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NON-DESTRUCTIVE ASSESSMENT OF LEAF CHLOROPHYLL AND EPIDERMAL FLAVONOIDS IN TWO TOMATO CULTIVARS (*SOLANUM LYCOPERSICUM* L.) GROWN UNDER DIFFERENT *TRICHODERMA* SPP. TREATMENTS

MIRJANA BOJOVIĆ[1], *ZORICA MRKONJIĆ*[1], *ZORANA SREĆKOV*[1], *GORDANA RACIĆ*[1], *VEDRANA PROROK*[2], *DANKA RADIĆ*[3], *DEJANA PANKOVIĆ*[1*]

[1]Faculty of Ecological Agriculture, Educons University, 21 208 Sremska Kamenica, Serbia
[2]Faculty of Technology, University of Novi Sad, 21 000 Novi Sad, Serbia
[3]Institute of General and Physical Chemistry, University of Belgrade, 11 158 Belgrade, Serbia
**Corresponding author*: dejanapankovic62@gmail.com

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SUMMARY

The purpose of this study is to examine the effects of different Trichoderma spp. treatments (T1: Trichoderma harzianum, T2: Trichoderma brevicompactum, and T3: T1 + T2) on two tomato cultivars ('Narvik' and 'Gružanski zlatni') by the non-destructive monitoring of chlorophyll (Chl) and epidermal flavonol (Flav) indices and nitrogen balance index (NBI) in their leaves. The main objectives were to compare the responses of tomato cultivars considered (grown in a greenhouse) to different Trichoderma strains and a combination of Trichoderma strains. Variations in the Chl, Flav, and NBI values obtained were significantly influenced by nearly all the factors examined (namely the tomato cultivar, experimental week, and treatment), the interaction of which was found significant for all the parameters analyzed. The results obtained indicate that the combinations of strains in the T3 treatment markedly enhanced the Chl content in the 'Narvik' cultivar in some experimental weeks. Moreover, reduced NBI values were determined in the Trichoderma-treated 'Narvik' plants due to the increased flavonol content, which indicates a shift from primary to secondary metabolism. Further research, including the same cultivars and treatments, is warranted under field conditions to evaluate the physiological responses of the 'Narvik' and 'Gružanski zlatni' tomato cultivars in a multiple-stressor environment.

Key words: *Trichoderma, Solanum lycopersicum L., Dualex optical sensor, chlorophyll, flavonols, nitrogen balance index, genotype-strain interaction*

Abbreviations: Chl – indice of chlorophyll (relative); Flav – indice of epidermal flavonols (relative); NBI – nitrogen balance index (relative)

INTRODUCTION

Nowadays, the concept of sustainable agriculture is largely in focus, implemented by eco-friendly strategies with a tendency to use fewer chemicals for land and water resource protection (Szekeres et al., 2004). One of the main components of such sustainable agriculture is the application of biocontrol agents (BCAs) for plant protection and fertilization.

One of the most researched fungal biocontrol agents belongs to the genus of *Trichoderma*, a few species of which have already had commercial success as bio-pesticides or bio-fertilizers (Alfiky & Weisskopf, 2021). As a root symbiont, *Trichoderma* spp. create chemical communication with a plant, changing the plant's physiology and reprogramming its gene expression (Zaidi et al., 2014).

The tomato (*Solanum lycopersicum* L.), which belongs to the Solanaceae family, represents the second most important vegetable crop in the world. The current global production of tomatoes is about 182.3 million tons, with 4.85 million ha devoted to tomato cultivation each year (FAOSTAT, 2019). Numerous previous studies suggest that the *Trichoderma* spp. root colonization in tomato plants causes modifications to their physiological systems, including photosynthesis or other metabolic processes (Alexandru et al., 2013; Racić et al., 2018; Vukelić, 2022). Accordingly, chlorophylls and flavonoids are the types of molecules indicative of the tomato-*Trichoderma* interaction.

