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Correlation and path analysis of Tobacco (*Nicotiana tabacum* L.) yield *vs* root traits and relative water content as affected by *Azotobacter*, mycorrhizal symbiosis and biochar application under dry-land farming conditions

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Abstract. The global approach in agriculture is to reduce the use of chemical fertilizers and the supply of nutrients from available sources which are environmentally friendly. In order to evaluate the feasibility of tobacco products without chemical fertilizer inputs, this research was carried out as a factorial experiment based on a 3×2×2 randomized complete block design which included biochar applied at three levels (0, 4, and 8 t ha^{-1}), mycorrhiza, and *Azotobacter* at two levels (with and without application) with four replications. According to the results, 4 t ha^{-1} biochar increased the dry yield by 22%, the relative water content by 6%, and the root length by 41% compared to the zero level. However, there was no statistically significant difference between the 4 and 8 t ha^{-1} application of biochar with regard to most traits. The application of mycorrhiza improved the leaf area index as well as the tobacco root length. *Azotobacter* significantly increased the root length and nicotine content. The tobacco yield in rain-fed conditions is lower than usual, therefore the combined use of biochar and these biofertilizers may be considered as a viable solution. With increasing interest in the use of environmentally friendly sources of fertilizers and in terms of economic considerations, the use of 4 t ha^{-1} of biochar along with mycorrhiza and *Azotobacter* achieved an acceptable yield.

Keywords: bio-coal, biofertilizers, chemical quality, nicotine content, tobacco

INTRODUCTION

In recent years, the excessive consumption of chemical fertilizers in combination with increasing production costs has led to the destruction of soil, water and biological resources (Gebhardt *et al.*, 2017). Hence, in order to reduce the traces of chemical fertilizers in the environment and maximize the economic use of fertilizers, biofertilizers are considered to be a promising alternative approach to maintain and impr ove agroecosystems (Gao *et al.*, 2020). These biofertilizers are mainly based on beneficial microorganisms which have the effect of enhancing soil fertility and plant growth by increasing the number and biological activity of useful microorganisms in the rhizosphere (Gao *et al.*, 2020).

Arbuscular mycorrhizal fungi (AMF) are the most effective microbial symbiotic organisms for improving the growth and yield of the majority (90%) of plants (Ardakani *et al.*, 2009; Ahanger *et al.*, 2014; Tarnabi *et al.*, 2019). The symbiotic relationship between plants and mycorrhizal constitutes a link between the biotic and the geochemical portions of the ecosystem, and such a relationship may be

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considered to be a bridge connecting the root with the surrounding soil microhabitats (Larsen *et al.*, 2017). Inoculating soil with AMF results in the formation of more constant masses and significantly higher extra-radical hyphal mycelium than the non-AMF-treated soils (Samarbakhsh *et al.*, 2009; Syamsiyah *et al.*, 2018). In low moisture conditions in the soil, the available water for plants is limited (Dai, 2012). Hence, all plant physiological processes such as cell turgidity, photosynthetic processes, growth of the root, tissue and organs are influenced (Sheteiwy *et al.*, 2021). AMF can enhance plant tolerance to various environmental stresses by improving the acquisition of mineral nutrients and water (Baum *et al.*, 2015) and it can also affect the water balance of both amply watered and drought-stressed host plants (Sheteiwy *et al.*, 2021). Moreover, AMF improves the physical and chemical properties of the soil, and in particular, the soil structure. Additionally, AMF symbiosis enhanced the activity of soil microbial enzymes (El-Sawah *et al.*, 2021). The plant growth-promoting rhizobacteria (PGPR) was used for the first time at the end of the 1970s in many key ecosystem processes, in such forms as bio-fertilizers and bio-pesticides (Gao *et al.*, 2020). Recent studies have reported that bio-fertilizers can promote plant growth through nitrogen fixation, phytohormone, phosphate (P), and potassium solubilization (Bashan and de-Bashan, 2005). To reduce the harmful effects of agrochemicals with regard to tobacco leaf quality, the use of biofertilizers and nature-based compounds such as biochar are becoming established as essential agroecological practices for plant production. Biochar is a carbon-rich material obtained by pyrolysis using various biomasses (Major *et al.*, 2010; Soliman *et al.*, 2020). The positive effects of biochar application in improving plant growth are manifested in multiple forms, including the enhancement of the uptake and transport of nutrients (Mehari *et al.*, 2015). Biochar enhances soil properties (soil physicochemical characteristics like pH, CEC, soil structure), water holding capacity and immobilizes soil environmental pollutants (Abbas *et al.*, 2017; Moosavi *et al.*, 2020). However, the properties of biochar are closely related to its physical and chemical properties. In recent times, biochar has been developed to improve crop production as an environmentally friendly solution to reduce water scarcity problems (Oppong Danso *et al.*, 2020).

