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Response of Coker (flue-cured) tobacco (*Nicotiana tabacum*) to inoculation with *Azotobacter chroococcum* at various levels of nitrogen fertilization

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Abstract

A field study was carried out during a two-year period (2008 to 2009) in order to investigate on response of Coker 347 (flue-cured) tobacco plant (*Nicotiana tabacum* L.) in vegetative growth stages to inoculation with *Azotobacter chroococcum* at various levels of nitrogen (N) fertilization. In a factorial trial and randomized completely block design (RCBD), four levels of N fertilizer (non-fertilizer, 15, 30 and 45 kg ha^{-1}) and three levels of *Azotobacter chroococcum* (non-inoculation, 1 and 2 kg ha^{-1}) in Coker (flue-cured) tobacco with three replications per treatments were applied. Sampling was done in three positions of leaves (priming, cutter and tip). Results revealed that the effect of year of experiment was significant on quantitative characteristics (except leaf length), N uptake and qualitative characteristics (except nicotine in priming). Application of N had significant effect on quantitative characteristics (except number and width of Leaf), N uptake and qualitative characteristics. Inoculation of roots with bacterium had significant effect on Cured leaf weight of cutter, yield, N uptake and qualitative characteristics. Furthermore, the lowest content of leaf length and nicotine in priming and the highest content reducing sugar in priming and tip were observed in non-fertilizer treatment. On the other part, the highest content of nicotine in priming and the lowest content of sugar in priming and tip were observed in treatment with 45 $kg.ha^{-1}$ N and 2 kg ha^{-1} bacterium. In general, *Azotobacter chroococcum* is a suitable inoculant for tobacco cultivation and it could be a strategy to achieve sustainable agriculture.

Keywords: Biofertilizer; nicotine; nitrogen fixation; reducing sugar; seedling inoculation
Abbreviations: CFU- colony forming units; PGPR- plant growth promoting rhizobacteria.

Introduction

The high cost of fertilizers, particularly nitrogen (N) fertilizers, is often prohibitive for farmers; therefore, it is important to explore alternative sources of N to partly meet the N requirement. Hence, there is a wide interest in biological N fixation. Microorganisms in the rhizosphere can increase or decrease the absorption of inorganic nutrient by plant roots and these seem to be significant interactions that occur between these organisms in certain conditions (El-Raheem et al., 1988). Plant growth promoting rhizobacteria (PGPR) represents a wide variety of soil bacteria which, growing in association with a host plant, result in stimulation of growth of their host plant (Vessey, 2003). Many different genera of plant growth promoting rhizobacteria such as Azospirillum, Azotobacter, Bacillus, Enterobacter and Pseudomonas have been used as biofertilizers for economically important. The first species of the genus Azotobacter, named *Azotobacter chroococcum*, was isolated from the soil in Holland in 1901 (Mrkovacki and Milic, 2001). For a number of years *Azotobacter chroococcum* was used in the former Soviet Union to inoculate seeds or roots of crop plants (Mishustin and Naumova, 1962). Bacteria of the Azotobacter genus synthesize auxins, cytokinins, and gibberelin acid–like substances, and these growth materials are the primary substances controlling the enhanced growth (Mrkovacki and Milic, 2001). The effect of *Azotobacter chroococcum* on vegetative growth and yields of maize has been studied by numerous authors (Nieto and Frankenberger, 1991; Mishra et al., 1995; Pandey et al., 1998), as well as the effect of inoculation with this bacterium on wheat (El-shanshoury, 1995; Fares, 1997). Meshram and Shende (1982) were reported that *Azotobacter chroococcum* inoculation was economically most efficient for maize at lower doses of fertilizer N and increased yield. N is the most important mineral nutrient affecting the yield and quality of flue-cured tobacco (Marchetti et al., 2006; Ju et al., 2008). Breland et al. (1961) showed that increased rates of N increased the concentration of N, Ca, Mg and K in flue-cured tobacco. Over 2500 compounds have been found in tobacco, among which alkaloids and terpenoids are the major groups (Nugroho and Verpoorte, 2002). Nicotine is a major alkaloid in tobacco leaf tissues. Nicotine concentration is a key index for evaluating the quality of tobacco, and is closely correlated with the amount of N supplied since N is 17.3% of the molecular weight of nicotine (Collins and Hawks, 1994). Despite numerous studies about the effect of *Azotobacter chroococcum* on vegetative growth and yields of various plants, however, there is no study about effect of *Azotobacter*

Table 1. Some physical and chemical characteristics of experimental soils.

