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Article

Non-Destructive Assessment of Leaf Chlorophyll, Flavonols and Anthocyanins Content in Sweet Corn Hybrids Grown Under Different *Trichoderma* spp. Treatments

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Abstract: Increasing interest in sustainable crop production has emphasized the need for biostimulants capable of enhancing plant physiological parameters while reducing dependence on chemical inputs. In this context, the present study assessed the effects of two *Trichoderma* strains (*T. harzianum* and *T. brevicompactum*) on chlorophyll, epidermal flavonols, anthocyanins, and the nitrogen balance index (NBI) in two sweet corn hybrids under field conditions. Non-destructive measurements were performed using the Dualex Scientific sensor every two weeks across six phenological stages, and data were analyzed through a three-way ANOVA to evaluate week, hybrid, and treatment effects. Week was the dominant source of variation for all parameters. Both *Trichoderma* strains increased chlorophyll indices, with the most significant differences recorded in week three, where chlorophyll content increased by up to 68% in Bonanca and 84% in Union compared with the control. Flavonol content increased significantly in later stages, particularly in weeks three and five, while anthocyanin responses were hybridspecific and expressed through significant interaction effects. NBI values also improved, mostly in week six in Bonanca and week five in Union. Overall, the findings show that *Trichoderma* spp. induce specific physiological responses in sweet corn and highlight the value of non-destructive sensing for monitoring these effects in field conditions.

Keywords: *Sweet corn; Trichoderma spp.; chlorophyll; flavonols; anthocyanins; nitrogen balance index; Dualex; non-destructive sensing; phenological stages; physiological responses.*

1. Introduction

Sustainable development of crops is highly dependent on soil health status and use of various fertilization technologies. It is estimated that 64% of global agricultural land is at risk of pesticide pollution, negatively impacting soil and water quality, biodiversity, and human health [1]. To address these challenges, sustainable environmental resource management has been identified as a cornerstone for sustainable economic growth, aligning with the European Green Deal's (EGD) objectives [2]. In addition to the environmental degradation caused by intensive agriculture, factors such as insecure production due to climate change, growing demand for safe food, and an unstable global food trade are threatening major staple crops like wheat, maize, and rice. It is estimated that about 35% of human's calories intake comes from these crops [3].

Sweet corn (*Zea mays* L. *saccharata* Sturt.) is an important cultivated species due to its high nutritional and economic importance. By the presence of easily digestible sugars, proteins, vitamins and minerals, it is widely used in human nutrition and industrial processing. The quality and chemical composition of sweet corn can be significantly influenced by agrotechnical measures, including the application of microbiological fertilizers. Such fertilizers, based on beneficial strains of bacteria and fungi, improve the availability of nutrients in the soil and encourage plant growth. Their application can affect the increase in the content of sugar, protein and antioxidant compounds in the grain, which improves the nutritional value and quality of the yield of sweet corn [4-6].

Non-destructive measurement methods in plant production play an increasingly important role in modern research, as they enable rapid, repeatable, and reliable monitoring of the physiological state of plants without damaging them. One of the most used devices in that context is the Dualex Scientific sensor (Force-A, Orsay, France), which is based on optical measurements of leaf fluorescence and reflection, enabling the simultaneous determination of the relative content of chlorophyll, flavonol, anthocyanin, as well as the calculation of the plant nitrogen balance index (NBI). The application of the Dualex in corn plants proved to be particularly significant in assessing the status of nitrogen nutrition [7] as well as in monitoring physiological responses of maize to abiotic stress, such as low temperatures, with tolerant genotypes showing higher content of photosynthetic pigments and protective secondary metabolites compared to more sensitive lines [8]. The particular importance of such measurements is reflected in research on maize hybrids, which are characterized by a specific metabolic profile and pronounced genotypic differences in pigment accumulation and nitrogen balance index, which allows early detection of photosynthetic efficiency differences and hybrid adaptive potential between [9,10].