The present study hypothesized that biofertilizers and their combination with biochar could increase the tobacco yield in dryland farming conditions. According to limited research concerning the response of tobacco plants to abiotic stresses, including water deficit stress, the combination of three types of non-synthetic and environmentally friendly substances were studied with regard to their effects on tobacco growth, water holding capacity and nicotine content without the use of chemical fertilizers.

MATERIALS AND METHODS

This study was performed during the spring and summer seasons of 2017 and 2018 at the experimental farm of the Tirtash Tobacco Research and Education Centre, located at (44°53′22″ E; 36°42′12 N) north of Iran. According to the 30-year meteorological statistics, the average rainfall is 622 mm per year, rain mainly falls in the second half of the year outside the growing season. The average annual maximum and minimum temperature occurs in July (30°C) and January (5°C), respectively.

To determine the soil's physical and chemical properties, soil sampling was performed from a depth of 0 to 30 cm (Table 1).

Biochar chemical analysis results are shown in Table 2.

Azotobacter chroococcum and AMF inoculum were obtained from the Laboratory of Soil and Water Research Institute, Karaj, Iran. The mycorrhizal inoculum included three species *Funneliformis mosseae*, *Rhizophagus irregularis*, and *Clariodeoglomus etunicatum*, with a total population of 70 active spores per gram of biological fertilizer and *Azotobacter* inoculum with a minimal bacterial density of 10^7 CFU g^{-1}.

Table 1. Soil physical and chemical properties

Year	Organic carbon (%)	Electrical conductivity (ds m^{-1})	Chloride (%)	Absorbable potassium (ppm)	Phosphorus (ppm)	Total nitrogen (%)	Soil texture	pH
2017	1.03	0.37	0.97	220	6.9	0.089	Sandy loam	7.2
2018	0.95	0.49	0.65	238	10.1	0.093	Sandy loam	7.6

Table 2. Chemical analysis of biochar

Elements weight percentage									
Silicon	Aluminum	Nitrogen	Iron	Calcium	Potassium	Phosphorous	Magnesium	Oxygen	Carbon
1.43	0.19	0.31	0.43	4.27	0.38	0.18	0	43.71	48.83

Source: Razi Metallurgical Research Center, Karaj, Iran.

The experiment was carried out as a factorial based on a randomized complete block design (RCBD) with three factors including biochar (B) at three levels: 0, 4 t ha^{-1} (taking the border effect into account, 11 kg per experimental plot), and 8 t ha^{-1} (taking the border effect into account, 22 kg per experimental plot), mycorrhiza (M), and *Azotobacter* (A) at two levels (with and without application) in four replications for dry yield, root traits, leaf area index, mycorrhizal symbiosis, relative water content and three replications for nicotine content. The flue-cured tobacco pellet seeds (Var. K326) were sown in 220-cell trays with dimensions of 57 × 37 cm. For the mycorrhizal treatments, each tray was filled with 2000 g of soil media containing 10% of myc orrhizal inoculum before sowing. In treatments containing *Azotobacter*, ten days before transplantation, the seedling roots were inoculated with an equal volume of inoculum and water solution for an hour. The biochar was scattered over the soil surface and then mixed to a 20 cm soil depth. Tobacco transplantation and harvest were conducted in May and September (2017 and 2018), respectively. Each plot, with dimensions of 5 × 4 m, was formed by five rows and 55 tobacco plants spaced at 50 × 100 cm.

Four to five leaves were harvested at a time and then cured with bulk-curing using the Virginia tobacco curing method. At the end of each harvesting and curing period, tobacco leaves from each plot were weighed separately and the dry weight of the tobacco of each plot was recorded as the dry leaf yield from that particular plot.

In the mid-growing season, relative water content (*RWC*) was measured using five 1 × 1 cm surfaces of new fully expanded leaves of the 13th tobacco leaf. At first, the fresh weight of the samples was determined and then, the samples were floated on distilled water for 12 h in darkness. The turgid weight was measured. The leaf samples were oven-dried at 75°C for 24 h to calculate their dry weight. *RWC* was measured using Eq. (1) (Smart and Bingham, 1974):

$$RWC = \frac{fresh\ weight - dry\ weight}{turgid\ weight - dry\ weight} \times 100. \qquad (1)$$

During the growing season from 45 days after transplanting with a time interval of about 20 days to the last harvest, in five stages for each experimental plot, the plant was randomly selected and, all of its leaves were separated. After measuring its length and width, the leaf area was calculated by applying Eq. (2):

$$Leaf\ area = length \times width \times 0.6. \qquad (2)$$

After calculating the total area of the plant leaves, the leaf area index was determined in terms of square metres of leaf area per square metre of land. The highest value of the leaf area index was considered to be the maximum leaf area index for analysis.