Owing to their efficient and non-destructive features, optical instruments for the *in situ* monitoring of chemicals in plants are considered very useful (Agati et al., 2016). The Dualex sensor detects plant leaf flavonoid (Flav) and chlorophyll (Chl) contents non-destructively, representing the indicator of the plant's nitrogen status based on the chlorophyll/flavonols ratio, i.e. the nitrogen balance index (NBI) (Kaniszewski et al., 2021). The chlorophyll content of a plant was shown to be positively correlated with the nitrogen content, whereas the epidermal flavonol content was conversely correlated with the nitrogen content (Guler et al., 2016).

The main objective of our study was to compare the physiological responses of tomato cultivars considered to different Trichoderma strains and their combinations through the magnitude of change in the following parameters: the chlorophyll content (Chl), epidermal flavonol content (Flav), and nitrogen balance index (NBI) (which were obtained by non-destructive measurements performed on the ‘Narvik’ and ‘Gružanski zlatni’ tomato plant leaves grown under different *Trichoderma* spp. treatments (T1: *Trichoderma harzianum*, T2: *Trichoderma brevicompactum*, and T3: T1 + T2). Moreover, the physiological responses obtained were utilized to recognize and define the positive effects of different *Trichoderma* strains and their combinations on the tomato cultivars considered. We hypothesized that our results would provide scientific evidence for the physiological benefits of *Trichoderma* spp. application to tomato plants grown under greenhouse conditions, based on the Dualex ability to pre-screen the reaction of tomato plants treated with different *Trichoderma* isolates.

MATERIAL AND METHODS

Plant material and fungal strains

‘Narvik’ and ‘Gružanski zlatni’ are mid-late tomato cultivars with good fruit quality and disease resistance, thus often used for both industrial and marketing purposes. The ‘Gružanski zlatni’ tomato cultivar is resistant to *Fusarium oxysporum* f. sp. *Licopersici*, *Verticillium albo-atrum*, and *Phytophthora infestans*, whereas ‘Narvik’ is resistant to *Fusarium oxysporum* f. sp. *Licopersici* and *Verticillium albo-atrum* (Vukelić, 2022).

A total of two *Trichoderma* strains, isolated from agricultural soils, were used in this study. They were identified as *Trichoderma harzianum* and *Trichoderma brevicompactum*, and deposited in the Szeged Microbiological Collection (www.szmc.hu) as SZMC 20660 and SZMC 22661, respectively. Both strains were isolated from the A horizon of the experimental soils (5-30 cm) and maintained on a potato-dextrose agar medium at 4^oC. Prior to the preparation of the fungal suspension, the strains were preincubated at 25^oC in the dark. The suspensions were prepared as follows: the pure cultures of *T. harzianum* and *T. brevicompactum* were taken from a Petri plate, resuspended in 100 mL of sterile tap water, and then shaken for 2 hr at 50 rpm (TalBoys, Kingwood, Houston, TX, USA). The density of the suspensions for plant treatments was 1.75×10^6 colony forming units (CFU) mL^{-1} (Racić et al., 2018).

Experimental design

The experiment was conducted in a randomized block design under greenhouse conditions (located in the Agricultural High School in Futog 45°14′17″N, 19°42′22″E) in three replications. A total of 30 plants of each tomato cultivar were measured per treatment: C - control, T1 - *T. harzianum*, T2 - *T. brevicompactum*, and T3 - *T. harzianum* + *T. brevicompactum*. The suspensions of *Trichoderma* isolates were applied to the root zone of the tomato plants when three established leaves were observed on each plant.

Physiological measurements

The content of chlorophyll (Chl), epidermal flavonols (Flav), and nitrogen balance index (NBI) in the tomato leaves were measured *in vivo* by a non-destructive method, using the Dualex Scientific sensor (Force-A, France) once a week during 84 days (12 weeks) of the plants’ growth (12 measurement points in total). The measurements were done on 10 plants per replication per treatment. The Chl-content estimation is based on a difference in the transmission of two distinct wavelengths: visible (VIS) (650 nm) and near-infrared (NIR) (710 nm). The epidermal flavonols content is determined by comparing the absorbance at ultraviolet A (UVA) (375 nm) and 650 nm. Both wavelengths excite Chl fluorescence, but only UVA is affected by flavonols. The difference in Chl fluorescence measured at 710 nm is directly proportional to the amount of epidermal flavonols. The nitrogen balance index (NBI) is calculated as the Chl/Flav ratio (Racić et al., 2018).

