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Effect of bio-inoculation of AM fungi and PGPR on the growth, yield and quality of FCV tobacco (*Nicotiana tabacum)* in Vertisols

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ABSTRACT

Applications of beneficial microbes, biofertilizer, are well known as an alternative to chemical fertilizer application in sustainable crop production. The objective of this field study was to evaluate the effect of co-inoculation with bacteria and arbuscular mycorrhizal (AM) fungi on the growth, yield and quality of tobacco (*Nicotiana tabacum* L.) in vertisols for two years (2008 and 2009). Sixty days old tobacco seedlings were inoculated with single, double and triple inoculations. The results revealed that triple inoculation of *Glomus intraradices* + *Psuedomonas flourescens* and *Azotobacter chroococum* stimulated increased root colonization, plant growth, plant biomass, gas exchange parameters, nitrogen, phosphorus and potassium content of leaf, cured leaf yield, grade index and quality significantly over the double and single inoculation treatments. Comparative analysis showed that the presence of *Pseudomonas fluorescens* inoculant alone and in combination of treatments played an effective role in stimulating growth, yield and quality grades of FCV tobacco leaf. Tobacco crop is grown for leaves instead of seed as in many crops.. The association of bacteria and AM fungi indicated that rhizosphere bacteria are involved in the beneficial effects of AMF on plant growth and enhanced tobacco yield and quality.

Key words: Arbuscular mycorrhizal fungi, *Azotobacter, Pseudomonas fluorescens,* Tobacco

Chemical nitrogen fertilizers are used worldwide to sustain and enhance the crop yields. Inspite of its efficiency in promoting crop yields they have proved to be hazardous for soil health and well being of human and animal populations (Abasi *et al*. 2011). Recent advances in agriculture are focused on the reduction of the use of inorganic fertilizers, search for alternative ways to improve crop yield in sustainable agriculture (Zaidi *et al*. 2009). Plant growth promoting rhizobacteria (PGPR) play an important role in mineralization and immobilization of nutrients needed for crop growth. They are assumed to be an alternative to the use of chemicals (Fischer *et al.* 2007). PGPR may benefit the host by causing plant growth promotion or biological disease control (Chen 2006). PGPR activity has been reported in strains belonging to several genera, such as *Azotobacter, Azospirillum, Pseudomonas, Acetobacter* and *Bacillus* (Kloepper 1993).

Providing a direct physical link between the soil and plant roots, arbuscular mycorrhizal fungi (AMF) are important rhizospheric microorganisms and there is well documented evidence that AMF contribute to increasing availability and uptake of P and micronutrients (Maiti 2010). The AMF symbiosis enhances plant growth and increases the plant's access to forms of nitrogen that are unavailable to non-mycorrhizal plants (Khade and Rodrigues 2009). The effect of co-inoculation of PGPR and arbuscular mycorrhizal fungi on plant growth and nutrient content has been studied by Sabannavar and Lakshman (2011). Various *Pseudomonas* species have been shown to be effective in controlling pathogenic fungi and stimulating plant growth by a variety of mechanisms including production of siderophores, synthesis of antibiotics, production of phytohormones, enhancement of phosphate uptake by the plant, nitrogen fixation and synthesis of enzymes that regulate plant ethylene levels (Subhashini and Padmaja 2009). Synergistic positive interactions have been reported between AM fungi and plant growth promoting bacteria (PGPB) such as N fixers, fluorescent pseudomonads, and sporulating bacilli (Khan and Zaidi 2007).