Root sampling was performed using an auger with a diameter of 10 and a height of 30 cm (volume: 2 355 cm^3). Soil samples from each plot were soaked in plastic trays for 24 h to allow the roots to separate more easily from the soil during washing (Antony *et al*., 2004). The root washing step was performed under water pressure using a 50 mesh plastic filter (pores per $inch^2$). Their fresh weight was measured in mg (fresh root weight refers to roots that, after sampling the roots from the soil, are immediately washed with plenty of water, then the root surface water is carefully dried and finally weighed). For the root length trait, only root lengths with a diameter of one millimetre and above were measured.

At the end of the growing season, four root samples from each treatment (one sample from each replication) were isolated and coloured using Phillips and Hayman's (1970) method, and the percentage of colonization was determined using Giovannetti and Mosse's (1980) method.

Leaf nicotine content was measured using the CORESTA recommended method no. 35 (CORESTA, 1994).

The normality of the variables was checked using the Kolmogorov-Smirnov test, and Levine's test was also used to examine the equality of the variances. Duncan's multidomain test at $p<0.05$ level was also used to separate the averages of the dependent variables, which were affected to a significant extent by the treatment. Compound data analysis covering the two-year experiment was completed using SPSS software (Ver. 25). The mean values were compared using Duncan's multiple range test at a 5% probability level. Correlation and stepwise regression were analysed with SPSS software (Ver. 25), and path analysis was implemented using PATH software.

RESULTS

The year and the addition of biochar had a significant effect ($p<0.01$) on tobacco dry yield (Table 3). In the second year, dry leaf yield was 14% higher than in the first year (Table 4). As the level of biochar consumption increased, the leaf yield also increased. Biochar addition had a gradual beneficial effect on tobacco dry yield however, no significant difference was observed between the 4 (B4) and 8 (B8) t ha^{-1} levels. A comparison between the B4 and B8 additions showed that the improvement rate in the dry yield due to a doubling in the consumption of biochar was only 7%. Although mycorrhiza and *Azotobacter* application did not have a significant effect on dry yield, these additions nevertheless increased dry yield by 22 and 3.4%, respectively.

Relative water content was significantly ($p<0.01$) affected by the year being studied and biochar application (Table 3). The greater average value of *RWC* in the second year could be due to the higher rainfall during the growing season and the lower average monthly temperature compared to the first year. For treatments containing biochar, the average *RWC* was more than 78%, while the level of this

Table 3. Analysis of variance for the effect biochar, mycorrhiza and *Azotobacter* and their interactions on dry yield, relative water content, leaf area index, root fresh weight, root length, mycorrhizal colonization and nicotine (%)

S.O.V.	d.f.	Dry yield	Relative water content	Leaf area index	Root fresh weight	Root length	Mycorrhizal colonization	d.f.	Nicotine (%)
Year	1	28×10^{6}**	374.5**	11.57**	1030**	526.9 ^{ns}	70.60 ^{ns}	1	0.561**
r(year)	6	48×10^{3}	1.897	0.052	6.58	318.3	28.70	4	0.006
B: Biochar	2	41×10^{5}**	320.5**	0.935 ^{ns}	1362*	25839*	72.80**	2	0.051 ^{ns}
B×Y	2	43×10^{2} ^{ns}	0.544 ^{ns}	0.225**	71.23**	456.0*	9.80 ^{ns}	2	0.009 ^{ns}
M: Mycorrhiza	1	65×10^{5} ^{ns}	78.20 ^{ns}	2.714**	1266 ^{ns}	18349*	17888*	1	0.028 ^{ns}
M×Y	1	13×10^{4} ^{ns}	83.76**	0.007 ^{ns}	158.5 ^{ns}	73.10 ^{ns}	46.50 ^{ns}	1	0.000 ^{ns}
A: *Azotobacter*	1	19×10^{4} ^{ns}	1.919 ^{ns}	0.018 ^{ns}	2.00 ^{ns}	3966**	13.80 ^{ns}	1	2.141*
A×Y	1	17×10^{3} ^{ns}	1.517 ^{ns}	0.033 ^{ns}	30.63 ^{ns}	0.100 ^{ns}	11.80 ^{ns}	1	0.006 ^{ns}
B×M	2	17×10^{4} ^{ns}	12.43*	0.111 ^{ns}	29.23 ^{ns}	1411 ^{ns}	78.20**	2	0.020**
B×M×Y	2	19×10^{4} ^{ns}	0.612 ^{ns}	0.056 ^{ns}	5.26 ^{ns}	271.4 ^{ns}	10.50 ^{ns}	2	0.000 ^{ns}
B×A	2	78×10^{3} ^{ns}	16.73 ^{ns}	0.003 ^{ns}	0.90 ^{ns}	538.4 ^{ns}	1.80 ^{ns}	2	0.014*
B×A×Y	2	16×10^{3} ^{ns}	16.00**	0.033 ^{ns}	9.15 ^{ns}	1422**	20.70 ^{ns}	2	0.000 ^{ns}
M×A	1	18×10^{4} ^{ns}	36.66 ^{ns}	0.002 ^{ns}	145.9 ^{ns}	5.9 ^{ns}	9.70 ^{ns}	1	0.031*
M×A×Y	1	65×10^{4} ^{ns}	42.11**	0.019 ^{ns}	131.3**	257.9 ^{ns}	19.20 ^{ns}	1	0.000 ^{ns}
B×M×A	2	22×10^{3} ^{ns}	7.898 ^{ns}	0.044*	32.21^{ns}	145.4 ^{ns}	19.90 ^{ns}	2	0.026**
B×M×A×Y	2	76×10^{3} ^{ns}	6.284*	0.002 ^{ns}	19.59**	279.8 ^{ns}	16.30 ^{ns}	2	0.000 ^{ns}
Error	66	11×10^{4}	1.833	0.026	3.54	141.2	11.60	44	0.007
C.V. (%)	-	10.50	1.750	9.340	6.07	11.31	18.18	-	4.320