Statistical analyses

Statistical analysis was performed using the STATISTICA 13 software (TIBCO Software Inc., 2017). A three-way factorial analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test (for a significance level of $\alpha = 0.05$) were performed for the purpose of statistical data analysis. The following main factors were considered: week, cultivar, and treatment.

RESULTS AND DISCUSSION

The ANOVA results obtained indicate that variations in the leaf Chl, Flav and NBI values of the 'Narvik' and 'Gružanski zlatni' tomato cultivars were significantly influenced by all the factors examined (namely the tomato cultivar, experimental week, and treatment), Tab.1. However, the effect of tomato cultivar was found non-significant relative to the leaf Chl content. The effect of factor interactions (Week*Cultivar, Week*Treatment, Cultivar*Treatment, and Week*Cultivar*Treatment) was significant for all the parameters analysed.

Table 1. Results of a three-way ANOVA analysis showing the effects of week, tomato cultivar, and treatment, and their interactions (Week*Cultivar, Week*Treatment, Cultivar*Treatment and Week*Cultivar*Treatment), on the physiological parameters observed

Variables	df	Chl F	Chl p	Flav F	Flav p	NBI F	NBI p
Week	11	203.79	<0.001	118.36	<0.001	117.61	<0.001
Cultivar	1	3.29	0.070	100.86	<0.001	57.73	<0.001
Treatment	3	10.92	<0.001	71.04	<0.001	36.17	<0.001
Week*Cultivar	11	5.27	<0.001	2.48	0.004	10.27	<0.001
Week*Treatment	33	2.26	<0.001	3.75	<0.001	5.74	<0.001
Cultivar*Treatment	3	13.21	<0.001	13.39	<0.001	6.36	<0.001
Week*Cultivar*Treatment	33	2.92	<0.001	2.70	<0.001	5.05	<0.001
Error	1824						

Legend: Chl - indice of chlorophyll (relative); Flav - indice of epidermal flavonols (relative); NBI - nitrogen balance index (relative); df - degrees of freedom

Indice of chlorophyll (Chl)

Relative to the control, the Chl contents of both tomato cultivars considered mostly exhibited no significant variations in plants grown in the presence of *Trichoderma* (Fig. 1). Similar results were also obtained by Vukelić et al. (2020), who reported no statistically significant differences between *Trichoderma*-treated tomato plants and the control. Moreover, Aleksandru et al. (2013) found that differences between the content of chlorophylls a and b and carotenoids in tomato plants treated with different *Trichoderma* species were also negligible. However, the presence of *Trichoderma* positively affected the Chl content in the 'Narvik' cultivar, as a significant increment of 13.26% was recorded in week 11 of the experiment after the T2 treatment (compared to the control). Furthermore, the 'Narvik' Chl content was increased in Treatment T3 (T3: T1+T2) by 18.67%, 14.3%, and 32.10% at the 3rd, 11th, and 12th measurement points, respectively. These results indicate that the combination of *T. harzianum* (SZMC 20660) and *T. brevicompactum* (SZMC 20661) was effective since the 'Narvik' chlorophyll content increased significantly. Our findings are consistent with the results of Singh et al. (2014), who reported that the combination of *T. harzianum* (BHU-51) and *T. harzianum* (BHU-105) showed a significant increase in the Chl content of tomato-treated plants alongside a higher mineral content and effective decrease in plant disease.

