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Development of bioconsortia for optimizing nutrient supplementation through microbes for sustainable tobacco production

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Abstract

Increased interests in low-input agriculture in recent years has seen the growing development in the use of commercial biological inoculants to increase the mobilization of key nutrients such as nitrogen (N), phosphorus (P) and potassium (K) to enhance their availability to crop plants. The objectives of this field experiment with tobacco were to determine i) reduced rates of inorganic fertilizer coupled with microbial inoculants that produce plant growth, ii) yield and nutrient acquisition levels equivalent to those with full rates of fertilizers and iii) the minimum level to which fertilizer could be reduced with the use of bioinoculants. The microbial inoculants used were plant growth promoting bacteria viz., *Azospirillum, Azotobacter, Bacillus subtilis* and *Frateuria aurantia* alone or a mixture of them in combination with 75% chemical fertilizer. Results showed that supplementing 75% of the chemical fertilizer rate with inoculants produced plant growth, yield and nutrient (N, P and K) acquisition that were statistically equivalent to the full fertilizer rate without inoculants. When inoculants were used in single, double or triple with 75% RDF the beneficial effects were usually not consistent. However, inoculation with the mixture of PGPR (N, P and K mobilizers) at 75% RDF produced significantly superior yield better than the full fertilizer dose without inoculants. Without inoculants use of fertilizer rates lower than the recommended resulted in significantly less plant growth, yield and nutrient uptake. The results suggest PGPR based inoculants can be used and should be further evaluated as components of integrated nutrient management strategies.

Keywords: *Azotobacter*; *Bacillus subtilis*; *Bioinoculants*; *Frateuria aurantia*; Tobacco.

Introduction

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 Rs 8,200 crores as excise revenue and Rs. 1362 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 can be reduced by exploiting the potential of bio-inoculants which are inexpensive and eco-friendly (Subhashini, 2013). Among the different tobacco types FCV tobacco is very important which is grown in an area of 2.0 lakh ha in Andhra Pradesh and Karnataka. FCV tobacco is cultivated in different soils ranging from sandy loams to

clay soils and available P content in the soils varies from low, medium to high. The chemistry and fertility of soils greatly influence the tobacco plant growth, physical, chemical, manufacturing and smoking properties of tobacco leaf. Balanced nutrition is a pre-requisite to get quality leaf (Subhashini, 2014).

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). Utility of microorganisms that improve soil fertility and enhance plant nutrition has continued to attract the attention due to the increase in cost of fertilizers and their negative impact on environment. Plant growth promoting rhizobacteria (PGPR) play an important role in mineralization and immobilization of nutrients needed for growth of tobacco crop (Subhashini, 2012). They are assumed to be an alternative to the use of chemicals (Hayat et al., 2010). PGPR may benefit the host by causing plant growth promotion or biological disease control (Subhashini, 2011). PGPR activity has been reported in strains belonging to several genera, such as *Azotobacter, Azospirillum, Pseudomonas, Acetobacter* and *Bacillus* (Kloepper, 1993). 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). 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).

Biofertilizer has been identified as an alternative to chemical fertilizer to increase soil fertility and crop production in sustainable tobacco farming. The objective of this field study is to evaluate the effects of four biofertlizers containing free living non symbiotic N- fixer (*Azospirillum brasiliensis* or *Azotobacter chroococcum*), P-solubiliser (*Bacillus subtilis*) and K-solubiliser (*Frateuria aurantia*) on soil properties and on the growth, yield and quality of tobacco. The interactions among the microorganisms were also investigated.

Materials and Methods

Soil

A field experiment was conducted at ICAR-Central Tobacco Research Institute farm, Katheru during 2010-11 and 2011-12. The basic properties of the soil are as follows. pH-7.4, organic carbon content 1.62%, available P content 17.34 kg ha^{-1} and available K 540.2 kg ha^{-1}. The plot size of each treatment in field trial is 16.4 m^2.

