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Yield and chemical composition of oriental tobacco as affected by biostimulant application

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Absract

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Biostimulants have been identified as an alternative to chemical fertilizer to increase crop production in sustainable farming. The aim of the study was to investigate the effects of two commercial biostimulants (protein hydrolysate (PH) Trainer® and microbial inoculant Europlus®) on the growth, yield and quality of oriental tobacco. The field experiment was conducted in 2020 and 2021 at Tobacco and Tobacco Products Institute, Markovo, Bulgaria on Rendzic Leptosol. The experimental design was a randomized complete block replicated three times. The doses of both biostimulants were used based on the manufacturer's recommendations. The plants were sprayed two times with PH Trainer four and six weeks after transplanting. The microbial product Europlus was incorporated in the top soil layer three weeks after transplanting. The control plants were not treated with biostimulants.

The application of microbial inoculant Europlus and PH Trainer improved the yield of tobacco by 5.5% and 6.7%, respectively, in comparison with untreated plants. The enhancement of the yield of sun-cured leaves was mainly due to the higher leaf area. Biostimulant treatments had no significant effect on macro and micronutrient concentrations in tobacco leaves. The application of PH Trainer and microbial inoculant Europlus increased the N, P, and K concentrations in leaves. The concentration of Ca, Mn, Zn and Cu were slightly higher than the control treatment when microbial inoculant Europlus was applied. The addition of protein hydrolysate Trainer and microbial inoculant Europlus increased nicotine content by 13.5% and 8.6%, respectively, compared to the control. The reducing sugars/nicotine ratio was better in tobacco from treatments with biostimulants compared to non-treated control. The results indicated that the use of biostimulants (microbial inoculant and protein hydrolysate) in sustainable tobacco farming was effective in increasing the yield of oriental tobacco and the quality of cured leaves.

Keywords: protein hydrolysate; microbial inoculant; tobacco; growth parameters; yield; macronutrients; micronutrients; chemical constituents

Introduction

Fertilization is an important agricultural practice for ensuring high crop production. While the use of chemical fertilizers is not recommended for quality Oriental cultivation, they are becoming more commonly applied to increase crop yields (Gilchrist, 1999). With organic tobacco cultivation, the quality and yields of oriental varieties have improved significantly (Tabaxi et al., 2021). The same authors noted that there is a growing interest in the cultivation of organic tobacco worldwide, since, in addition to being the final product of conventional tobacco, in terms of concentration of toxic and hazardous substances, it also has advantages such as increasing soil fertility, minimizing producers' exposure to toxic chemicals.

In the last decades, several technological innovations were proposed in order to enhance the sustainability of production systems through a significant reduction of chemicals. A promising and effective tool would be the use of biostimulants (Rouphael et al., 2015). Plant biostimulants which are defined as substances (humic acids, protein hydrolysates and seaweed extracts) and/or microbial inoculants (plant growth promoting rhizobacteria, arbuscular mycorrhizal fungi and *Trichoderma* spp.) can improve nutrient uptake and translocation (De Pascale et al., 2018). The same authors emphasized that plant biostimulants were initially used in organic production, but now they are adopted in several cropping systems such as conventional and integrated crop production. Numerous reports described that plant biostimulants have a positive effect on crop productivity (Rouphael et al., 2010; Colla et al., 2013; Caruso et al., 2019; Di Mola et al., 2020). The stimulation of biomass production in response to biostimulant application has been often associated to the action of specific signaling molecules on plant metabolism and physiology (Colla et al., 2020).