*0.05, **0.01 significant probability levels, ns non-significant.

Table 4. Main (± SD) of year, biochar, mycorrhiza and *Azotobacter* on dry yield, relative water content, leaf area index, root fresh weight, root length, mycorrhizal colonization and nicotine percent

Parameter		Dry yield (kg ha^{-1})	Relative water content (%)	Leaf area index	Root fresh weight (mg cm^{-3})	Root length (mm cm^{-3})	Mycorrhizal colonization (%)	Nicotine (%)
Year	2017	2455 ± 437^{b}	75.1 ± 3.8^{b}	1.39 ± 0.30^{b}	11.8 ± 2.12^{b}	0.44 ± 0.15^{a}	17.8 ± 3.8^{a}	1.86 ± 0.19^{b}
	2018	2798 ± 588^{a}	79.1 ± 3.6^{a}	2.08 ± 0.27^{a}	14.5 ± 3.85^{a}	0.46 ± 0.12^{a}	19.5 ± 4.8^{a}	2.04 ± 0.21^{a}
Biochar	B0	2228 ± 397^{b}	73.5 ± 3.1^{c}	1.55 ± .047^{a}	10.1 ± 2.23^{b}	0.32 ± 0.05^{c}	16.9 ± 2.6^{b}	1.91 ± 0.25^{a}
	B4	2723 ± 496^{a}	78.2 ± 3.1^{b}	1.77 ± 0.42^{a}	13.9 ± 2.65^{a}	0.45 ± 0.10^{b}	19.8 ± 5.5^{a}	1.95 ± 0.22^{a}
	B8	2928 ± 507^{a}	79.5± 2.8 ^{a}	1.89 ± 0.40^{a}	15.5 ± 2.76^{a}	0.56 ± 0.10^{a}	19.3 ± 4.9^{a}	2.00 ± 0.17^{a}
Mycorrhiza	M0	2365 ± 396^{a}	76.2 ± 4.4^{a}	1.57 ± 0.40^{b}	11.6 ± 2.65^{a}	0.39 ± 0.10^{b}	5.0 ± 1.8^{b}	1.93 ± 0.20^{a}
	M1	2887 ± 546^{a}	78.0 ± 3.4^{a}	1.91 ± 0.43^{a}	14.7 ± 3.37^{a}	0.50 ± 0.14^{a}	32.3 ± 5.5^{a}	1.97 ± 0.24^{a}
Azotobacter	A0	2581 ± 553^{a}	76.9 ± 3.7^{a}	1.73 ± 0.46^{a}	13.2 ± 3.76^{a}	0.42 ± 0.13^{b}	18.3 ± 4.1^{a}	1.78 ± 0.13^{b}
	A1	2671 ± 532^{a}	77.2 ±4.3^{a}	1.75 ± 0.44^{a}	13.2 ± 3.01^{a}	0.47 ± 0.14^{a}	19.0 ± 4.7^{a}	2.12 ± 0.13^{a}

Mean (± SD) with a common letter in the same column do not differ significantly at $p<0.05$.

parameter in non-biochar treatments was 73.5% (Table 4). The interaction between biochar and mycorrhiza was significant at a level of 5% (Table 3), and with the increasing consumption of biochar and mycorrhiza, the relative water content also increased. The lowest and highest values with 72.5 and 80.1%, are related to BM0 and B8M1, respectively (Table 5). There was no significant difference in *RWC* between the application of mycorrhiza and *Azotobacter* (Table 3).