Texture (%)			SP [a]	pH	ECe [b]	OC [c]	Total N	Available P	Exchangeable K	CEC [d]
Sand	Silt	Clay			($ds.m^{-1}$)	($g.kg^{-1}$)	($g.kg^{-1}$)	($mg.kg^{-1}$)	($mg.kg^{-1}$)	($cmol+.kg^{-1}$)
56	26	18	28	6.5	0.29	5.9	0.6	73	241	12

[a] SP= soil moisture percentage; [b] ECe= Electrical conductivity; [c] OC= Organic carbone; [d] CEC= Cations exchangeable capacity.

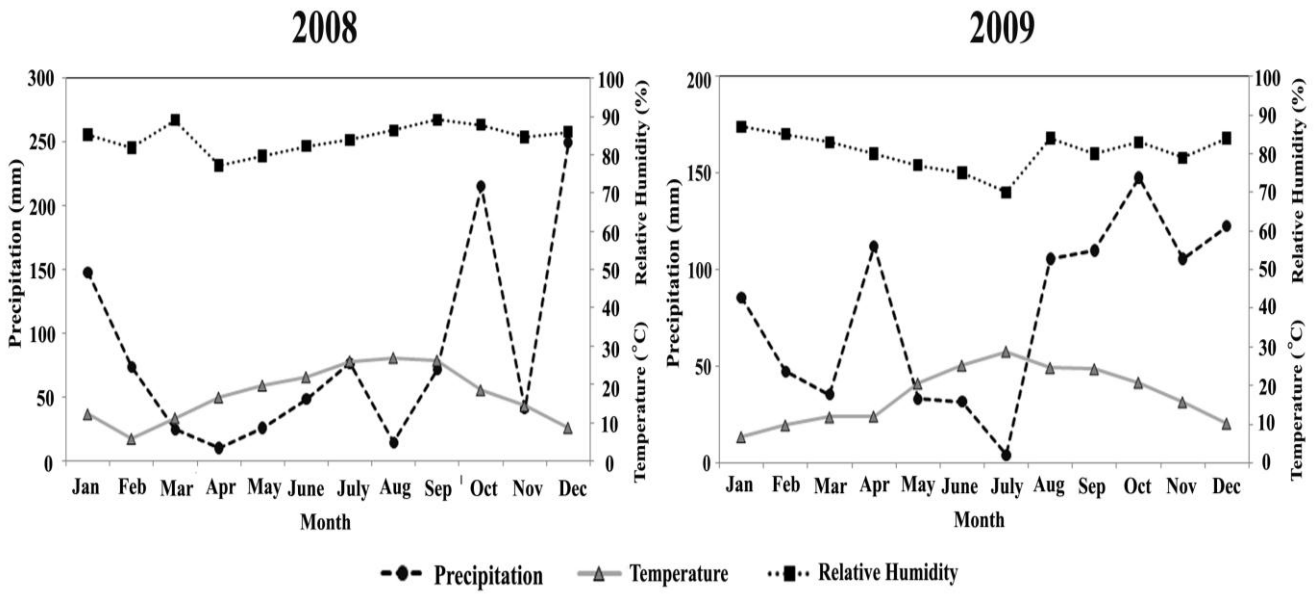

Fig 1. The amount of precipitation, temperature and relative humidity in 2008 and 2009 at the experimental site.

chroococcum on tobacco. The main purpose of this research was investigating the response of Coker (flue-cured) tobacco to inoculation with *Azotobacter chroococcum* at various levels of N fertilization.

Results

Effects of nitrogen fertilizer and Azotobacter Chroococcum on quantitative characteristics of tobacco

The result of analysis of variance (ANOVA) for the major effect of year (Y), nitrogen (N) and bacterium (B) on quantitative characteristics of Cured leaf weight in priming, cutter and tip, yield, plant height, leaf number, leaf length and leaf width showed that the year of experiment had significant effect on all of quantitative characteristics expect leaf length (Table 2). N fertilizer had significant effect on all of quantitative characteristics expect number and width of leaf, while the bacterium (*Azotobacter chroococcum*) had significant effect on cured leaf weight of cutter and yield of tobacco. Furthermore, interaction effect between year and bacterium (Y × B) had significant effect ($p < 0.05$) on cured leaf weight of priming and yield of tobacco. Interaction effect between N and bacterium (N × B) had significant effect ($p < 0.05$) on leaf length. The results of comparison of mean for the years of experiment (Table 3) showed that cured leaf weight and yield in 2008 year were higher and decreased in next year (2009), while, plant height and leaf number were more in second year (2009). Moreover, increasing N fertilizer in soil significantly increased cured leaf weight in three parts of priming, cutter and tip, yield, plant height and leaf length in comparison with control treatment. The increase N fertilizer in soil had not any influence on leaf number and leaf width. The result of comparison of mean for the major effect of bacterium showed that on cured leaf weight of cutter and yield showed that application 1 $kg.ha^{-1}$ of bacterium had maximum cured leaf weight of cutter and yield. The comparison of mean for the interaction effect between nitrogen and bacterium (N × B) on leaf length showed that treatments of N_4B_2 (with 45 $kg.ha^{-1}$ N and inoculation with 1 $kg.ha^{-1}$ bacterium) and N_1B_1 (without N fertilizer and inoculation with bacterium) had maximum and minimum of leaf length (Fig. 2).