To promote soil fertility and decrease environmental pollution, minimizing use of chemical fertilizers while introducing beneficial microorganisms is recognized [11]. Fungi of the genus *Trichoderma* spp. are known in agriculture for various purposes with emphasis in promoting plant health and yield and as biocontrol agents [12]. *Trichoderma* strains exert indirect biocontrol against fungal plant pathogens by competing for nutrients and space, altering environmental conditions, and promoting plant growth and defense responses. They can also induce systemic resistance in plants and produce antimicrobial compounds involved in antibiosis [13]. Studies show that the beneficial effects of many *Trichoderma* spp. are linked to their ability to produce secondary-metabolite compounds that exhibit antimicrobial activity and/or positively influence plant growth, yield, and other beneficial characteristics. Today, these fungi are widely used as active ingredients in biopesticides, biofertilizers, plant growth enhancers, and natural resistance stimulants [14]. This is due to their ability to protect plants, enhance vegetative growth and refrain from pathogen populations under numerous agricultural conditions, as well as to act as soil amendments/inoculants for improvement of nutrient ability, decomposition and biodegradation. Nowadays, there are more than 400 available *Trichoderma*-containing products found on the international market, counting that more than 60% of registered biopesticides are *Trichoderma* based [15].

The aim of the present study was to evaluate the effects of two *Trichoderma* strains on chlorophyll content, epidermal flavonols, and nitrogen balance index in two sweet corn hybrids under field conditions. By integrating non destructive physiological measurements during plant development, this study aims to provide insight into the temporal and cultivar-specific responses of sweet maize to *Trichoderma* application, contributing to understanding of *Trichoderma*–sweet corn interactions in sustainable agricultural production systems.

2. Materials and Methods

2.1. Plant material and fungal strains

Two sweet corn hybrids (*Zea mays* var. *saccharata*), Bonanca and Union,, were used in the experiment. Both hybrids belong to the FAO 400 maturity group and reach technological maturity in approximately 85 days. Bonanca F1 is resistant to powdery mildew and ear rot, while Union F1 is drought-tolerant.

A total of two *Trichoderma* strains, *Trichoderma harzianum* and *Trichoderma brevicompactum,* previously isolated from the A horizon of the experimental soils (5-30 cm) were used in this study. These strains were identified and deposited in the Szeged Microbiological Collection (www.szmc.hu) as SZMC 20660 and SZMC 22661, respectively. Prior to fungal suspension preparation, the strains were pre-incubated on potato dextrose agar at 25°C under dark conditions. The suspensions were prepared as follows: the pure cultures of *T. harzianum* and *T. brevicompactum* were taken from a Petri dish, resuspended in 100 mL of sterile tap water, and then shaken for 2 h 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} [16].

2.2. Experimental design

The field experiment was established according to a completely randomized design with three replications at a single location (Selenča, latitude 45.4097° N, longitude 19.3006° E). A total of 30 plants of each sweet maize hybrid were measured per treatment: C - control, T1 - *T. harzianum*, T2 - *T. brevicompactum*. The *Trichoderma* suspension was applied two times: after emergence, and during the flowering stage.

2.3. 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) every two weeks during 12 weeks of the plants' growth (6 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 [17].

2.4. Statistical analyses

Statistical analyses were conducted using IBM SPSS Statistics V26 (SPSS Inc., Chicago, USA). A three-way factorial analysis of variance (ANOVA) was applied to assess the effects of the examined factors, followed by Tukey's Honestly Significant Difference (HSD) post hoc test at a significance level of $\alpha = 0.05$. The primary factors included in the analysis were week, cultivar and treatment.

3. Results

The results of the analysis of variance indicate the significant influence of the week of measurement on all examined physiological parameters of sweet corn (chlorophyll content, epidermal flavonols and anthocyanins, and nitrogen balance index), at a significance level of 0.001 (Table 1). The significance of the hybrid and treatment effect depended on the indicator. The content of anthocyanins, as well as the nitrogen balance index, was not significantly influenced by the studied hybrid, while for the content of chlorophyll and flavonoids, statistical significance was at the significance level of 0.01 (chlorophyll content) and 0.001 (flavonoid content). The treatments showed a significant effect at the 0.001 level on the variation of all studied physiological parameters, except for the anthocyanin content.