The combined analysis of variance showed that the effect of the year being studied and mycorrhiza on leaf area index was significant at 1% (Table 3). Accordingly,

Table 5. Mean (± SD) of biochar and mycorrhiza interaction effects on relative water content, nicotine percent and mycorrhizal colonization

Experimental treatments		Relative water content (%)	Nicotine (%)	Mycorrhizal colonization (%)
Biochar	Mycorrhizal status			
No application	-AMF	72.5 ± 3.36^{d}	1.91 ± 0.18^{abc}	5.07 ± 1.89^{c}
	+AMF	74.5 ± 2.34^{c}	1.92 ± 0.29^{bc}	28.83 ± 5.02^{b}
4 t ha^{-1}	-AMF	76.8 ± 3.39^{ab}	1.87 ± 0.20^{c}	4.94 ± 1.64^{c}
	+AMF	80.0 ± 2.58^{a}	1.98 ± 0.22^{ab}	34.63 ± 5.07^{a}
8 t ha^{-1}	-AMF	79.3 ± 3.44^{a}	1.99 ± 0.17^{ab}	5.04 ± 1.55^{c}
	+AMF	80.1 ± 2.08^{a}	2.01 ± 0.16^{a}	34.49 ± 4.85^{a}

Mean (± SD) with a common letters in the same column do not differ significantly at $p<0.05$. AMF – arbuscular mycorrhizal fungi. Other explanations as in Table 4.

the average of this parameter in the second year was about 50% higher than in the first year (Table 4). The use of biochar did not have a significant effect on LAI_{max} (Table 4). Mycorrhiza application also resulted in a 22% increase in LAI_{max} compared to its non-application (Table 4). The B × M × A interaction effect was also significant for LAI_{max} at a 5% level (Table 3). However, the lowest LAI_{max} level belonged to B0M0A1 with a value of 1.40, and the highest level was related to B8M1A0 with a value of 2.13 (Table 5).

The results showed that the year being studied and biochar application had a significant effect on the fresh weight of the root at 1 and 5%, respectively (Table 3). The average fresh root weight in the first year was 11.8 and in the second year it was 14.5 mg cm^{-3} (Table 4). Even though there was no statistically significant difference between B4 and B8, the highest root fresh weight was obtained in treatments with the application of 8 t ha^{-1} of biochar producing, a value of 15.5 mg cm^{-3} and the lowest value of 10.1 mg cm^{-3} for the non-biochar treatments (Table 4). The root fresh weight for B8 compared to B4 and B0 showed an increase of 11 and 52%, respectively (Table 4). Although the root fresh weight was 27% (Table 4) higher for the mycorrhiza-containing treatments, this increase was not statistically significant (Table 3). In this study, biochar and mycorrhiza application had a significant effect on root length, increasing it by 5% (Table 3). Root length was continually enhanced by increasing the biochar application dosage from 4 to 8 t ha^{-1} in the soil which caused a 41 and 75% increase compared to the no biochar application, respectively (Table 4). The application of mycorrhiza increased root length by 30% (Table 4). Tobacco root length showed a significant increase ($p<0.01$) in response to *Azotobacter* application (Table 3), and treatments containing *Azotobacter* produced root lengths approximately 13% longer than the non-*Azotobacter* treatments (Table 4).

The colonization rate was positively affected and increased ($p<0.01$) by biochar application (Table 3) but not by enough, with results varying from 16.9 to 19.8%. There was no statistically significant difference between B4 and B8 (Table 4). The highest root mycorrhizal colonization rate with no significant difference was observed in B4 and B8. The mycorrhizal inoculum significantly ($p < 0.05$) increased the extent of root colonization as compared to the non-inoculated treatments (Table 3). In our study, although symbiosis was found to occur to a limited extent in natural soil conditions, fungal inoculum increased this symbiosis with roots by a factor of several times. In this regard, the application of mycorrhiza greatly enhanced the average colonization rate from 5 to 32% (Table 4). Also, the simultaneous application of biochar and mycorrhizal inoculum significantly ($p<0.01$) improved the colonization of tobacco root with fungi and caused approximate increases in root colonization from 5.1 to 34.5% (Table 5).

The year of the study had a significant effect ($p<0.01$) on nicotine content. The average percentage of nicotine in the first year was 1.86, which was enhanced by approximately 10% in the second year to 2.04% (Table 4). Also, *Azotobacter* had a significant ($p<0.05$) influence and the most considerable effect on nicotine content was observed with an increase of 20% (Table 4). The simultaneous use of biochar and mycorrhiza, biochar and *Azotobacter*, as well as mycorrhiza and *Azotobacter* had a significant effect on leaf nicotine content (Table 3). For treatments containing biochar and mycorrhiza, biochar and *Azotobacter*, and also mycorrhiza and *Azotobacter*, the highest amount of nicotine is related to B8M1 (Table 5), B8A1 (Fig. 1), and M1A1 (Fig. 2), respectively. The triple effect of experimental factors had a significant effect on nicotine content at the level of 1% (Table 3), which varied between 1.66 and 2.17% in B0M1A0 and B4M1A1, respectively (Table 6).