Stamenković et al. (2018) summarized that plant growth promoting microorganisms (PGPM) are an important group of microbial inoculants, which exist in rhizosphere and have the ability to inhabit the root of the plants and improve their development. Their positive influence is achieved through solubilization of phosphorus, nitrogen fixation, production of plant nutrients and phytohormones, protection from pathogens and recovery from stressful environmental conditions. Arbuscular mycorrhizal fungi (AMF) support plant nutrition by absorbing and translocating mineral nutrients beyond the depletion zones of plant rhizosphere and induce changes in secondary metabolism leading to improved nutraceutical compounds (Rouphael et al., 2015). The inoculated plants with arbuscular mycorrhizal biofertilizer containing *Glomus intraradices* had higher total, marketable yield, and total biomass than noninoculated plant (Rouphael et al., 2010). According to Radhakrishnan et al. (2017) the plant-beneficial *Bacillus* spp. produce plant growth-promoting substances (hormones and solubilizing enzymes) to increase plant growth. The use of biofertilizer (*G. mosseae* and three bacterial species) resulted in the highest biomass and increased the nutritional assimilation of maize plant (Wu et al., 2005). The success of microbial inoculants has been variable. The use of AMF inoculum in agricultural soil had no impact on above-ground biomass in wheat (Elliott et al., 2021). The authors suggest that one possibility for the lack of growth response, despite increases in tissue P, is that the plants were N, rather than P, limited. Michałojć et al. (2015) also reported that there was no effect of AMF on the total and marketable yield of tomatoes, as well as on the number of fruits per plant.

Protein hydrolysates (PHs) are an important group of plant biostimulants based on a mixture of peptides and amino acids that have received increasing attention in the recent years due to their positive effects on crop performances. PHs are mainly produced by enzymatic and/or chemical hydrolysis of proteins from animal- or plant-derived raw materials (Colla et al., 2015). The foliar application of PH Trainer could help in reducing the fertilizer application and consequently the environmental pollution in the production of high-quality vegetables (Colla et al., 2013). Many studies reported that the application of plant-derived PHs improved plant nutrition and increased biomass accumulation and crop productivity. The legume-derived protein hydrolysate resulted in the highest biomass of the vegetative plant parts and gave a significantly higher yield compared to non-treated control (Colla et al., 2017; Caruso et al., 2019). Di Mola et al. (2020) found that the marketable yield of baby spinach and lamb's lettuce was positively affected by PH foliar application and concluded that the application of protein hydrolysates can be a sustainable practice in intensive greenhouse cropping systems to enhance crop productivity and NUE under both optimal and sub-optimal (low-input conditions) N regimes.

Biofertilizer has been identified as an alternative to chemical fertilizer to increase soil fertility and crop production in sustainable tobacco farming (Subhashini et al., 2016). Triple inoculation of *Glomus intraradices* + *Psuedomonas flourescen*s and *Azotobacter chroococum* stimulated increased root colonization, growth, plant biomass, gas exchange parameters, nitrogen, phosphorus and potassium content in Virginia tobacco leaf, cured leaf yield, grade index and quality significantly over the double and single inoculation treatments (Subhashini, 2013). Increasing the application rate of microbial fertilizer (FZB42) could obviously promote the growth of flue-cured tobacco plants and improve the internal quality and smoking quality of leaves (Cheng et al., 2012). Supplementation of P and inoculation of AMF significantly enhanced growth, biomass accumulation and root activity of Virginia tobacco plants under normal conditions and mitigated the drought induced decline (Begum et al., 2020). Earlier research by Bozhinova & Hristeva (2020) found that with the exception of manganese and zinc, concentrations of macro- and microelements in Virginia tobacco leaves were significantly increased by the application of microbial product Europlus. There is little information about the effect of biostimulants (PHs and microbial inoculants) on the growth, crop performance and quality of oriental tobacco. De Pascale et al. (2018) emphasize that additional studies are required for defining the optimal dose, application time and method for each species and environmental conditions.

The aim of this study was to investigate the effects of two commercial biostimulants (protein hydrolysate Trainer® and microbial inoculant Europlus®) on growth parameters, yield, mineral and chemical composition of oriental tobacco.

Materials and Methods

The field trial was carried out in 2020 and 2021 with oriental tobacco (*Nicotiana tabaccum* L. cv. Krumovgrad 58). The experiment was conducted at Tobacco and Tobacco Products Institute, Markovo, Bulgaria (42°06′ N and 24°70′ E) on Rendzic Leptosol. The soil was heavy sandy loam with an alkaline reaction (pH_{H2O} – 7.7-7.9) and humus content – 2.74%-2.85%.

The available soil contents of N, P_2O_5, K_2O, Ca, Mg, Fe, Mn, Zn, and Cu are presented in Table 1.