Bioinoculants

The bioinoculants *Azotobacter, Azospirillum* and *Bacillus subtilis* were obtained from Tamil Nadu Agricultural University, Coimbatore and *Frateuria aurantia* was

obtained from Gokulum Biotech, Pondicherry. The bacterial cultures were mixed with Farm Yard Manure (FYM) and applied to the plant before transplanting. Three strains N- fixing bacteria (*A. chrocooccum*), P-solubiliser (*B. subtilis*) and K- solubiliser (*F. aurantia*) were used. Mutants of rhizobacterial strains marked with antibiotic resistance were obtained after plating of the parental strain on sucrose-minimal salts agar amended with rifampicin (150 mg L^{-1}). After incubation for 4 days at 28 ^{o}C the rifampicin resistant strains were selected (Subhashini, 2014).

Effective microbial counts (EFU) were enumerated before applying to the field. Selected culture of *A. chroococcum* 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^{-1} and 1000 ml of such cell suspension was used to inoculate one acre of tobacco field at the time of transplantation. King's B broth was prepared without addition of agar and *B. subtilis* was inoculated aseptically to the broth in 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^{-1} and used as bacterial inoculum.

Preparation of experimental site

The experiment was conducted in a completely randomized block design with twelve treatments and three replications. The treatments were T_1 - control; T_2 - 100 % recommended dose of fertilizer (RDF); T_3 - 75% recommended dose of fertilizer (RDF)*;* T_4 - 75% RDF+ *Azospirillum* @ 1×10^8*;* T_5 - 75% RDF+ *Bacillus subtilis* @ 1×10^9 / g; T_6 - 75% RDF+ *Frateuria aurantia* @ 1×10^9 CFU*;* T_7 - 75% RDF (*Azospirillum* + *B. subtilis*)*;* T_8 - 75% RDF (*Azospirillum* + *F. aurantia*); T_9 - 75% RDF (*B. subtilis* + *F. aurantia*); T_{10} - 75% RDF (*Azospirillum* + *B. subtilis* + *F. aurantia*); T_{11} - 75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia*; T_{12} - 75% RDF+ *Azotobacter* + *F. aurantia.*

Sixty days old tobacco seedlings (var. Siri) were transplanted in the field. The bio-inoculants were applied at the time of transplantation. Before transplanting the tobacco seedlings, a thin layer of bacterial inoculants was placed 2 cm below the soil surface. Establishment of bioinoculants in the rhizosphere of FCV tobacco plants grown in vertisols was estimated after 60 days of transplantation.

Gas exchange and plant growth parameters

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 vanidomolybdate method and potassium by flame photometry. Soil analysis was carried out after completion of the crop (Jackson, 1973).

Statistical Analysis

The data of two years were pooled and analysed statistically according to Panse and Sukhatme (1978). The test of significance level was < 0.05.

Results

The present data demonstrates that the population size of the inoculated bacteria in tobacco rhizosphere varied in accordance with the levels of fertilization and the combination of microorganisms used (Table 1). The low level of fertilization resulted in a higher level of microbial population in the rhizosphere and a larger community of *A. chroccoccum* or *Azospirillum* in the rhizosphere. There is an enhancement in photosynthetic rate, transpiration rate and foliage index due to triple inoculation (Table 4) and chlorophyll content was also significantly improved due to dual or triple inoculation (Table 5). The triple inoculation of rhizobacteria resulted in a significant increase of cured leaf, bright leaf yield and grade index (Table 3). The dry matter of the plants (i.e. Cured Leaf Yield, CLY) after 120 days of transplantation ranged from 1264 to 2199 kg ha^{-1}. The control plants showed very poor growth which may be attributed to nutrient deficiency. Moderate increases in plant biomass were due to increase of nutrients either in chemical or organic form. The fertilizer effect on plant growth was much more pronounced after inoculation of N- fixer and its combination with beneficial rhizobacteria. The maximum yield of cured leaf (2199 kg ha^{-1}), bright leaf (1190 kg ha^{-1}) and grade index (1747) were obtained with the treatment 75% RDF+ *Azospirillum* + *B. subtilis* + *F. aurantia* followed by 75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia*. With regard to the increase in plant biomass leading to higher cured leaf yield, bright leaf and grade index, *Azospirillum* seemed to be more effective than *Azotobacter* at the 75% recommended fertilization level. However, the effect of the two N- fixers at 75% of the recommended fertilization level on leaf yields was not significantly different ($P>0.05$). These results suggest that the triple inoculation of beneficial bacteria could at least to some extent compensate the deficiency in soils, besides making the unavailable form of P and K available to the plant. Triple inoculation also resulted in significant increase in quality parameters, such as nicotine and sugars compared to dual or single inoculation of beneficial rhizobacteria (Table 7).