Tobacco (*Nicotiana tabacum* L.) is an important commercial crop which plays a significant role in Indian economy. Tobacco continues to be an important industrial crop in India providing employment to 36 million people including 6 million tobacco farmers fetching ₹ 8 200 crores as excise revenue and ₹ 1 362 crores as foreign exchange. As leaf is the economic product, indiscriminate use of chemical fertilizers and pesticides effects the quality and export potential of the commodity. The use of chemical fertilizers

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can be reduced by exploiting the potential of bio-inoculants which are inexpensive and eco-friendly (Subhashini and Padmaja 2011). Plant-growth promoting effects of endomycorrhizal and bacterial inoculation have been demonstrated in sesamum (Sabannavar and Lakshman 2008). Arbuscular mycorrhizal fungi (AMF) facilitate higher water absorption and nutrient uptake in plants, which in turn helps to combat various diseases and enhances plant growth AMF are also known for increasing phosphorus uptake and plant growth promotion at low P levels in the soil solution (Baslam *et al.* 2011). Phosphorus, nitrogen, zinc, and copper are the most commonly reported elements whose uptake is enhanced by AMF in plants; however, acquisition of other mineral nutrients required for plant growth may also be enhanced (Subhashini and Padmaja 2010). Significant efforts have been made to elucidate the role of soil microbiota in relation to mycorrhizal association and their effects on development of the host plant and its productivity. Beneficial effects of AMF, such as growth promotion, increased root branching in lengths of lateral roots, specific root length, root diameter, transplant performance, protection against pathogens, and tolerance to abiotic stresses could be due to positive interactions between mycorrhizae and associated microorganisms (Sabannavar and Lakshman 2011). Several bacteria described as good root colonizers are also capable of attaching to hyphal surfaces and the efficiency of microorganisms residing in the rhizosphere depends on the soil type, which provides nutrients and habitat (Kamlesh *et al*. 2010). Amongst the abiotic factors, soil plays a major role in the interaction of soil microorganisms. The present study was conducted to evaluate the interaction of AMF and *P. fluorescens* and *A. chroococcum* on the growth, yield, nutrient content and quality of FCV tobacco grown with conserved soil moisture in vertisols.

MATERIALS AND METHODS

A field experiment was conducted at Central Tobacco Research Institute farm, Katheru during 2008-09 and 2009-10. The soil is clay with a pH of 7.6, EC 2.95, OC 0.41%, available P 14 kg/ha, K 633 kg/ha. The experiment was conducted in a completely randomized block design with eight treatments and three replications. The treatments were VAM @ 30 spores/g ; *Pseudomonas fluorescens*@ 1×10^9/ ml*; Azotobacter chroococcum* @1×10^8/ml*;* VAM + *Pseudomonas fluorescens*; VAM + *Azotobacter chroococcum; Pseudomonas fluorescens* + *Azotobacter chroococcum;* VAM + *Pseudomonas fluorescens* + *Azotobacter chroococcum)* and uninoculated control.

Selected culture of *Azotobacter chrococcum* was grown on Jensen's N_2 – free medium for 3 days. The cells were centrifuged, washed thrice in sterile distilled water and suspended in 0.15 M phosphate buffer at pH 7.0. The cell suspension was having 10^8 cells/ml and 1 000 ml of such cell suspension was used to inoculate one acre of tobacco field at the time of transplantation. *Glomus intraradices* was multiplied on maize plant roots under sterile conditions. Soil including root bits containing 30 viable arbuscular micorrhizal fungi propagules per g soil were used as inoculum. King's B broth was prepared without addition of agar and *P. fluorescens* was inoculated aseptically to the broth Erlenmeyer flasks and allowed to multiply in a rotary shaker for 48 hr at room temperature. The cultures were centrifuged at 600 rpm for 10 minutes and bacterial cells were resuspended in phosphate buffer and concentration was adjusted to 1×10^9 cfu/ml and used as bacterial inoculum. Sixty days old tobacco seedlings (var VT 1158) were transplanted in the field. The bio-inoculants were applied at the time of transplantation. Before transplanting the tobacco seedlings, a thin layer of *Glomus intraradices* inoculum was placed 2cm below the soil surface along with other bacterial inoculants. Establishment of bioinoculants in the rhizosphere of FCV tobacco plant grown in vertisols was estimated after 60 days of transplantation. The AM fungal spores were counted after removing them from the soil by wet sieving and decanting (Gerdemann and Nicolson 1963).