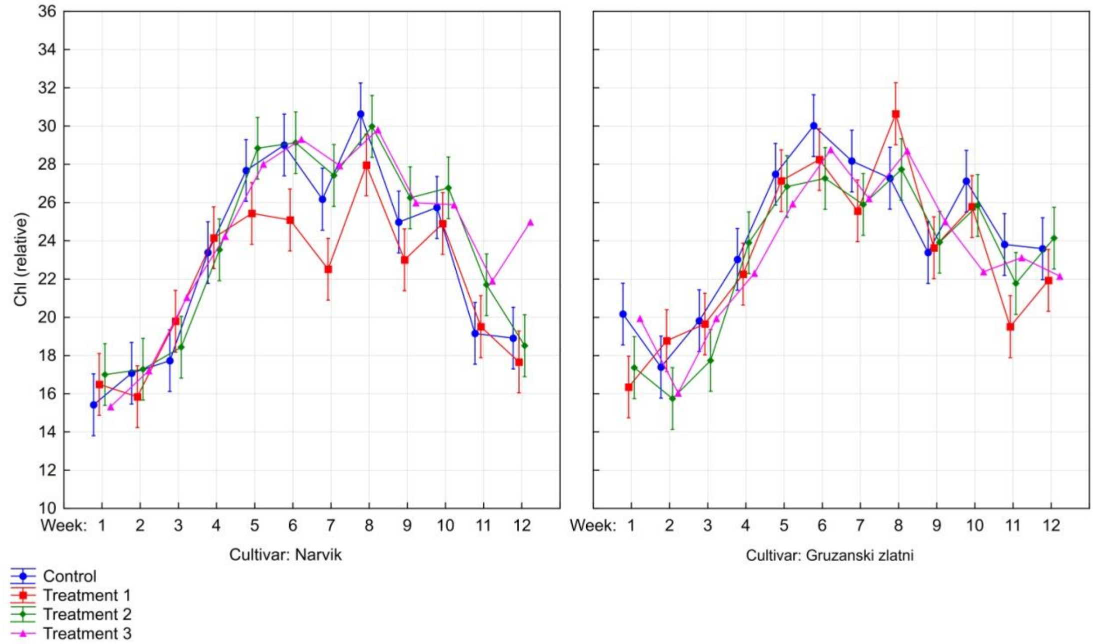

Figure 1. Mean values and standard deviation of mean indices of leaf chlorophyll (Chl) determined in 30 plants of the'Narvik' and 'Gružanski zlatni' tomato cultivars under three treatments over an experimental period of 12 weeks (Control, Treatment 1 - *T. harzianum*, Treatment 2 - *T. brevicompactum,* Treatment 3 - *T. harzianum* + *T. brevicompactum*)

Indice of epidermal flavonols (Flav)

In response to all the *Trichoderma* treatments (T1-T3) considered, the epidermal flavonol content in the 'Narvik' tomato cultivar increased significantly in weeks 2, 4 and 9 of the experiment, which was not the case in the 'Gružanski zlatni' cultivar (Fig. 2). Moreover, a considerable increase in the content of flavonols was observed in the 'Narvik' cultivar during the T2 and T3 treatments for 7 weeks continuously (from the 4^{th} to 10^{th} measurement points), compared to the control. The relative Flav content in the 'Gružanski zlatni' cultivar mostly did not show significant variations in plants grown in the presence of *Trichoderma* relative to the control. It is commonly known that plants contain a variety of polyphenols, which can be divided into three classes. Flavonoids are exclusively induced by symbionts and respond to purified signaling molecules of these organisms (Hasan & Matezius, 2012). They are carbon-based secondary metabolites with a content that rises with the decreasing N availability (Padilla et al., 2014). Therefore, our findings for the 'Narvik' tomato cultivar are consistent with previous research suggesting that plants treated with several *Trichoderma* spp. individually (Ortega-Garcia et al., 2005; Mayo-Prieto et al., 2019), or in consortium (Şesan et al., 2020), accumulated more polyphenols and flavonoids than the control.