Effects of nitrogen fertilizer and Azotobacter Chroococcum on nitrogen uptake and qualitative characteristics of tobacco

The result of ANOVA for the major effect of year (Y), nitrogen (N) and bacterium (B) on nitrogen uptake and qualitative characteristics (nicotine and reducing Sugar) showed that the year of experiment had significant effect on N uptake and reducing sugar content in three parts of leaves (priming, cutter ad tip) and nicotine content in leaves of cutter and tip. The results of comparison of mean for the years of experiment (Table 4) showed that N content in leaves of cutter and tip were maximum in second year (2009), whereas, N content in leaves of priming was maximum in first year. Moreover, nicotine content in leaves of cutter and tip and reducing sugar were maximum in the first year and decreased in second year, but nicotine content showed no significant difference among the years of experiment. Increasing N fertilizer in soil significantly decreased reducing sugar in comparison with control treatment. In addition, bacterium inoculation significantly increased N uptake and nicotine and significantly decreased reducing sugar in comparison with control treatment, like application of N fertilizer. The comparison of mean for the interaction effect

Table 2. Analysis of variance (mean square and significance) for the effect nitrogen fertilizer and *Azotobacter chroococcum* on quantitative characteristics of tobacco.

S.O.V	df.	Cured leaf weight			Yield	Plant height	Leaf number	Leaf length	Leaf width
		Priming	Cutter	Tip					
Replicate	2	6495**	90141*	126924**	136318*	806.98*	5.71	54.11*	25.39*
Year (Y)	1	8320*	3297312**	41424**	4454615*	2617.86**	15.12*	41.25	218.06**
Nitrogen (N)	3	24249**	197331**	75583**	752109**	3845.47**	4.98	84.53**	4.88
Bacterium (B)	2	754	81390*	25153	177070*	433.06	2.04	25.20	21.23
Y×N	3	1398	45640	24528	74742	353.89	1.49	14.15	3.43
Y×B	2	5245*	49350	29757	181105*	47.77	3.29	6.01	1.68
B×N	6	2910	25759	8564	66557	432.98	10.95	57.92*	15.62
Y×B×N	6	953	6027	9223	14877	383.51	4.61	13.98	4.81
Error	46	1668	24558	10202	40003	251.60	2.46	18.64	7.68
CV		12.8	25.2	20.1	13.9	15.8	6.8	9.6	13.3

* and ** significant at level of 5 and 1%, respectively. Values that do not have any symbol are non-significant.

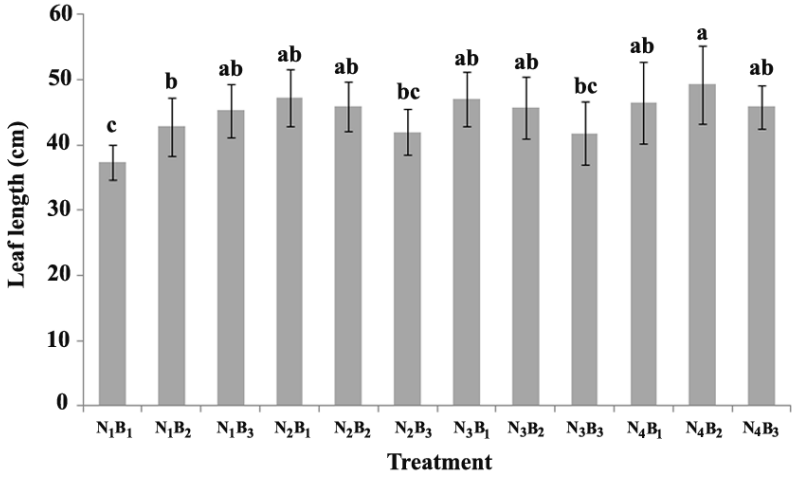

Fig 2. the effects of different levels of nitrogen fertilizer (N_1= 0, N_2=15, N_3= 30 and N_4= 45 kg.ha^{-1}) and inoculation of *Azotobacter chroococcum* (B_1= non inoculation, B_2=1 and B_3= 2 kg.ha^{-1}) on leaf length. Vertical error bars shows standard deviation (± SD).