DISCUSSION

For tobacco, the quantitative yield is one of the most critical indicators for evaluation, so the utilization of correct management methods to achieve this may be considered to be a scientific solution. In this study, biochar application promoted growth, but for higher amounts of

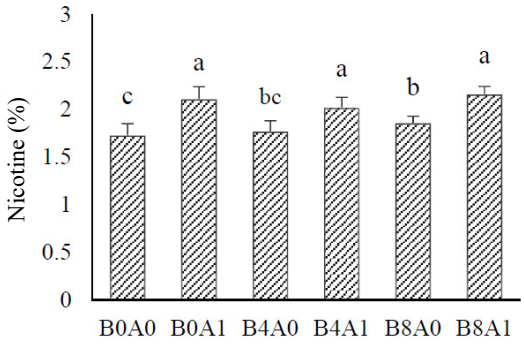

Fig. 1. Effect of biocbar and *Azotobacter* on nicotine (%). BO – no biochar application, B4 – application 4 t ha^{-1} of biochar, B8 – application 8 t ha^{-1} of biochar, AO – no *Azotobacter* application and Al – *Azotobacter* application. Means with the same letter are not significantly different.

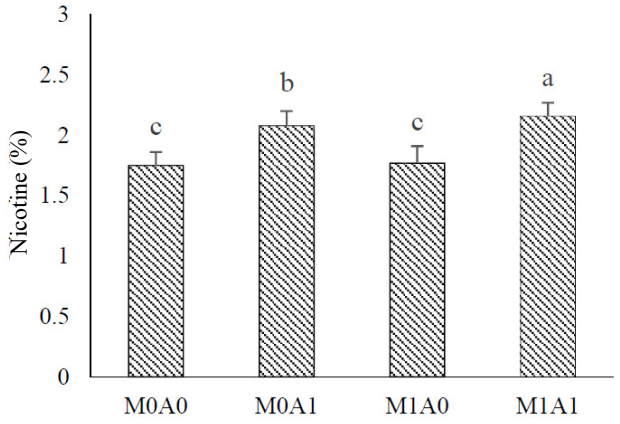

Fig. 2. Effect mycorrhiza and *Azotobacter* interaction on leaf nicotine content, MO – no mycorrhiza application, Ml – mycorrhiza application, AO – no *Azotobacter* application and A1 – *Azotobacter* application. Means with the same letter are not significantly different.

Table 6. Mean (± SD) of biochar, mycorrhiza and *Azotobacter* interaction effects on leaf area index and nicotine percent

Biochar	Experimental treatments		Leaf area index	Nicotine (%)
	Status			
	Mycorrhiza	*Azotobacter*		
No application	-AMF	-Az	1.47 ± 0.489^{cd}	1.78 ± 0.10^{bc}
		+Az	1.40 ± 0.464^{d}	2.06 ± 0.13^{a}
	+AMF	-Az	1.63 ± 0.492^{cd}	1.66 ± 0.14^{c}
		+Az	1.71 ± 0.466^{bc}	2.16 ± 0.14^{a}
4 t ha^{-1}	-AMF	-Az	1.58 ± 0.506^{cd}	1.72 ± 0.12^{bc}
		+Az	1.66 ± 0.391^{bcd}	2.04 ± 0.11^{a}
	+AMF	-Az	1.93 ± 0.418^{ab}	1.83 ± 0.11^{bc}
		+Az	1.92 ± 0.308^{ab}	2.17 ± 0.11^{a}
8 t ha^{-1}	-AMF	-Az	1.61 ± 0.198^{cd}	1.86 ± 0.07^{b}
		+Az	1.70 ± 0.344^{bc}	2.14 ± 0.10^{a}
	+AMF	-Az	2.13 ± 0.367^{a}	1.89 ± 0.85^{b}
		+Az	2.12 ± 0.406^{a}	2.15 ± 0.88^{a}

Explanations as in Table 4.

biochar addition (8 t ha^{-1}), no significant increment was observed. Some researchers have reported that biochar increases plant growth, biomass, and the absorption of nutrients in water deficit conditions (Kim *et al.*, 2019). Under low humidity conditions, biochar modification limited these adverse effects through its enormous surface area resulting in enhanced soil porosity and aeration, improvements in water holding capacity and the conservation of the water due to the porosity of biochar (Suliman *et al.*, 2017). Despite the insignificant effect of mycorrhiza on increasing tobacco yield revealed by this study, an increase of more than 500 kg ha^{-1} of dry yield was achieved with the application of mycorrhiza. Several previous studies have shown that mycorrhiza plays the role of secondary roots and can reduce the effects of water deficit stress by raising the water and nutrient content in plant tissues (Behrooz *et al.*, 2019).

