.383	2.147
75% RDF + *Azotobacter* + *F. aurantia*	2.873	0.263	1.947
SEm ±	0.028	0.011	0.028
CD (P=0.05)	0.081	0.032	0.082
CV%	1.58	6.99	2.61

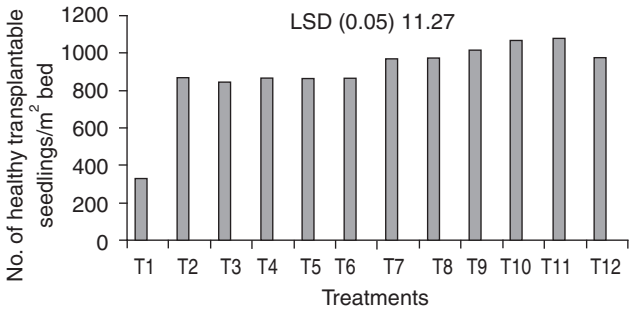

Fig 3 Effect of bio-inoculants on the number of healthy transplantable seedlings

transplantable seedlings (Fig 3) compared to the individual application or combination of two bioinoculants and uninoculated control supporting the contention of earlier workers.

Leaf area and chlorophyll content

Application of nitrogen fixing, phosphorus solubilizing and K mobilizing bacteria significantly improved leaf area and total chlorophyll content in 60 days old tobacco seedlings with maximum enhancement in all the parameters under dual and triple inoculation (Table 2). These observations are in conformity with those of Baqual *et al.* (2005) who reported higher chlorophyll content and photosynthesis in banana upon inoculation with nitrogen fixing bacteria. The enhancement in plant growth and biomass production might be due to increased rate of photosynthesis and higher metabolic activities, mobilization of some movable forms of important nutrients especially phosphorus, production of growth regulators and inhibition of soil borne pathogens by the bioinoculants .

There was significant increase in dry weight of tobacco seedlings due to combined inoculation of all the three beneficial microorganisms. Application of *Azospirillum*/ *Azotobacter* or *B. subtilis* or *F. aurantia* separately did not significantly increase seedling dry weight.

Nutrient content

Nitrogen, phosphorus and potassium content were significantly improved in tobacco seedlings upon inoculation with consortia of microorganisms. Higher amount recorded was due to the synergistic effect leading to improved biomass and higher nutrient concentration (Table 3) in the inoculated plants. Nitrogen content was maximum in plants inoculated with nitrogen fixing bacterium, while phosphorus content was more with phosphorus solubilizing bacteria and potassium content was more with K-mobilizing bacteria (Wani *et al.* 2007). Multiple inoculation significantly improved NPK content of the plant (Table 3). The results are in conformity with those of Douds (2007) who had observed higher uptake of phosphorus in different fruit and forest crops upon inoculation with P-solubilizers. The enhancement in growth might be due to the production of siderophores by phosphorus solubilzing bacteria (PSB) and its activity in biological control of soil-borne pathogens (Subhashini and Padmaja 2009), ability of PSB for the uptake of immobile ions and enhanced absorbing surface area of the roots (Hamel and Strullu 2006). It is well known that the magnitude of plant response to any microbial inoculation greatly affects NPK content and uptake of the plant (Subhashini 2014). Moreover the combined inoculation effects were greater than the individual inoculation effects suggesting synergism beyond simple additive effects (positive multiplication interaction) (Khan and Zaidi 2007).

From the results, it is suggested that the consortia of free living non-symbiotic N- fixer (*Azospirillum brasiliensis* or *Azotobacter chroococcum*), P-solubiliser (*Bacillus subtilis* and K-mobilizer (*Frateuria aurantia*) could be successfully used in tobacco nurseries to produce healthy, vigorous and more number of transplantable tobacco seedlings.

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