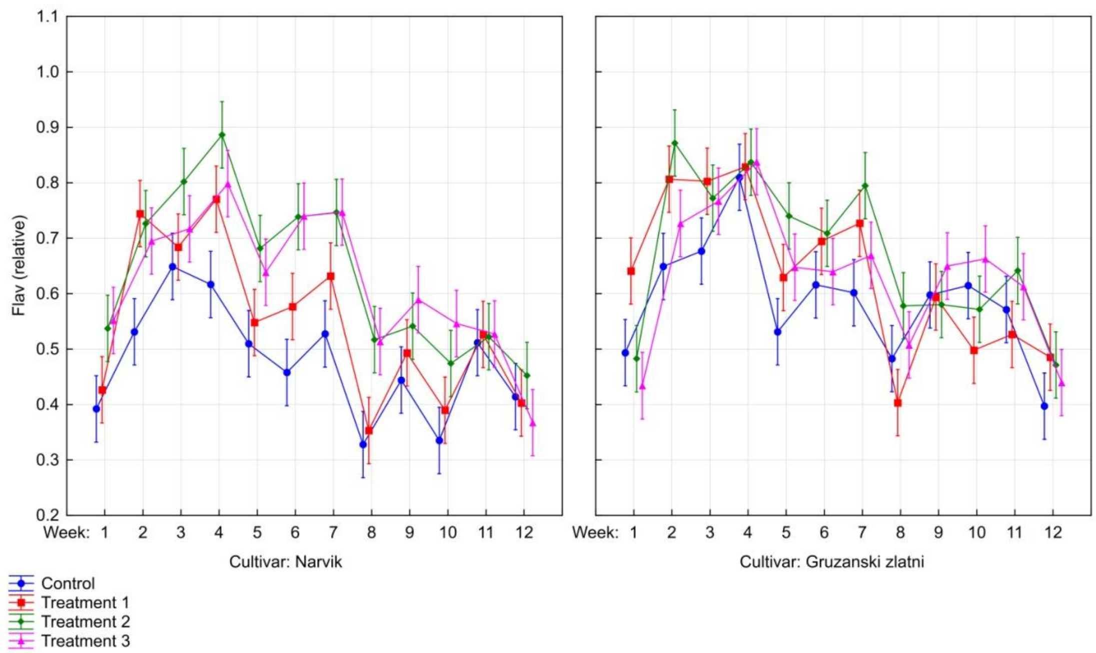

Figure 2. Mean values and standard deviation of mean indices of leaf epidermal flavonols (Flav) determined in 30 plants of the 'Narvik' and 'Gružanski zlatni' tomato cultivars under three treatments over an experimental period of 12 weeks (Control, Treatment 1 - *T. harzianum*, Treatment 2 - *T. brevicompactum*, Treatment 3 - *T. harzianum* + *T. brevicompactum*)

Nitrogen Balance Index (NBI)

On balance, the NBI was found to be lower in the 'Gružanski zlatni' tomato cultivar than in the 'Narvik' cultivar (Fig. 3). However, in response to the *Trichoderma* treatments (T1-T3) considered, the NBI values obtained in the 'Narvik' cultivar were reduced from the 4^{th} to 10^{th} measurement points (7 weeks), compared to the control. Conversely, the NBI values in the 'Gružanski zlatni' cultivar mostly did not show significant variations in the plants grown in the presence of *Trichoderma* relative to the control. The NBI (Chl/Flav) index reacts significantly less to phenology and shows the N availability superior to both of the two indicators used (Cerović et al., 2015). According to Agati et al. (2013), the Chl-to-Flav ratio index is a better predictor of N status than chlorophyll alone since it is independent of leaf mass per area and has a wider response range because Chl and Flav react oppositely to N. Currently, it is well known that phenolics rose in response to nitrogen deprivation, whereas chlorophyll had the reverse reaction (Cerović et al., 2005). In our study, the NBI values in the 'Narvik' cultivar were reduced by 7 weeks compared to the control, which was a consequence of an increase in the flavonol index. Moreover, the decreased NBI values in the 'Narvik' plants treated with *Trichoderma* suggest a switch from primary to secondary metabolism, which is consistent with numerous experimental studies done on tomato plants treated with *Trichoderma* (Vukelić et al., 2021; Vukelić et al., 2022). As the 'Narvik' tomato cultivar had lower NBI values due to its higher flavonol content, it might be considered more resistant to pests and diseases (Agati et al., 2013).