It has been reported that biochar mixing with the soil can affect soil physical properties such as structure, pore distribution and density, water holding capacity and plant growth (Downie *et al.*, 2009). The positive effects of biochar application to agricultural soils are linked to changes in the physical and chemical parameters of the soil leading

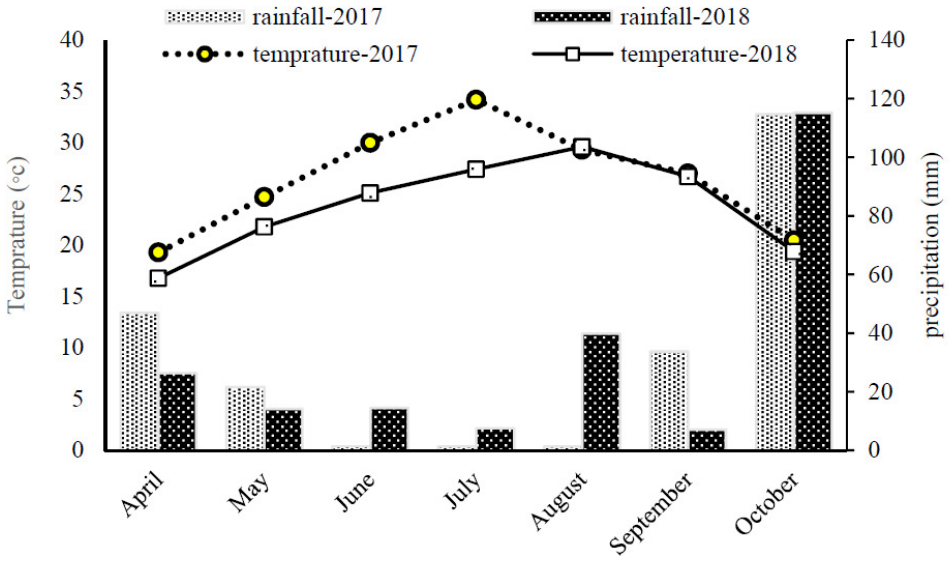

Fig. 3. Temperature and precipitation distribution (2017-2018).

to increased water-holding capacity, which improves the plant's access to soil water resources and thus improves the plant's water status (Laird, 2008).

An increase in *RWC* due to mycorrhiza application may be attributed to primary drought-avoidance mechanisms, such as increased water uptake related to mycorrhizal changes in root morphology or the active water transfer from mycorrhizal fungi to the host (Miri *et al.*, 2013). The combined application of biochar and AMF had a positive and significant effect on the relative water content (Table 3). Similar results have been reported by Hashem *et al.* (2019), showing that the combined application of AMF and biochar significantly increased the relative water content.

The results obtained in the present study revealed that all of the variables related to leaf area increased due to mycorrhizal inoculation. The observed enhancement in leaf area characteristics due to AMF inoculation confirms the results of previous studies. In particular, mycorrhizal inoculation increased the leaf area and leaf area ratio (LAR) of *Carica papaya* by 64 and 54%, respectively (Alarcón *et al.*, 2002). Moreover, the leaf area of *Leucaena leucocephala* increased by 161% due to inoculation (Dixon *et al.*, 1993). Increasing the water absorption capacity of mycorrhizal plants increases the extent of swelling of their cells, which is a stimulus for cell elongation (Wu *et al.*, 2009). In this study, the triple interaction of biochar addition, as well as mycorrhiza and *Azotobacter* on leaf area index was also positive and significant. Simultaneous biochar and biofertilizer application can also be a beneficial decision in drought conditions to stimulate host plant growth and thus is effective in increasing leaf area index (Ilkaee *et al.*, 2011; Sharma, 2002).

Improvements in root-related traits in the second year may be due to the prevailing optimal growth conditions as compared to the first year, including rainfall during the growing season and the low average monthly temperature (Fig. 3). In this study, the positive effect of biochar application on fresh weight and root length was observed. In this regard, it was shown that biochar increases the root length in annual plants, but the increase in root length depends on the time of use of the biochar and the species of plant in question (Xiang *et al.*, 2017). Other studies have shown that drought stress significantly reduced root length. Moreover, the use of biochar improves root length and depth, so it may be considered as a suitable alternative for increasing plant growth and productivity under drought stress (Hashem *et al.*, 2019). Results indicate that the co-amendment of biochar with other organic fertilizers has a more significant and positive influence on plant root growth and increased root biomass by 20-28% (Gul and Whalen, 2016). Biofertilizers effectively increase root length by increasing nutrient uptake, this is followed by more leaf photosynthesis and the allocation of more carbon to the roots (Azimi *et al.*, 2013).

The symbiosis of mycorrhizal fungi with plant roots, which has been identified by a sequence of biological functions, has a positive and beneficial impact on agroecosystems (Mardukhi *et al.*, 2015; Van der Heijden *et al.*, 2015). In this study, biochar and mycorrhiza application demonstrated a significant effect on the cloning rate of the fungus within the tobacco root system. The change in the frequency of mycorrhiza in the presence of biochar has been explained by certain mechanisms: biochar changes the ability of plants to gain access to soil nutrients, it plays a role in altering the activity of other microorganisms, or changes the signalling processes between the plant and the fungus. The effect of biochar in root colonization is related to improvements in the growth of fungal hyphae under water deficit conditions (Mickan *et al.*, 2014). The indirect