The treatments with biostimulants were as follows: (1) Control (non-treated); (2) Application of protein hydrolysate Trainer; (3) Application of microbial inoculant Europlus.

The experimental design was a randomized complete-block design with three replications.

The biostimulant Trainer® (Italpollina, Italy) is produced using the innovative LISIVEG production system, starting from protein substances of vegetable origin. It contains organic nitrogen (5%), vegetal peptides (31%), organic matter (35.5%) and organic carbon (17.8%).

The biostimulant Europlus® (Eurovix, Italy) is a microbial consortium containing *Glomus* spp. (2%) and *Bacillus* spp. 1.2×10^9 CFU/g^{-1}.

Tobacco seedlings were transplanted at a 0.5 x 0.12 m distance on 18 May and 14 May in 2020 and 2021, respectively. No chemical fertilizers were added. The doses of both biostimulants were used based on the manufacturer's recommendations: Trainer (400 ml.da^{-1}) and Europlus (1000 g.da^{-1}). The plants were sprayed two times with PH Trainer four and six weeks after transplanting. The microbial product Europlus was incorporated in the top soil layer three weeks after transplanting. The control plants were not treated with biostimulants. All cultural practices were in accordance with those used by the growers for oriental tobacco production.

The following measurements were taken: plant height and number of leaves per plant (as measured at flowering), dimensions of the middle and upper leaves, 14^{-th} and 21^{-st} leaves, respectively, and yield of sun-cured leaves.

The concentration of N, P, K, Ca, Mg, Fe, Mn, Zn and Cu in mature leaves from middle stalk position was determined. All samples were washed with tap water to remove any adhering soil particles and rinsed afterwards with distilled water. Following drying at 75°C for 12 h samples were ground. Total nitrogen in the plants was determined by the Kjeldahl method. The preparation of plant samples for analysis of P, K, Ca, Mg, Fe, Mn, Zn and Cu was made by means of dry ashing and dissolution in 3 M HCl. Phosphorus was determined colorimetrically by the molybdovanadate procedure. An atomic absorption spectrometer "SpektrAA 220" (Varian) was used for determination of K, Ca, Mg, Fe, Mn, Zn and Cu content in the plant samples.

The random samples of sun-cured leaves were analyzed for nicotine content (ISO 15152:2003) and soluble carbohydrates (ISO 15154:2003).

Statistical processing of data was done by means of the analysis of variance. The differences were assessed with the Duncan's multiple range test at the 0.05 probability level.

Results and Discussion

Growth parameters were positively affected by biostimulant application (Table 2). The application of microbial inoculant Europlus and PH-based biostimulant Trainer resulted in higher plant height compared to non-treated control (+4.6% and +3.1%, respectively). The microbial inoculant increased the number of leaves by 1.5% compared to untreated plants. Similarly, Yari et al. (2019) reported that the number of the middle tobacco leaves was increased by mycorrhizal inoculation. Our results are somewhat in line with those of Nakmee et al. (2016), who found that AM fungal inoculation did not greatly affect the sorghum height, but significantly increased the number of leaves. In our study the number of leaves per plant was slightly affected by foliar application of PH Trainer. On the other hand, Rouphael et al. (2017) demonstrated that application of protein hydrolysate at 5.0 ml L^{-1} enhanced leaf number of tomato by 21.8% compared to untreated plants.

The biostimulants used in the experiment had significant effect on the dimensions of the middle leaves. The microbial inoculant Europlus increased the length and width of middle

Table 1. Available nutrients in the soil, mg.kg^{-1}

Year	$N\text{-}NH_4$+$N\text{-}NO_3$	P_2O_5	K_2O	Ca	Mg	Fe	Mn	Zn	Cu
2020	12.51	31.0	472	4375	264	12.0	33.3	10.7	5.0
2021	22.76	13.8	437.5	4126	237	9.8	28.2	12.8	6.1

N_{min} (extracted with 1% solution of KCl); P_2O_5 (Olsen method); K_2O (in 2N HCl); available Ca and Mg (method with 1N KCl); Fe, Mn, Zn and Cu mobile forms were extracted from *soil* using diethylene triamine pentaacetic acid (*DTPA*)

Table 2. The effect of biostimulant treatments on tobacco growth parameters and yield of cured leaves (2-year average)