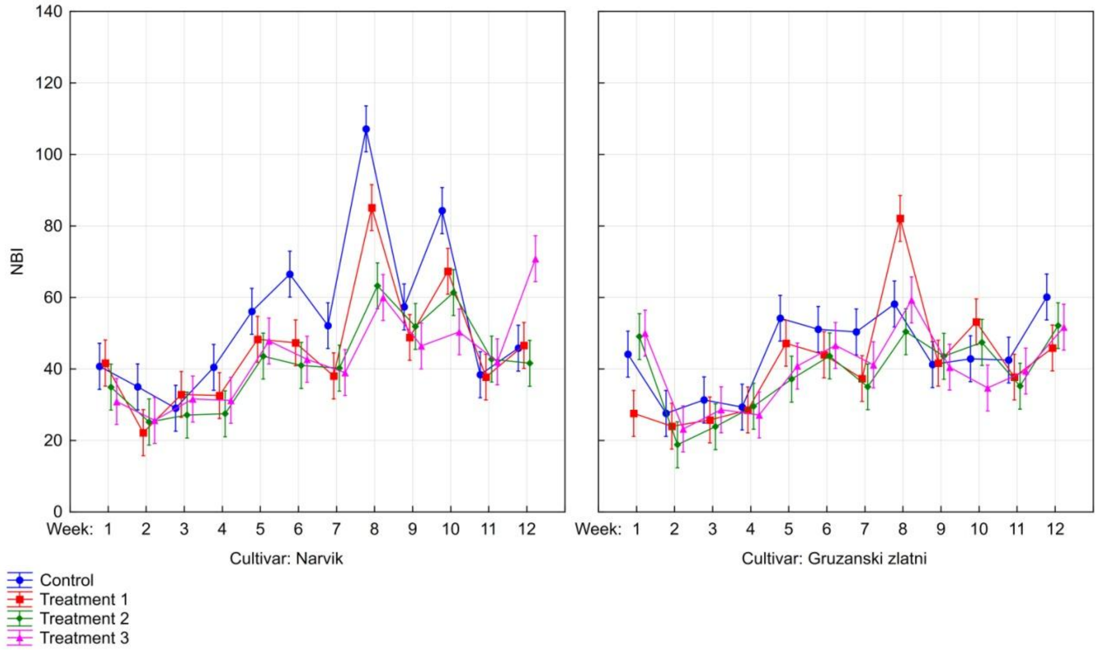

Figure 3. Mean values and standard deviation of the mean nitrogen balance index (NBI) values determined in 30 plants of the ‘Narvik’ and ‘Gružanski zlatni’ tomato cultivars under three treatments over an experimental period of 12 weeks (Control, Treatment 1 - *T. harzianum*, Treatment 2 - *T. brevicompactum*, Treatment 3 - *T. harzianum* + *T. brevicompactum*)

CONCLUSION

The non-destructive *in vivo* monitoring of chlorophyll (Chl) and epidermal flavonol (Flav) indices, and nitrogen balance index (NBI) in the leaves of the ‘Narvik’ and‘Gružanski zlatni’ tomato cultivars indicates that variations in the Chl, Flav, and NBI values obtained were significantly influenced by nearly all the factors examined (namely the experimental week, tomato cultivar, and treatment), the interaction of which was found significant for all the parameters analyzed. Our results indicate that the consortium of compatible isolates in the T3 treatment, in some experimental weeks, significantly enhanced the Chl content in the ‘Narvik’ tomato cultivar, compared to single isolates. In that respect, using a combination of strains of helpful microorganisms like *Trichoderma* (such as the T3 mix used herein) is more advantageous than using individual strains because two compatible isolates from different species have a greater impact when used together. Furthermore, the *Trichoderma* presence was found to significantly affect the Flav content and NBI values in the ‘Narvik’ leaves, which indicated a shift from primary to secondary metabolism. Finally, the results obtained suggest that the *Trichoderma* treatments applied are warranted to be tested on the ‘Narvik’ and ‘Gružanski zlatni’ tomato cultivars (for the physiological responses) under field conditions.

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Conflict of interest: The authors declare that they have no conflict of interest.

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