Triple inoculation with rhizobacteria seemed to be the most effective treatment combination to improve the acquisition of plant nutrients (Table 6). N concentration in plants under different treatments ranged from 1.55 (control) to 2.69 (dual inoculation with *Azospirillum* and *B. subtilis*). Although triple inoculated plants with rhizobacteria showed unexpectedly low N concentration in the plant tissue compared to dual inoculated treatments, N- fixer still assisted the host to assimilate the maximum total N and resulted in a higher biomass and showed more stimulating effect with one another.

The patterns of P and K acquisition by tobacco plants under different treatments were similar to N assimilation. The lowest P and K acquisition was detected in plants grown in uninoculated and unfertilized plants. Either single treatment of chemical fertilizer or biofertilizer inoculation resulted in an increase in P and K uptake to different degrees when compared with the control. The maximum P and K assimilation was obtained with the triple inoculation of N, P and K- contributing microbes.

The triple inoculation of rhizobacteria resulted in a significant increase of soil organic matter content (Table 2). The Organic matter content in treatments of 75% RDF+ *Azospirillum* + *B. subtilis* + *F. aurantia* and 75% RDF+ *Azotobacter* +

B. subtilis + *F. aurantia* increased in comparison to uninoculated control. These results imply that the increase is induced by the activity of the N- fixing microorganisms. Single or dual or triple inoculation treatments with N- fixers resulted in a higher total N content in soil. Phosphorus is also a major nutrient for plants and microorganisms. The soil for this experiment is fairly good in available P; however, available P (Olsen, 1954) in soil was significantly increased with inoculation of *B. subtilis* alone or in combination with other rhizobacteria. The inoculation of beneficial microbes exerted a stimulating effect on K-acquisition by the tobacco plants.

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

Treatments	Microbial Population (1×10^5 cfus g^{-1})			
	Bacillus subtilis	*Frateuria aurantia*	*Azospirillum*	*Azotobacter*
Control	20.33	17.83	14.66	6.66
100% recommended dose of fertilizer (RDF)	19.66	22.00	16.33	7.33
75% recommended dose of fertilizer (RDF)	22.16	27.00	16.66	6.50
75% RDF + *Azospirillum* @ 1×10^8 cfu g^{-1}	19.50	25.17	46.50	9.50
75% RDF + B. *subtilis* @ 1×10^9 cfu g^{-1}	55.33	24.33	19.16	5.33
75% RDF + *F. aurantia* @ 1×10^9 cfu g^{-1}	32.50	101.83	21.16	9.17
75% RDF + *Azospirillum* + *B. subtilis*	108.50	28.16	40.66	9.50
75% RDF + *Azospirillum* + *F. aurantia*	34.83	79.16	36.83	10.16
75% RDF + *B. subtilis* + *F. aurantia*	80.66	81.33	18.50	12.00
75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia*	73.83	106.66	32.16	9.33
75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia*	71.00	115.83	19.50	15.66
75% RDF + *Azotobacter* + *F. aurantia*	21.66	72.83	21.83	20.00
Seasons				
2010-11	49.00	60.72	26.25	9.22
2011-12	44.33	56.30	24.42	10.97
General Mean	46.67	58.51	25.33	10.10
SEm ± seasons	1.61	1.42	0.58	0.92
SEm ± Treatments	0.77	1.22	1.42	0.68
CD at 5% Seasons	NS	NS	NS	NS
CD at 5% Treatments	2.14	3.39	3.95	1.89
CD at 5% Interactions	NS	4.79	5.58	2.67
CV% Seasons	20.65	14.52	13.65	54.79
CV% Treatments	4.05	5.12	13.77	16.51