between nitrogen and bacterium (N × B) on nicotine content in priming showed that treatments of N_4B_3 (with 45 kg.ha^{-1} N and inoculation with 2 kg.ha^{-1} bacterium) and N_1B_1 (without N fertilizer and inoculation with bacterium) had maximum and minimum of nicotine concentration, respectively (Fig. 3). The concentration of reducing sugar in priming and tip leaves follow almost the same trend. According to results, treatments of N_1B_1 (without N fertilizer and inoculation with bacterium) and N_4B_3 (with 45 kg.ha^{-1} N and inoculation with 1 kg.ha^{-1} bacterium) had maximum and minimum of reducing sugar concentration in priming and tip leaves (Fig. 4 and 5).

Discussions

This distribution of N fertilizer significantly increased cured leaf weight and yield. The lowest rate of N fertilizer (15 kg N.ha^{-1}) had significant effect on cured leaf weight and yield in comparison with control treatment (non-N fertilizer). While, there were not any significant difference in fertilizer treatments. Effects of N fertilizer on growth and yield of tobacco were associated with N content in roots and leaves. The amount of N in the plant increased in response to N fertilization. Sifola and Postiglione (2003) and Karaivazoglou et al. (2007), were reported that N fertilization had significant effects on both yield and cured leaf weight. Effect of *Azotobacter chroococcum* on yield was pronounced (11% for 1 kg.ha^{-1} treatment) without fertilizer application. Narula et al. (2005) were reported that *Azotobacter chroococcum* increased yield of wheat with less N fertilizers. Mrkovacki et al. (1997) were reported that *Azotobacter chroococcum* increased dry weight of sugar beet. Although information is scant about effect of *Azotobacter chroococcum* on tobacco now. With increasing amounts of N fertilizer, length and width of leaves increased (leaf width was not significant). It can be related to N stimulates the biosynthesis and export of cytokinins hormone from roots to leaves that causes increasing cell division and increasing length and width of the leaves (Haghighi et al., 2011). In a similar study, on Coker 347 cultivar, Haghighi et al (2011) observed that application of N fertilizer had significant effect on plant height, leaf number, leaf length and leaf width. Application of *Azotobacter chroococcum* had not significant effect on quantitative characteristics. The amount of N in the plant increased in response to N fertilization. N uptake significantly increased with increasing of N fertilizer in priming, cutter and tip leaves. In flue-cured tobacco, Goenaga et al. (1989) showed that N partitioning between plant parts

Table 3. Mean comparison (means ± standard deviation (SD)) effects nitrogen fertilizer and *Azotobacter Chroococcum* on quantitative characteristics of tobacco.

S.O.V		Cured leaf weight (kg.ha^{-1})			Yield (kg.ha^{-1})	Plant height (cm)	Leaf number	Leaf length (cm)	Leaf width (cm)
		Priming	Cutter	Tip					
Year (Y)									
	2008	330 ± 58 a	834 ± 151 a	525 ± 171a	1689 ± 271 a	127.27 ± 24 b	22.33 ± 2.0 b	43.93 ± 5.3 a	22.56 ± 3.6 a
	2009	308 ± 51 b	406 ± 108 b	477 ± 112 b	1192 ± 245 b	139.33 ± 11a	23.25 ± 1.8 a	45.44 ± 4.9 a	19.08 ± 2.2 b
Nitrogen (N)									
	0	264 ± 40 b	464 ± 111 b	406 ± 121 b	1135 ± 221 b	116.57 ± 26 b	22.05 ± 2.1 a	41.83 ± 5.0 b	20.05 ± 3.5 a
	15	333 ± 60 a	656 ± 211 a	521 ± 134 a	1522 ± 423 a	126.79 ± 11 b	23.22 ± 1.8 a	44.72 ±4.2 ab	20.95 ± 4.1 a
	30	338 ± 46 a	667 ± 302 a	528 ± 155 a	1546 ± 288 a	139.98 ± 14 a	22.77 ± 1.7 a	45.10 ± 4.9 a	21.12 ± 3.6 a
	45	341 ± 37 a	691 ± 297 a	550 ± 137 a	1559 ± 334 a	149.85 ± 17 a	23.11 ± 2.1 a	47.08 ± 5.3 a	21.16 ± 2.7 a
Bacterium (B)									
	0	321 ± 77 a	567 ± 182 b	470 ± 162 ab	1359 ± 311 b	133.50 ± 17 a	22.66 ± 2.4 a	44.42 ± 5.9 a	20.02 ± 3.9ab
	1	313 ± 54 a	682 ± 334 a	534 ± 161 a	1530 ± 219 a	137.44 ± 26 a	23.12 ± 1.9 a	45.81 ± 5.1 a	21.85 ± 3.5 a
	2	323 ± 26 a	610 ± 242 ab	499 ± 107 ab	1432 ± 302ab	128.96 ± 11 a	22.58 ± 1.4 a	43.81 ± 4.2 a	20.6 ± 2.7 ab

Means, in each column, with similar letters are not significantly different at the 5% probability level using Tukey's test.