Biostimulant	Plant height, cm	Number of leaves per plant	Dimensions of the 14-th leaf, cm		Dimensions of the 21-st leaf, cm		Yield, kg.da^{-1}
			Length	Width	Length	Width	
Control	126.7b	27.2	23.5b	14.8b	16.5	10.5	140.6
Trainer	130.6ab	27.4	24.9a	16.3a	17.3	11.1	150.0
Europlus	132.5a	27.6	25.2a	15.9a	17.2	11.1	148.4
Significance	*	NS	**	***	NS	NS	NS

NS – Nonsignificant; Significant at *P < 0.05, **P < 0.01 or ***P < 0.001. Different letters within each column indicate that the means are significantly different (P < 0.05)

leaves by 7.2% and 7.4%, respectively, compared with the plans from the control. The protein hydrolysate enhanced length of middle leaves by 6% and the width by 10.1%. The biostimulants effect on length and width of upper leaves was positive, but not statistically confirmed. It is known that except in extreme heat and drought conditions, no irrigation is recommended to quality Oriental production. Physiological and biochemical processes in *Nicotiana tabacum* were affected by drought stress resulting in altered growth (Begum et al., 2020). The authors concluded that AMF inoculation allevaited the drought induced decline in photosynthesis by up-regulating the antioxidant metabolism and osmolyte accumulation. Colla et al. (2014) indicated that the greater biomass of plants treated with PH Trainer containing amino acids and small peptides could be attributed to an increase in leaf N content, which may account for improved photosynthesis and enhanced translocation of synthates to the sinks.

The application of microbial inoculant Europlus and PH Trainer improved the yield of sun-cured leaves by 5.5%, and 6.7%, respectively, in comparison with untreated plants. The higher productivity of tobacco treated with a biostimulants was mainly due to the increased dimensions of the leaves from different stalk position. Our results on PH were consistent with the findings of Colla et al. (2017), who observed that the foliar application of legume-derived PH enhanced the marketable yield of tomato by 7%, compared with the control. The positive effects of the PH biostimulant on marketable fresh yield of baby spinach and lamb's lettuce were likely derived from the signaling molecules (such as small peptides) as a result of augmented leaf nitrate content, SPAD index and pigments synthesis (Di Mola et al., 2020). According to Rouphael et al. (2017) improved yield performance with protein hydrolysate foliar applications at the highest rate was related to improved leaf nutritional status of tomato plants and higher net assimilation of CO_2. Rouphael et al. (2010) concluded that the highest yield production in inoculated cucumber plants grown under alkaline conditions seems to be related to the capacity of maintaining higher net photosynthesis and to a better nutritional status in response to bicarbonate stress with respect to nonmycorrhizal plants.

Table 3 shows the macronutrient concentrations in mature leaves from middle stalk position as dependent on biostimulant treatments, averaged over the period studied.

No significant influence of biostimulants was found regarding the leaf content of nitrogen. The application of microbial inoculant Europlus increased the N concentrations in leaves by 4.5% compared to untreated plants. According to Battini et al. (2017) AMF after establishing symbiosis produce extensive underground extra-radical mycelia ranging from the roots up to the surrounding rhizosphere, thereby helping in improving the uptake of nutrients specifically N. Saia et al. (2015) found that under unfertilized conditions, inoculation of durum wheat with AMF and AMF + PGPR upregulated the expression of nitrate transporter genes and AMT1-type ammonium transporter genes in roots increasing the nitrogen in the aboveground biomass. However, Farzaneh et al. (2011) reported that AMF colonization significantly reduced the N concentrations of chickpea in compared to the control and this might be attributed to a dilution effect due to higher dry matter production in the AMF treatment. The applications of protein hydrolysate Trainer increased the concentration of nitrogen in tobacco leaves by 3.4% com-

Table 3. The effect of biostimulant treatments on macronutrient concentrations in tobacco leaves (2-year average)

Biostimulant	N	P	K	Ca	Mg
	% of dry weight				
Control	1.78	0.15	0.76	2.11	0.20
Trainer	1.84	0.16	0.84	2.01	0.19
Europlus	1.86	0.16	0.99	2.29	0.19
Significance	NS	NS	NS	NS	NS

NS – Nonsignificant at P < 0.05

pared to control. The high nitrogen uptake observed in plants treated with Trainer could be explained by the extensive root apparatus and the increase of nitrogen assimilation process (Colla et al., 2014). The high nitrate and glutamine synthetase activities together with the high induction of glutamine synthetase isoforms indicate a stimulatory effect of the protein hydrolyzate–based fertilizers on the assimilation of nitrate by maize plants (Ertani et al., 2009).