Discussion

Inoculum establishment in the tobacco rhizosphere

The present data reveals that combination of all the three bioinoculants resulted in the highest population of introduced microorganisms exhibiting synergistic effect and positive interaction among the beneficial bacteria in the rhizosphere of tobacco. Similar findings were reported by earlier workers (Subhashini, 2013; Dodd and Ruiz-Lozano 2012). The mechanisms by which these bacteria stimulate one another 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 development of a large population thus leading to a higher infection percentage (Glick, 2012). Some PGPR endophytic species are known to have cellulase and pectinase (Meenakshi and Rajni, 2013) and these activities could no doubt aid in more infection. The present results demonstrated that the population size of the inoculated rhizobacteria varied in accordance with the treatment in the rhizosphere (Table 1). Lack of P and K fertilization resulted in a higher level of *B. subtilis* and *F. aurantia* 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 Bhattacharya and Jha (2012) who noted that the population size of N-fixing bacteria in soil deceased significantly after N fertilizer was used. It implies that tobacco is likely to be more dependent on the symbiosis with P solubilizers under the condition of insufficient nutrient supply or when no P fertilizer is applied.

Soil properties

Triple, double and single inoculation of rhizobacteria resulted in a significant increase in soil organic matter content when compared to uninoculated control (Table 2). Similar findings were reported in sesame (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 K mobilizing bacteria. The significant correlation ($P<0.05$) between soil organic matter content and plant dry biomass (Tables 2 and 3) suggests that the organic matter content in the rhizosphere was mainly influenced by plant growth, metabolism and physiological activities.

Table 2. Effect of biofertilisers on nutrient status of soil.

Treatments	pH	O.C	P	K
Control	7.2	0.28	24.89	516
100% recommended dose of fertilizer (RDF)	6.9	0.41	50.79	521
75% recommended dose of fertilizer (RDF)	7.1	0.40	48.20	558
75% RDF + *Azospirillum* @ 1×10^8 cfu g^{-1}	7.3	0.26	59.31	529
75% RDF + *B. subtilis* @ 1×10^9 cfu g^{-1}	7.3	0.42	47.03	564
75% RDF + *F. aurantia* @ 1×10^9 cfu g^{-1}	7.3	0.39	49.84	631
75% RDF + *Azospirillum* + *B. subtilis*	7.2	0.45	33.39	529
75% RDF + *Azospirillum* + *F. aurantia*	7.2	0.43	41.25	584
75% RDF + *B. subtilis* + *F. aurantia*	6.9	0.38	43.55	615
75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia*	7.0	0.53	102.87	813
75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia*	7.0	0.51	101.38	839
75% RDF + *Azotobacter* + *F. aurantia*	7.2	0.47	55.00	830
S Em±	0.05	0.01	1.03	18.82
CD at 5 %	0.14	0.03	3.01	55.19
CV%	1.12	3.70	3.05	5.14

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 resulted in a slightly higher total N content in soil (Table 2) compared to those with PSB or KMB inoculation only. The application rate of organic fertilizer as farm yard manure also influenced soil N content. It could be attributed not only to N but also organic C contained in the manure. Subhashini (2013) 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.

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 the combination of beneficial bacteria. The maximum cured leaf yield of 2290 kg /ha was recorded in the treatment 75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia*, while the 75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia* achieved the yield of 2212 kg ha^{-1}. The effect of bioinoculants on yield characters recorded is given in Table 3. 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, *B.subtilis* seemed to be on par with *F. aurantia*. However, the effect of the *F. aurantia* and *B. subtilis* 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 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 workers 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 and chlorophyll content index were found in uninoculated plants and those inoculated with *Azotobacter* (Subhashini and Padmaja, 2010).

Table 3. Effect of biofertilisers on yield of FCV tobacco (kg/ha).

Treatments	Green leaf	Cured leaf	Bright grade	Grade Index
Control	10636	1520	103	767
100% recommended dose of fertilizer (RDF)	12790	2025	666	1435
75% recommended dose of fertilizer (RDF)	11819	2015	710	1403
75% RDF + *Azospirillum* @ 1×10^{8} cfu g^{-1}	10891	1780	945	1202
75% RDF + *B. subtilis* @ 1×10^{9} cfu g^{-1}	11387	1723	887	1163
75% RDF + *F. aurantia* @ 1×10^{9} cfu g^{-1}	12804	2052	1040	1372
75% RDF + *Azospirillum* + *B. subtilis*	12762	1971	1184	1295
75% RDF + *Azospirillum* + *F. aurantia*	12414	1967	1132	1345
75% RDF + *B. subtilis* + *F. aurantia*	13470	2161	1247	1477
75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia*	13541	2212	1299	1430
75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia*	13669	2290	1320	1595
75% RDF + *Azotobacter* + *F. aurantia*	13407	2151	1257	1509
Seasons				
2010-11	12313	1873	948	1463
2011-12	12619	2105	1017	1202
General Mean	12466	1989	982	1333
SEm ± seasons	323.87	83.78	50.19	63.94
SEm ± Treatments	521.48	84.98	46.44	72.34
CD at 5% Seasons	NS	NS	NS	251.01
CD at 5% Treatments	1445.46	235.51	128.79	200.52
CD at 5% Interactions	NS	333.11	182.05	283.58
CV% Seasons	15.59	25.27	30.64	28.77
CV% Treatments	10.25	10.46	11.57	13.29