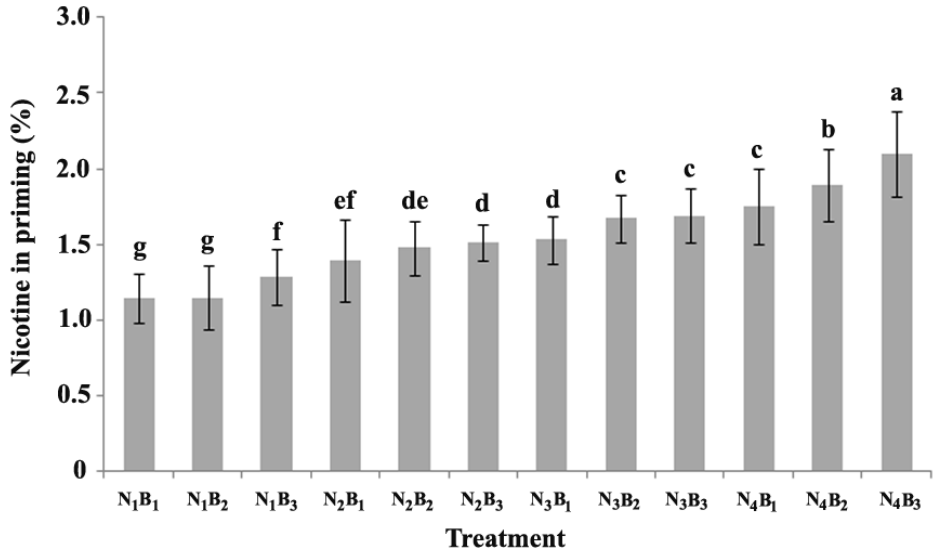

Fig 3. The effects of different levels of nitrogen fertilizer and inoculation of *Azotobacter chroococcum* on nicotine content in priming. Vertical error bars shows standard deviation (± SD).

Table 4. Analysis of variance (mean square and significance) for the effect nitrogen fertilizer and Azotobacter chroococcum on nitrogen uptake and qualitative characteristics of tobacco.

S.O.V	df.	Nitrogen			Nicotine			Reducing Sugar		
		Priming	Cutter	Tip	Priming	Cutter	Tip	Priming	Cutter	Tip
Replicate	2	0.08**	0.11**	0.04*	0.02*	0.18**	0.04	18.3**	21.2**	18.2
Year (Y)	1	0.05*	2.21**	0.53**	0.01	1.34**	7.26**	460.3**	46.9**	309.6**
Nitrogen (N)	3	1.00**	1.83**	1.41**	1.66**	3.05**	3.06**	139.9**	323.5**	191.7**
Bacterium (B)	2	0.12**	0.25**	0.19**	0.22**	0.45**	0.41**	25.0**	42.3**	22.0**
Y×N	3	0.05**	0.21**	0.08**	0.01	0.08*	0.09	0.8	2.29	1.0
Y×B	2	0.014	0.01	0.00	0.20	0.05	0.03	0.1	0.5	0.2
B×N	6	0.015	0.01	0.01	0.02*	0.01	0.04	4.4**	3.8	4.9*
Y×B×N	6	0.01	0.01	0.01	0.01	0.03	0.05	0.8	1.0	1.5
Error	46	0.01	0.02	0.01	0.01	0.03	0.03	1.3	2.3	1.8
CV		5.1	5.1	5.1	6.4	7.5	6.6	14.9	9.8	14.8

* and ** significant at level of 5 and 1%, respectively. Values that do not have any symbol are non-significant.