P concentration was positively affected by biostimulants compared to non-treated control, with no significant difference between them. Concentrations of P in tobacco leaves were increased by 6.7% when different biostimulants were applied. Plant tissue phosphorus increased in inoculated plants potentially because of changes induced by inoculation in microbial community composition and/or nutrient cycling within the rhizosphere (Elliott et al., 2021). According to Rouphael et al. (2017) the biostimulant-mediated joint effects of increased root surface area and enhanced nutrient transporter activity may explain the improved uptake of P, K, and Mg found in the leaves of tomato plants treated with PH Trainer.

The use of microbial inoculant led to higher contents of K in tobacco leaf tissue compared to control. Similar results have been reported for cucumber (Rouphael et al., 2010). The same authors supposed that the beneficial effect of AM fungi may be due to enhanced nutrient acquisition and transport to the plant. The data from our trial showed that K was positively affected by application of PH Trainer compared to non-treated control, with no significant difference between them. Caruso et al. (2019) also found that the application of a PH-based biostimulant resulted in higher K content in cherry tomato fruit. Rouphael et al. (2017) demonstrated that the application of protein hydrolysate at 5.0 ml L^{-1}, and to a lesser degree at 2.5 ml L^{-1}, elicited an increase in mineral composition (K and Mg), thereby increasing the nutritional quality of the tomato fruits.

The PH biostimulant treatment was not significantly affected the Ca concentrations in leaf tissue. These findings are in agreement with those of Rouphael et al. (2017) and Caruso et al. (2019), who reported that the influence of legume-derived PH application on Ca concentrations in tomato leaves and fruits was no significant. Compared to the untreated plants, Ca concentration was increased by 8.5% in response to application of microbial inoculant Europlus. Positive influence of AMF inoculation on calcium content in tomato fruits was reported by Michałojć et al. (2015). In contrast, Baslam et al. (2011) found that AMF improved growth of lettuce, thus producing a dilution effect on the concentrations of some mineral nutrients (e.g., Ca and Mn).

We have not found any significant difference in Mg concentrations between different treatments. Similar, Farzaneh et al. (2011) reported that Mg concentrations in chickpea were unaffected by mycorrhizal inoculation. Application of PH extracts caused an increase in Mg concentrations in tomato leaves and fruits (Rouphael et al., 2017; Caruso et al., 2019), which was not confirmed in the present study.

The concentrations of Fe, Mn, Zn and Cu were not significantly affected by biostimulant treatmens (Table 4).

The biostimulants tested did not increase the concentrations of Fe in tobacco leaves. On the other hand, Rouphael et al. (2010) reported that inoculated plants accumulated more Fe than noninoculated plants and this suggests that inoculation with a biofertilizer containing *G. intraradices* enhance the uptake and translocation of Fe toward the shoot. Liu et al. (2000) explained that inconsistent responses of mycorrhizal plants in micronutrient uptake may be related to highly variable soil conditions, which influence AMF root colonization and extraradical hyphal development.

Mn concentration was higher (by 9.8%) than the control treatment when microbial inoculant was applied. Rouphael et al. (2010) also reported that the Mn concentration in leaves of inoculated cucumber was higher by 11% than in leaves of noninoculated plants. The effects of *G. intraradices* on Zn, Cu, Mn, and Fe uptake varied with micronutrient and P levels added to soil (Liu et al., 2000). The same authors found that at the highest dose of micronutrients, mycorrhizal plants had lower Mn contents in shoots than non-mycorrhizal plants, while at the lower dose of micronutrients or with no micronutrient addition, mycorrhizal and non-mycorrhizal plants had similar Mn contents.