Table 4. Effect of biofertilisers on physiological parameters.

Treatment	Net Photosynthetic rate (μ mol m^{-2} s^{-1})	Transpiration rate (m mol m^{-2} s^{-1})	Foliage Index
Control	16.07	3.95	1.95
100% recommended dose of fertilizer (RDF)	30.50	7.60	3.46
75% recommended dose of fertilizer (RDF)	25.27	7.41	3.13
75% RDF + *Azospirillum* @ 1×10^{8} cfu g^{-1}	30.67	7.63	3.70
75% RDF + *B. subtilis* @ 1×10^{9} cfu g^{-1}	30.33	8.22	3.39
75% RDF + *F. aurantia* @ 1×10^{9} cfu g^{-1}	31.57	9.15	3.80
75% RDF + *Azospirillum* + *B. subtilis*	33.23	7.77	3.35
75% RDF + *Azospirillum* + *F. aurantia*	31.27	7.26	3.63
75% RDF + *B. subtilis* + *F. aurantia*	31.47	7.21	3.41
75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia*	31.10	7.44	3.63
75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia*	31.48	8.45	3.85
75% RDF + *Azotobacter* + *F. aurantia*	32.30	8.21	3.39
S Em ±	0.97	0.53	0.31
C.D. (0.05)	2.83	1.56	0.90

Table 5. Effect of biofertilisers on physiological parameters.

Treatment	Chlorophyll a (mg g^{-1} f.wt.)	Chlorophyll b (mg g^{-1} f.wt.)	Total Chlorophyll (mg g^{-1}f.wt.)
Control	1.38	0.400	1.78
100% recommended dose of fertilizer (RDF)	2.18	0.517	2.69
75% recommended dose of fertilizer (RDF)	2.13	0.557	2.68
75% RDF + *Azospirillum* @ 1×10^8 cfu g^{-1}	2.14	0.587	2.72
75% RDF + *B. subtilis* @ 1×10^9 cfu g^{-1}	2.21	0.503	2.70
75% RDF + *F. aurantia* @ 1×10^9 cfu g^{-1}	2.20	0.513	2.71
75% RDF + *Azospirillum* + *B. subtilis*	2.20	0.547	2.75
75% RDF + *Azospirillum* + *F. aurantia*	2.25	0.563	2.81
75% RDF + *B. subtilis* + *F. aurantia*	2.38	0.623	3.01
75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia*	2.33	0.623	2.97
75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia*	2.12	0.547	2.67
75% RDF + *Azotobacter* + *F. aurantia*	2.32	0.650	2.99
S Em ±	0.10	0.034	0.13
C.D. (0.05)	0.30	0.100	0.39

Nutrient acquisition

Triple inoculation with 75% RDF + *Azotobacter*+ *B.subtilis* + *F. aurantia* and 75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia* seemed to be the most effective treatment combination to improve plant nutrient acquisition (Table 6). N concentration in plants under different treatments ranged from 1.95 (control) to 2.59% (triple inoculation with 75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia*). Although *F. aurantia* showed unexpectedly low N concentrations in the plant tissue, the bacteria still assisted the host to assimilate the maximum total N and resulted in a higher biomass. The inoculation with *Azospirillum* had a more stimulating effect on the assimilation of N than *F. aurantia* alone. However, *Azospirillum* performed better than *Azotobacter* in stimulating N and P acquisition, when combined with bacterial inoculation, especially at lower nutrient level. The pattern of P and K acquisition by plants under different treatments was similar to N assimilation (Wu et al., 2005). The lowest P and K acquisition was detected in plants grown in uninoculated and unfertilized plots. Single treatment with bacterial inoculation resulted in an increase in P and K acquisition to different degrees when compared to control. The maximum P and K assimilation were obtained with the triple inoculation of 75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia* (Ordookhani and Zare, 2011; Owen et al., 2014).