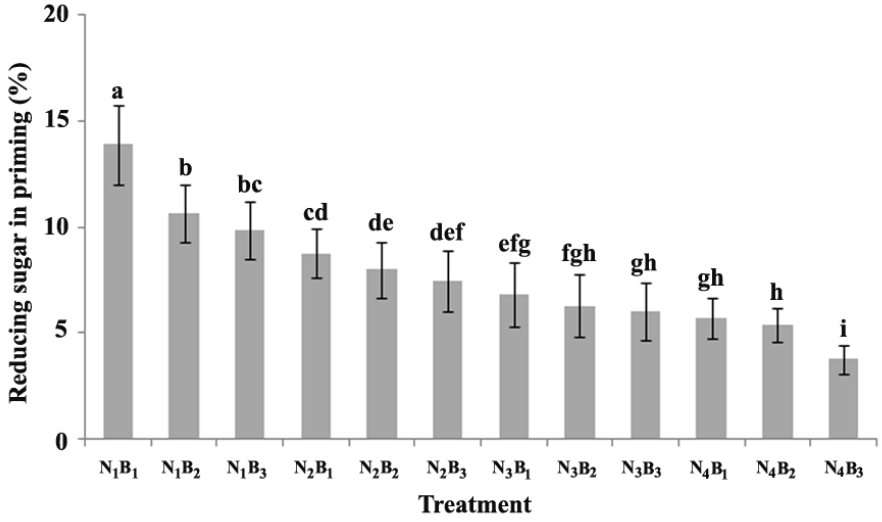

Fig 4. the effects of different levels of nitrogen fertilizer and inoculation of *Azotobacter chroococcum* on reducing sugar content in priming. Vertical error bars shows standard deviation (± SD).

reflected the growth rates of different tissues and that little remobilization from older to younger tissues occurred when N uptake from the soil was appreciable. N is an active element in plant and will be mobilized from the lower leaves and translocated to young leaves (Barker and Bryson, 2007). Inoculation with *Azotobacter chroococcum* like N fertilization significantly increased N uptake by tobacco plant. The increases in yield and N uptake with *Azotobacter chroococcum* inoculations may be explained with possible mechanisms of N fixation (Lakshminarayana et al., 1992) and growth hormone production (Azcon and Barea, 1976). *Azotobacter chroococcum* is an aerobic microorganism that fixes molecular N at different physiological conditions (Saribay, 2003). In relation to nicotine of leaves, our results showed that N fertilization and Inoculation with *Azotobacter chroococcum* significantly increased nicotine concentrations in leaves of tobacco. Application of 45 kg.ha^{-1} of nitrate ammonium fertilizer was most pronounced in comparison with control treatment. Application of this treatment increased 38, 36 and 31.5 % of nicotine concentrations in priming, cutter and tip leaves, respectively. Whereas, Inoculation with *Azotobacter chroococcum* (in 2 kg.ha^{-1} treatment) increased 12, 11 and 9.5 % of nicotine concentrations in priming, cutter and tip leaves, respectively. Nicotine content in the leaves of tip was more than other parts of leaves. This agrees with former studies made by Ju et al. (2008) who found similar results. It seems that there are correlations between N and nicotine of leaves, so that with increasing N due to consuming more N fertilizer causes increasing nicotine of leaves. Tso (1990) reported that in flue-cured tobacco leaf tissue N concentration was positively correlated with leaf nicotine content. Karaivazoglou et al. (2007) and Fuqua et al. (1976) were reported that leaf nicotine concentration was significantly affected by N fertilization. Soil N mineralization at late growth stages was an important factor affecting N accumulation and therefore the nicotine content in the upper leaves (Ju et al. 2008). The amount of reducing sugars in the leaves significantly decreased in response to N fertilization and bacterium inoculation. Reducing sugars can be produced both before and after harvest of tobacco (Gaines et al. 1983). Reducing sugars may appropriately vary from 15%–25% with a desirable value around 20% in the cured leaf (Maw et at., 2009). All values of reducing sugar in priming, cutter (except control treatment in cutter) and tip (table 5) were low in comparison with the desired value.

Table 5. Mean comparison (means ± standard deviation (SD)) effects nitrogen fertilizer and Azotobacter chroococcum on nitrogen uptake and qualitative characteristics of tobacco.