After 55 days of transplantation, observations on gas exchange parameters, viz. photosynthetic rate, transpiration rate and stomatal conductance were measured using Portable Photosynthetic system (LICOR-6400-40 model) and chlorophyll content index was measured using Chlorophyll content meter model CCM-200. Biometric observations on plant height. number of leaves, stem girth were recorded at grand growth period of the crop. Matured leaves were harvested and cured. Yield data such as cured leaf and bright leaf was recorded and grade index was calculated. Cured leaf samples collected were analysed for quality parameters such as nicotine, reducing sugars and chlorides (Harvey *et al*. 1969) and nutrient contents of leaf, nitrogen (AOAC 1950), phosphorus by vanadomolybdate method and potassium by flame photometry. Soil analysis was carried out after completion of the crop (Jackson 1973).

RESULTS AND DISCUSSION

Establishment of bioinoculants in the rhizosphere

The mycorrhizal inoculum significantly increased the number of VAM spores in the rhizosphere compared to the uninoculated control (Table 1). The inoculation with beneficial bacteria and 50 kg N/ha in the form of ammonium sulphate, with the omission of P and K chemical fertilizer increased root colonization by *G. intraradices*. It has already been noted that the rhizobacteria can act as mycorrhization helper bacteria which improve the ability of mycorrhizal fungi to colonize plant roots (Abasi *et al*. 2011). The mechanisms by which these bacteria stimulate AM colonization are still poorly understood. Specialized bacterial activities such as the production of vitamins, amino acids, and hormones may be involved in these interactions (Narula *et al.* 2009). The

presence of rhizobacterial inoculation might have assisted in the germination of a large number of spores thus leading to a higher infection percentage (Sabannavar and Lakshman 2011). Some PGPR endophytic species are known to have cellulase and pectinase (Parmar and Dufresne 2011) and these activities could no doubt aid in mycorrhizal infection. The present results demonstrated that the population size of the inoculated rhizobacteria varied in accordance with the treatment and AMF colonization in the rhizosphere (Table 1). Lack of P and K fertilization resulted in a higher level of mycorrhizal root infection and less population of *A. chroococcum* in the rhizosphere. According to the results the population of *A. chroococcum* was seriously inhibited when the nitrogen fertilizer was applied. This is in agreement with Alizadeh and Ordookhani (2011) who noted that the population size of N-fixing bacteria in soil decreased significantly after N fertilizer was used. However, a marked raise in the number of P solubiling Pseudomonads was observed with the increase of *G. intraradices* colonization. It implies that tobacco is likely to be more dependent on the symbiosis with *G. intraradices* than P solubilizers under the condition of insufficient nutrient supply or when no P fertilizer is applied. Chandanie *et al*. (2009) stated that mycorrhizal colonization with *G. intraradices* allowed the introduced populations of beneficial soil microorganisms like *Azotobacte*r, and *Pseudomonas* to maintain a higher abundance than non-mycorrhizal plants and thereby exerted a synergistic effect on plant growth.

Soil properties

The inoculation of rhizobacteria and mycorrhizae resulted in a significant increase of soil organic matter content (Table 2). The organic matter content in triple, double and single inoculated treatments when compared to uninoculated control (Sabannavar and Lakshman 2011). However, the results indicate that the increase was not directly induced by the activity of soil microorganisms. The treatments with N fertilizer and omission of P and K exhibited a larger population size of N-fixing bacteria, P solublising bacteria and higher mycorrhizal spores. Most soil microorganisms consume a considerable amount of organic matter, e.g. carbohydrates, to generate the energy for maintenance and growth. Thus, some organic carbon (C) is lost with the production of carbon dioxide. The significant correlation ($P<0.05$) between soil organic matter content and plant dry biomass (Table 2 and 3) suggests that the organic matter content in the rhizosphere was mainly influenced by plant growth, especially root exudates through the root metabolism and physiological activities. It has been reported that *Azotobacter* not only provides nitrogen, but also produces a variety of growth-promoting substances (Rekha *et al*. 2009), among them indole acetic acid, gibberellins and B vitamins (Bais *et al*. 2006). These substances stimulate, at least to some degree, the production of root exudates.