Inoculation with microbial product Europlus slightly increased Zn and Cu concentration in tobacco leaves (by 6.9%

Table 4. The effect of biostimulant treatments on micronutrient concentrations in tobacco leaves (2-year average)

Biostimulant	Fe	Mn	Zn	Cu
	mg.kg^{-1} dry matter			
Control	141.9	53.0	68.6	11.0
Trainer	133.2	49.3	62.3	10.9
Europlus	139.5	58.2	73.3	11.3
Significance	NS	NS	NS	NS

NS – Nonsignificant at $P < 0.05$

Table 5. The effect of biostimulant treatments on chemical composition of oriental tobacco (2-year average)

Biostimulant	Nicotine, %	Reducing sugars, %	Reducing sugars/Nicotine
Control	1.33	16.8	12.6
Trainer	1.51	13.7	9.1
Europlus	1.45	13.7	9.5

and 2.7%, respectively) compared with the control. Liu et al. (2000) reported that total Zn content in shoots was higher in mycorrhizal maize than non-mycorrhizal plants grown in soils with low P and low or no micronutrient addition. The authors also found that total Cu content in shoots was increased by mycorrhizal colonization when no micronutrients were added. In our study the application of microbial inoculant had no significant effects on the concentrations of Zn and Cu in leaves and this may be due to the high amount of available Cu and Zn in the soil. The foliar application of PH-based biostimulant Trainer have not influenced the concentrations of Zn and Cu in tobacco, while Ertani et al. (2019) reported that greater accumulation of micro (Cu, Zn) elements was evident in maize plants supplied with protein hydrolysates.

Table 5 shows the effect of different biostimulant treatments on chemical characteristics of sun-cured tobacco. Most of the chemical constituents of tobacco, although genetically controlled, have been found to change with growth stage and a number of cultural practices (Lolas et al., 1979). The normal nicotine content for oriental tobacco is within 0.6%-2.5% and the optimum reducing sugars' content is between 10% and 18%. Balance of chemical components is important for a correct assessment of tobacco quality. The ratio of sugars to nicotine serves as a good smoking quality indicator. The reducing sugars/nicotine ratio should range between 6 and 10 (Ghiuselev, 1983). The nicotine values for all treatments were within acceptable ranges. The lowest content of nicotine in the cured leaves was found in control treatment. Microbial inoculant Europlus increased the percentage of nicotine by 8.6% compared to control. The addition of protein hydrolysate Trainer increased nicotine content by 13.5%.

Our results correspond to the findings of Begum et al. (2020), who reported that nicotine content increased due to AMF inoculation. Adding microbial inoculum and protein hydrolysate resulted in decreased reducing sugars' content, when compared to control plants. Our results were consistent with the findings of Tabaxi et al. (2021), who found that as nicotine increases, sugars decrease with the application of organic fertilizers. The ratio of these components was better in tobacco from treatments with biostimulants compared to non-treated control. Given the above data we can point out that the use of biostimulants (microbial inoculum and protein hydrolysate) had favorable effect on chemical composition of the cured tobacco.

Conclusions

Growth parameters were positively affected by biostimulant application. The biostimulants used in the experiment significantly increased the length and width of the middle leaves compared to the control. The application of microbial inoculant Europlus and PH Trainer improved the yield of tobacco by 5.5% and 6.7%, respectively, in comparison with untreated plants. The enhancement of the yield of sun-cured leaves was mainly due to the higher leaf area.

Biostimulant treatments had no significant effect on macro and micronutrient concentrations in tobacco leaves. The application of protein hydrolysate Trainer and microbial inoculant Europlus increased the N, P, and K concentrations in leaves. The concentration of Ca in leaf tissues was positively affected by microbial inoculant. Mn, Zn and Cu concentrations were slightly higher than the control treatment when microbial inoculant Europlus was applied.

The addition of protein hydrolysate Trainer and microbial inoculant Europlus increased nicotine content by 13.5% and 8.6%, respectively, compared to the control. The reducing sugars/nicotine ratio was better in tobacco from treatments with biostimulants compared to non-treated control.

The results indicated that the use of biostimulants (microbial inoculant and protein hydrolysate) in sustainable tobacco farming was effective in increasing the yield of oriental tobacco and the quality of cured leaves.

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