S.O.V	Nitrogen (%)			Nicotine (%)			Reducing Sugar (%)		
	Priming	Cutter	Tip	Priming	Cutter	Tip	Priming	Cutter	Tip
Year (Y)									
2008	1.96 ± 0.19 a	2.39 ± 0.24 b	2.20 ± 0.23 b	1.53 ± 0.49 a	2.36 ± 0.30 a	2.94 ± 0.45 a	10.21 ± 3.3 a	16.29 ± 4.1 a	11.16 ± 2.8 a
2009	1.90±0.28 b	2.74 ± 0.41 a	2.37 ± 0.3 a	1.55 ± 0.34 a	2.09 ± 0.32 b	2.30 ± 0.31b	5.15 ± 1.2 b	14.67 ± 3.5 b	7.01 ± 2.4 b
Nitrogen (N)									
0	1.66 ± 0.15 d	2.17 ± 0.24 d	1.96 ±0.17 d	1.18 ± 0.33 d	1.70 ± 0.37 d	2.13 ± 0.36 d	11.38 ± 4.2 a	20.34 ± 6.3 a	13.40 ± 3.8 a
15	1.85 ± 0.10 c	2.48 ± 0.27 c	2.19 ± 0.16 c	1.44 ± 0.37 c	2.13 ± 0.36 c	2.49 ± 0.42 c	8.07 ± 3.5 b	16.83 ± 5.7b	9.42 ± 2.8 b
30	1.99 ± 0.11 b	2.68 ± 0.33 b	2.40 ± 0.17 b	1.62 ± 0.36 b	2.39 ± 0.40 b	2.75 ± 0.49 b	6.36 ± 3.3 c	14.49 ± 5.0 c	7.84 ± 3.0 c
45	2.22 ± 0.18 a	2.93± 0.41 a	2.61 ± 0.17 a	1.90 ± 0.59 a	2.67 ± 0.39 a	3.11 ± 0.55 a	4.91 ± 2.9 d	10.24 ± 3.9 d	5.68 ± 2.2 d
bacterium (B)									
0	1.86 ± 0.23 c	2.46± 0.42 c	2.20 ± 0.31 c	1.44 ± 0.51c	2.10 ± 0.46 c	2.50 ± 0.54 b	8.77± 4.6 a	16.87 ± 6.8 a	10.09 ± 4.5 a
1	1.93 ± 0.22 b	2.57± 0.41 b	2.29 ± 0.26 b	1.53 ± 0.5 b	2.21 ± 0.45 b	2.62 ± 0.56 b	7.54 ± 4.1 b	15.34 ± 5.6 b	8.98 ± 4.2 b
2	2.00 ± 0.27 a	2.67± 0.44 a	2.38 ± 0.27 a	1.63 ± 0.36 a	2.37 ± 0.46 a	2.76 ± 0.59 a	6.74 ± 4.2 c	14.22 ± 5.5 c	8.19 ± 4.0 c

Means, in each column, with similar letters are not significantly different at the 5% probability level using Tukey's multiple range test.

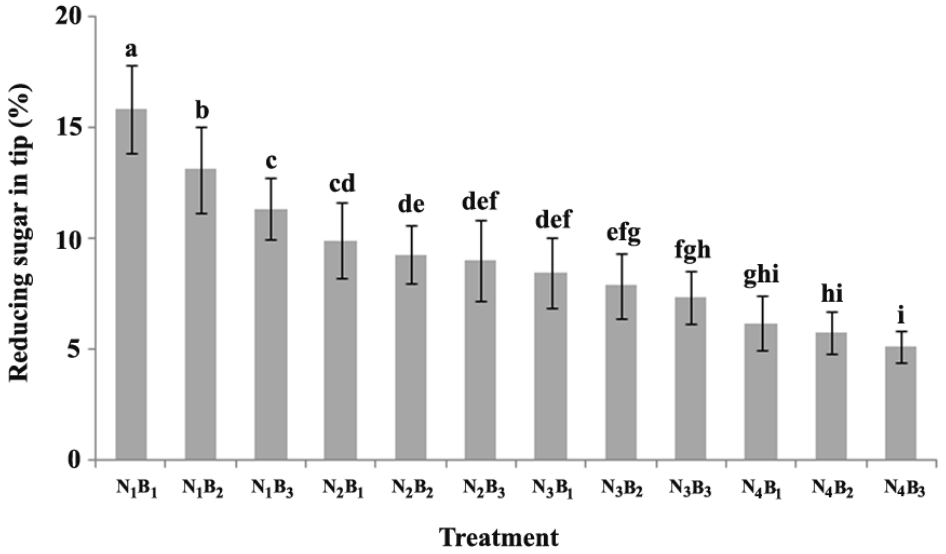

Fig 5. The effects of different levels of nitrogen fertilizer and inoculation of *Azotobacter chroococcum* on reducing sugar content in tip. Vertical error bars shows standard deviation (± SD).

Material and methods

Site description, weather conditions and soil properties

A field experiment was conducted at the experimental farm of the Rasht Tobacco Research Station lying in 37° 16´ northern latitude and 49° 31´ eastern longitude, in Guilan, Iran. Mean annual precipitation at the nearest meteorological station (Rasht synoptic station) is 1359 without any dry season. Average of annual temperature is 16°C, and average of annual relative humidity in percent is 81.5, with high relative humidity especially in the summer (94%). Fig. 1 shows the precipitation, temperature and relative humidity data for Guilan Tobacco Research Center during the growth season of tobacco as month period in cropping season, which was recorded in 2008 and 2009. The soil of experimental site has a Sandy loam texture. It is poor in organic matter, CEC and the pH of its saturated paste is 6.5 Table 1 shows some physical and chemical characteristics of used soil.