In addition, another important characteristic of *Azotobacter* associated with plant improvement is excretion of ammonia in the rhizosphere in the presence of root exudates (Narula *et al.* 2009), which could explain why the dual inoculation treatments with bacteria and AMF resulted in a slightly higher total N content in soil (Table 2) compared to those with AMF inoculation only. The application rate of organic fertilizer as farmyard manure also influenced soil N content. It could be attributed not only to N but also organic C contained in the manure. Hameeda *et al*. (2007) reported that the use of suitable farmyard manures, green manures and other organic manures and fertilizers may enhance the benefits of *Azotobacter* inoculation. This is due to the fact that the N-fixation reaction needs a lot of energy from available organic C to break the bonds between nitrogen atoms.

Table 1 Establishment of bioinoculants in the rhizosphere of TBS

Treatment	Microbial population		
	Pseudomonas (10^6 CFU/ g soil)	*Azotobacter* (10^6 CFU/ g soil)	VAM spores/ 100 g soil
Control	19	26	5
VAM	25	40	38
Pf (*Pseudomonas*)	108	22	10
Azotobacter	27	110	11
VAM + Pf	135	31	38
VAM+ *Azotobacter*	35	200	60
Pf+ *Azotobacter*	70	130	16
VAM + Pf + *Azotobacter*	160	207	134
SEm±	2.55	4.56	1.88
CD (P=0.05)	7.72	13.83	5.72
CV (%)	6.06	8.24	8.37

Table 2 Effect of biofertilizers on soil status of FCV tobacco in TBS

Treatment	OC (%)	P (kg/ha)	K (kg/ha)
Control	0.39	33	746
VAM	0.51	51	802
PF	0.45	52	854
Azoto	0.52	39	769
VAM+Pf	0.57	57	941
VAM+Azoto	0.52	52	802
Pf+Azoto	0.51	51	832
Pf + Azoto+VAM	0.55	61	955
SEm±	0.06	0.10	78.72
CD (P=0.05)	NS	NS	NS
CV (%)	20.80	37.88	16.27\

Initial soil characteristics

pH, 7.6; EC, 2.95; OC, 0.41%; P, 14 kg/ha; K, 633 kg/ha

Phosphorus is also a major nutrient for plants and microorganisms. The soil for this experiment is having sufficient P. However, available P (Olsen-P) in soil was significantly increased with the inoculation of AM fungi alone or in combination with rhizobacteria (Table 2). Native soil P is mostly unavailable to crops because of its low solubility. Therefore, the AMF colonization and P-solubilizing bacteria can play an important role in improving P bio availability. However, the effect of P-solubilizing bacteria was more significant when compared with AMF according to present results (Ahanthem and Jha 2007).

Plant biomass accumulation

Observations on plant growth were recorded 90 days after transplantation. The control plants showed very poor growth, which may be attributed to nutrient deficiency, e g the lack of available P in the unfertilized soil (Table 3). Bio-inoculation effect on plant growth was much more pronounced due to AMF and its combination with beneficial bacteria. The maximum cured leaf yield of 2 592 kg/ha was recorded in the treatment VAM + Pf + *Azotobacter,* while the VAM + Pf treatment achieved the yield of 2 569 kg/ha. The effect of bioinoculants on yield characters recorded is given in Fig 1. Cured leaf yield and grade index increased with triple inoculation followed by double inoculation. Single inoculation was better than uninoculated control. No significant differences were observed among double and single inoculated treatments. The results are in agreement with Baslam *et al.* (2011). With regards to the increase in plant biomass, *P. fluorescens* seemed to be more effective than *G. intraradices*. However, the effect of the mycorrhizal fungi and *P. fluorescens* on plant yield was significantly different. It was noted that plants grown with the application of bioinoculants produced more dry matter than plants grown in the uninoculated control. These results suggest that the triple or dual inoculation of beneficial bacteria and AMF could, contribute to the nutrient availability in vertisols. The low biomass of plants grown on the control treatments could be attributed to the disappearance of indigenous microbes, which may be essential to increase nutrient bioavailability and uptake in the rhizospheric soil. Stimulation of different crops by rhizobacterial inoculation has also been demonstrated by other studies both in laboratory and field trials (Zaidi *et al.* 2009).