Table 7. Pearson correlation coefficients for dry yield (DR), root fresh weight (RFW), root length (RL), leaf area index max (LAI_{max}), relative water content (*RWC*), mycorrhizal symbiosis (MS) and nicotine content (NC)

	DR	RFW	RL	LAI_{max}	*RWC*	MS	NC
DR	1						
RFW	0.740 **	1					
RL	0.667 **	0.762 **	1				
LAI_{max}	0.638 **	0.638 **	0.463 **	1			
RWC	0.312 **	0.352 **	0.519 **	-0.105	1		
MS	0.532 **	0.525 **	0.493 **	0.448 **	0.270 **	1	
NC	0.326 **	0.291 **	0.371 **	0.415 **	-0.088	0.140	1

*p<0.01 significant probability level.

effect of biochar addition on increasing fungal propagation in roots and soil may be due to the cumulative effect on root growth (Hammer *et al.*, 2015).

The chemical composition of tobacco leaves determines their quality. The most valuable quality assessment criterion in tobacco is nicotine content (Shang *et al.*, 2017), this substance is used in the pharmaceutical industry and agricultural pesticides (Baldwin, 2001). In tobacco, nicotine is synthesized by the roots and transferred to the aerial organs, especially to the leaves (Shang *et al.*, 2017). The tobacco leaf nicotine content is closely dependent on soil conditions. This alkaloid is affected by the soil nitrogen content provided by biofertilizers. Inoculation with *Azotobacter chroococcum,* like N fertilization significantly increased the N uptake by the tobacco plants. The increases in N uptake due to *Azotobacter* inoculations may be explained by considering the possible mechanisms of N fixation and growth hormone production (Azcon and Barea, 1976). *Azotobacter chroococcum* is an aerobic microorganism that fixes molecular N under different physiological conditions (Saribay, 2003). With regard to the nicotine of leaves, our results showed that N inoculation with *A. chroococcum* significantly increased nicotine concentrations in tobacco leaves. This result confirms the results of previous studies performed by Ju *et al.* (2008). Tso (1990) reported that in flue-cured tobacco leaf tissue, N concentration was positively correlated with leaf nicotine content.

The dry yield had significant positive correlations (p<0.01) with all of the evaluated traits (Table 7). The closest correlation was observed between RFW and dry yield, suggesting an elevated dry yield with increases in the fresh weight of the root due to greater access to water resources, improving the relative water content, providing more favourable conditions for tobacco leaf growth, and finally, leading to an elevated dry yield. Pasban Eslam *et al.* (2017) studied the correlation between yield and physiological traits and reported that rapeseed genotypes with a higher *RWC* produced relatively higher yields under drought stress conditions. Stepwise regression was used to eliminate the effects of ineffective or less effective traits on dry yield in the regression model. Among the studied traits, root fresh weight, leaf area index, and relative water content were entered into Eq. (3), respectively (Table 8):

$$Y = -1\,234 + 27X_1 + 465X_2 + 29X_3, \quad (3)$$

where: Y is the dry yield, and X_1, X_2, and X_3 denote root fresh weight, leaf area index, and relative water content, respectively. The model had an R^2 of 0.609, meaning that these traits account for more than 60% of dry yield variations. The root fresh weight alone accounts for 54% of dry yield variations (Table 8). The root fresh weight was introduced earlier in the model than the other traits and had the closest correlation (0.74) with seed yield (Table 7). The addition of other variables to the model did not significantly influence any further increase in R^2. Root fresh weight had the most significant direct and positive effect (0.402) on dry yield (Table 9). This trait had the most pronounced indirect effect

Table 8. Stepwise regression for dry yield (dependent variable) and the other traits (independent variable)

Added trait to model	1	2	3
Intercept	1070	906	-1234
RFW	50	39	27
LAI_{max}		301	465
RWC			29
R^2	0.55	0.58	0.61

Root fresh weight (RFW), leaf area index max (LAI_{max}), relative water content (*RWC*).

Table 9. Path analysis showing direct and indirect effects on the dry yield

Trait	RFW	LAI	*RWC*
RFW	<u>0.402</u>	0.264	0.074
LAI_{max}	0.275	<u>0.386</u>	-0.022
RWC	0.142	-0.041	<u>0.211</u>
	Residual effect = 0.625		

Underlined values have direct effects. Other explanations as in Table 8.

on dry yield through LAI (0.264) and *RWC* (0.074), respectively. *RWC* exerted the least direct influence (0.211) and the lowest indirect effect (0.142 and -0.041) on dry yield.

CONCLUSIONS

1. The results showed that biochar improved the tobacco dry yield by significantly increasing leaf water content, further leaf area expansion, and root development.

2. Application of mycorrhiza while improving plant water status increased tobacco yield. Also, root length was increased with the application of biofertilizers.

3. The *Azotobacter* also significantly increased the nicotine content of the leaves.

4. The results of this study showed that the application of biochar with improved yield and biofertilizers with increasing growth characteristics and chemical properties could be recommended for use in dryland conditions.

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