Plant culture

This study was conducted on flue-cured tobacco plant (*Nicotiana tabacum* L.) cultivar Coker 347. In order to prepare the soil for tobacco cultivation, the experimental site was ploughed at the depth of 30 cm. After application of Eradican herbicide in the level of 5 liter per hectare (2:1000) a rotary was applied. Seedlings of Coker 347 (flue-cured) tobacco cultivar were transplanted in experimental plots having 30 m^2 areas (5×6 m). The transplanting accomplished when the tobacco seedlings had the height of approximately 15 cm. Seedlings were transplanted with population of 20000 plants per hectare (the space between rows was 100cm and between plants on rows was 50cm). Basal fertilizer was applied annually over the whole experimental area and consisted of 96 kg P_2O_5 ha^{-1}, 200 kg $K_2O.ha^{-1}$ and N fertilizer as treatments. N and K fertilizer was divided by two stages (3 and 30 days after seedling). The commercial fertilizers used were ammonium nitrate (34.5% N), triple superphosphate (46% P_2O_5) and potassium sulfate (50% K_2O) and used in the middle of the ridge at 15 cm depth.

Seedling inoculation

A mixture of fine calcium carbonate neutralized peat as a carrier was packed into polyethylene bags (200 g carrier per bag), then sealed and sterilized with gamma irradiation (5.0×10^6 rads). *Azotobacter chroococcum* was grown on the medium of Hegazy and Neimela (1976), incubated for 48 hr at 28°C to ensure population density of 10^9 $cfu.ml^{-1}$ culture and then injected into the bags containing the sterilized carrier to have 10^8 $cell.g^{-1}$ carrier. Ultimately, microbial inoculant was prepared in Soil and Water Research Institute of Iran as powdery form. In addition, root segments of seedling from all treatments (except control treatment) were partially surface sterilized in absolute ethanol for 2 min and washed twice in sterilized distilled water. For applying Azotobacter, first a 20% solution of sucrose ($C_{12}H_{22}O_{11}$) was made, afterwards, powder of Azotobacter added to it and completely mixed, afterwards, seedling roots were placed in the solution for 30 minutes.

Experimental design and treatments

The experiment was performed as a 2×3×4 (Years × Bacterium levels × N levels) factorial experiment in a randomized complete block design (RCBD), with 3 replication and 24 treatments. This experiment was carried out during a two-year period (2008 to 2009). N fertilizer was applied in four levels (0, 15, 30 and 45 kg N ha^{-1}). Furthermore, Azotobacter fertilizer was added in three levels (0, 1 and 2 $kg.ha^{-1}$) as powder form.

Laboratory analysis

Leaves were harvested at five times in each year (2008 and 2009) from three leaf positions (priming, cutter and tip). Priming consists of the oldest, most mature leaves grown at the bottom of the stalk. Cutters are the middle leaves and are normally wider long than others leaves; and tips are narrow leaves from the top of the plant (Tassew, 2007). Fresh leaves were weighed, immediately. Afterwards, samples at 70 °C for 96h, in a forced-air oven were dried and dry weight was determined. Total N was analyzed employing the Kjeldahl procedure (Bremmer and Mulvaney, 1982). Nicotine and reducing sugars were measured using CORESTA recommended methods no. 35 (ISO/DIS 15152) (CORESTA, 1994a) and no. 38 (ISO/DIS 15154) (CORESTA, 1994b), respectively.

Statistical analysis

Statistical analysis of data including normality test, analysis of variance, and comparisons of means was performed by using SAS program (SAS Institute, 2002). Comparison of means was carried out using Tukey's tests at $P < 0.05$.

Conclusions

Bacterial inoculation can be added to the soil fertility and in turn to better crop production and good quality. Although all parts of the plant are colonized by microorganisms, the rhizosphere represents the main source of bacteria with plant-beneficial activities. In general, the quality of tobacco was intensity affected by the N and bacterium fertilization. From the results of this study, it is evident that the efficiency of fertilization on tobacco quality characteristics seems to depend on the rate of fertilization used. *Azotobacter chroococcum* inoculation was economically most efficient at lower doses of N fertilizer, which not only increased yields but also resulted in increasing of qualitative characteristics of tobacco. As a result, this study suggests that *Azotobacter chroococcum* is a suitable inoculant for tobacco cultivation due to its positive response and it could be a strategy to achieve sustainable agriculture. However, more studies and field trials should be run in the near future to reach the levels of recommendation for this bacterium and others.

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