Gas exchange parameters and chlorophyll content index

Significantly higher photosynthetic rates were observed with triple inoculation followed by the plants inoculated with dual inoculation or single inoculation of bacteria or VAM (Table 4). Lower rates of photosynthesis, transpiration

Table 3 Effect of biofertilisers on growth parameters of tobacco

Treatment	Plant height (cm)	No. of leaves/ plant	Stem girth (cm)	Stem dry weight (g)	Root dry weight (g)
Control	106.83	26	7.33	85.33	36.67
VAM	148.50	27	8.08	115.33	43.33
Pf (*Pseudomonas*)	140.00	27	8.75	123.33	40.00
Azotobacter	143.33	28	8.08	94.00	38.00
VAM + Pf	146.00	27	8.92	117.33	38.00
VAM+ *Azotobacter*	139.16	27	7.42	96.67	42.00
Pf+ *Azotobacter*	144.50	27	8.67	130.67	44.00
VAM + Pf + *Azotobacter*	149.83	28	8.42	142.00	43.33
SEm	9.68	0.87	0.53	23.27	6.91
CD (P=0.05)	NS	NS	NS	NS	NS
CV (%)	12.00	5.44	11.17	35.64	29.43

Table 4 Effect of bioinoculants on gas exchange parameters and CCI of FCV tobacco after 55 days of transplantation

Treatment	Photo-synthetic rate (μ mol/m²/s)	Stomatal conductance (mol/ m²/s)	Transpira-tion rate (mol/ m²/s)	Chloro-phyll content index (CCI)
Control	17.48	0.32	4.51	22
VAM	25.43	0.43	5.40	76
Pf	25.90	0.50	6.60	73
Azotobacter	21.56	0.42	5.04	52
VAM + Pf	23.16	0.41	4.06	55
VAM + *Azotobacter*	23.53	0.53	5.92	45
Pf + *Azotobacter*	22.67	0.51	6.56	75
VAM + Pf + *Azotobacter*	27.33	0.49	5.19	89
SEm	0.22	0.01	0.07	2.41
CD (P=0.05)	0.68	0.03	0.23	7.32
CV (%)	1.66	2.60	2.46	6.88

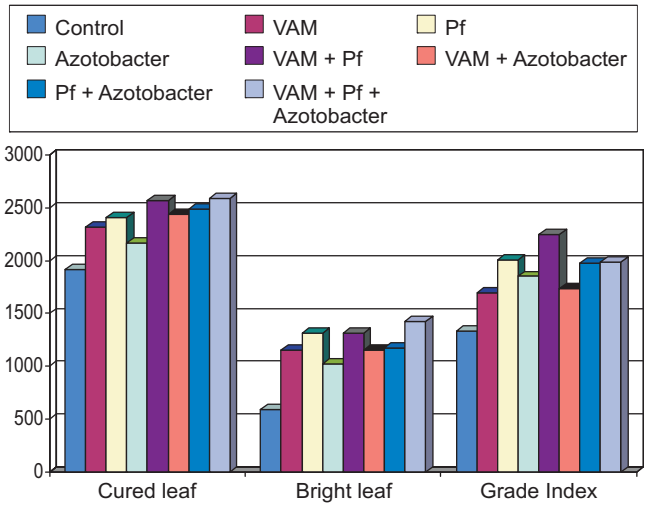

Fig 1 Effect of biofertilisers on the yield of FCV tobacco in TBS

and chlorophyll content index were found in uninoculated plants and those inoculated with *Azotobacter* (Subhashini and Padmaja 2010).