Table 6. Effect of biofertilisers on nutrient content of FCV tobacco.

Treatments	Nitrogen (%)	Phosphorus (%)	Potassium (%)
Control	2.12	0.21	0.56
100% recommended dose of fertilizer (RDF)	2.10	0.21	0.59
75% recommended dose of fertilizer (RDF)	1.96	0.23	0.59
75% RDF + *Azospirillum* @ 1×10^{8} cfu g^{-1}	2.51	0.23	0.68
75% RDF + *B. subtilis* @ 1×10^{9} cfu g^{-1}	2.35	0.25	0.55
75% RDF + *F. aurantia* @ 1×10^{9} cfu g^{-1}	1.95	0.26	0.60
75% RDF + *Azospirillum* + *B. subtilis*	2.59	0.26	0.59
75% RDF + *Azospirillum* + *F. aurantia*	2.49	0.27	0.59
75% RDF + *B. subtilis* + *F. aurantia*	2.38	0.27	0.69
75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia*	2.52	0.28	0.71
75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia*	2.53	0.28	0.72
75% RDF + *Azotobacter* + *F. aurantia*	2.44	0.26	0.72
Seasons			
2010-11	2.05	0.26	0.80
2011-12	2.60	0.24	0.47
General Mean	2.33	0.25	0.63
SEm ± seasons	0.06	NS	0.02
SEm ± Treatments	0.10	0.01	0.04
CD at 5% Seasons	0.25	NS	0.07
CD at 5% Treatments	0.29	0.02	0.10
CD at 5% Interactions	0.40	0.02	0.14
CV% Seasons	16.32	2.83	16.86
CV% Treatments	10.82	5.86	13.69

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. Subhashini (2013) reported that % nicotine in the treatment *P. fluorescens* alone and in combination with *A. chroococcum* showed increased level of nicotine.

Since both the yield and quality are very important for commercial crops like FCV tobacco, the present study concludes that inoculation of biofertilizers along with reduced rate of recommended dose of chemical fertilizer increased growth, yield and quality parameters of FCV tobacco as compared to individually applied biofertilizer and chemical fertilizer applied plants.

Table 7. Effect of biofertilisers on quality of FCV tobacco.

Treatments	Nicotine (%)	Reducing sugars (%)	Chlorides (%)
Control	2.82	9.37	3.26
100% recommended dose of fertilizer (RDF)	2.96	11.12	3.02
75% recommended dose of fertilizer (RDF)	3.13	10.41	3.17
75% RDF + *Azospirillum* @ 1×10^8 cfu g^{-1}	3.06	10.82	3.01
75% RDF + *B. subtilis* @ 1×10^9 cfu g^{-1}	3.06	12.14	2.77
75% RDF + *F. aurantia* @ 1×10^9 cfu g^{-1}	3.22	13.00	3.06
75% RDF + *Azospirillum* + *B. subtilis*	3.18	11.29	2.89
75% RDF + *Azospirillum* + *F. aurantia*	3.35	11.88	3.26
75% RDF + *B. subtilis* + *F. aurantia*	3.20	11.99	3.17
75% RDF + *Azospirillum* + *B. subtilis* + *F. aurantia*	3.37	11.82	3.20
75% RDF + *Azotobacter* + *B. subtilis* + *F. aurantia*	3.02	13.70	2.99
75% RDF + *Azotobacter* + *F. aurantia*	2.96	11.70	3.21
Seasons			
2010-11	2.79	11.68	3.28
2011-12	3.43	11.52	2.89
General Mean	3.11	11.60	3.09
SEm ± seasons	0.08	0.38	0.05
SEm ± Treatments	0.11	0.61	0.15
CD at 5% Seasons	0.32	NS	0.18
CD at 5% Treatments	0.31	1.68	NS
CD at 5% Interactions	NS	NS	NS
CV% Seasons	15.58	19.55	8.97
CV% Treatments	8.90	12.82	12.21

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