Nutrient acquisition

Dual inoculation with AMF and rhizobacteria seemed to be the most effective treatment combination to improve plant nutrient uptake (Table 5). N concentration in plants under different treatments ranged from 1.49 (control) to 2.50% (dual inoculation with rhizobacteria and *G. intraradices*). Although dually inoculated plants (with rhizobacteria and *G. intraradices*) showed unexpectedly low N concentrations in the plant tissue, the fungi still assisted the host to assimilate the maximum total N and resulted in a higher biomass. The inoculation with G. *intraradices* had a more stimulating effect on the assimilation of N than *G. intraradices* in the absence of bacterial inoculation. However, *G. intraradices* performed better than *P. fluorescens* in stimulating N and P uptake, when combined with bacterial inoculation, especially at lower nutrient level. The pattern of P and K uptake by plants under different treatments was similar to N assimilation. The lowest P and K uptake was detected in plants grown in uninoculated and unfertilized plots. Either single treatment with AMF/bacteria inoculation resulted in an increase in P and K uptake to different degrees when compared to control. The maximum P and K assimilation were obtained with the triple inoculation of *G. intraradices* and rhizobacteria (Ordookhani and Zare 2011).

Leaf quality

Tobacco is a quality conscious crop, leaf is the economic product and sensitive to applied chemical fertilizers such as nitrogen. Nicotine, reducing sugars and chlorides determine the quality of FCV tobacco leaf. Nicotine is an alkaloid which is synthesized in tobacco roots and is regulated more by nitrogen supply than any other nutrient (Collins and Hawks Jr 1993). Triple inoculation proved to be the best treatment in terms of quality recording highest percent reducing sugars and lowest chlorides. Regarding % nicotine, *A. chroococum* alone and in combination with *P. fluorescens* showed increased level of nicotine than in triple inoculation (Table 6).

Table 5 Effect of biofertilisers on nutrient content of tobacco leaf

Treatment	N (%)	P (%)	K (%)
Control	2.1	0.21	2.6
VAM	2.2	0.24	2.9
Pf (*Pseudomonas*)	2.3	0.24	2.9
Azotobacter	2.3	0.22	2.7
VAM + Pf	2.3	0.26	3.6
VAM + *Azotobacter*	2.3	0.24	3.6
Pf + *Azotobacter*	2.4	0.24	3.9
VAM +Pf + *Azotobacter*	2.5	0.28	4.1
SEm±	0.01	0.004	0.04
CD (P=0.05)	0.04	0.013	0.13
CV (%)	1.06	3.03	2.20

Table 6 Effect of biofertilisers on quality of TBS tobacco leaf

Treatment	Nicotine (%)	Reducing sugars (%)	Chlorides (%)
Control	2.56	10.12	1.78
VAM	2.73	12.42	1.29
Pf	3.10	12.81	1.42
Azotobacter	2.80	10.45	1.54
VAM + Pf	2.97	13.10	1.56
VAM + *Azotobacter*	2.36	12.63	1.63
Pf + *Azotobacter*	3.04	11.81	1.65
VAM + Pf + *Azotobacter*	2.84	15.62	1.40
SEm±	0.01	0.07	0.01
CD (P=0.05)	0.03	0.22	0.04
CV (%)	0.64	1.02	1.39

Since both the yield and quality are very important for commercial crop like FCV tobacco, the present study concludes that application of bioinoculants greatly influence the growth, gas exchange parameters, chlorophyll index, cured leaf weight, NPK content of leaf and quality parameters such as nicotine, reducing sugars and chlorides.

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