Table 1. Three-factorial ANOVA of physiological parameters studied.

Variables	**df**	**Chl (F)**	**Chl (p)**	**Flav (F)**	**Flav (p)**	**Anth (F)**	**Anth (p)**	**NBI (F)**	**NBI (p)**
Week	5	42,82	0,000	413,95	0,000	78,76	0,000	199,51	0,000
Hybrid	1	9,31	0,002	83,95	0,000	0,07	0,792	0,03	0,843
Treatment	2	171,74	0,000	141,99	0,000	0,26	0,768	14,21	0,000
Week*Hybrid	5	7,34	0,000	14,451	0,000	13,10	0,000	11,71	0,000
Week*Treatment	10	16,27	0,000	60,21	0,000	1,91	0,039	3,94	0,000
Hybrid*Treatment	2	10,56	0,000	23,67	0,000	2,64	0,071	2,58	0,076
Week*Hybrid*Treatment	10	9,04	0,000	3,33	0,000	3,15	0,001	4,52	0,000
Error	1044								

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

In addition to the main effects, most of the interactions showed a statistically significant effect on the parameters studied at the 0.001 level. Only the hybrid × treatment interaction did not significantly affect the variation of anthocyanin content and nitrogen balance index (Table 1).

3.1. Chlorophyll content (Chl)

Both biological treatments increased the chlorophyll content compared to the control in most measurement terms in both hybrids, with the effects being pronounced especially in the early weeks (Figures 1 and 2).

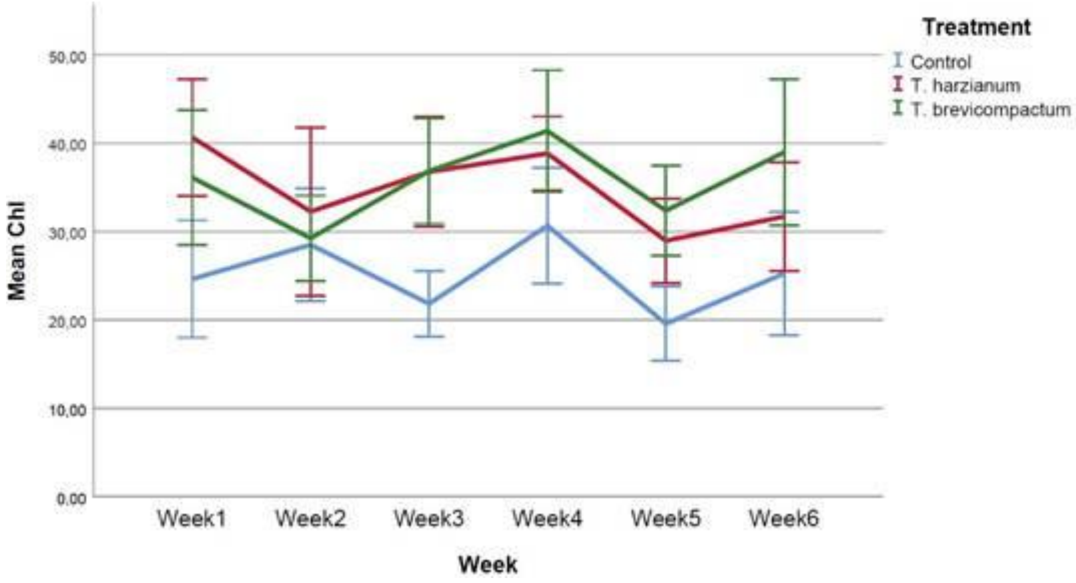

Figure 1. Mean values and standard deviations of chlorophyll content as affected by treatments in Bonanca across all measurement periods.

In both studied hybrids, across almost all measurement periods, a significant increase in chlorophyll content was observed under the effect of both applied treatments (*T. harzianum* and *T. brevicompactum*), except for Union, where a slight decrease in chlorophyll content was observed in the last measurement period (week 4) (Figure 2). The highest increase in chlorophyll content of Bonanca was observed in the third week, with a 68.26% rise when treated with *T. harzianum* and 68.71% rise when treated with *T. brevicompactum*, compared to the control.

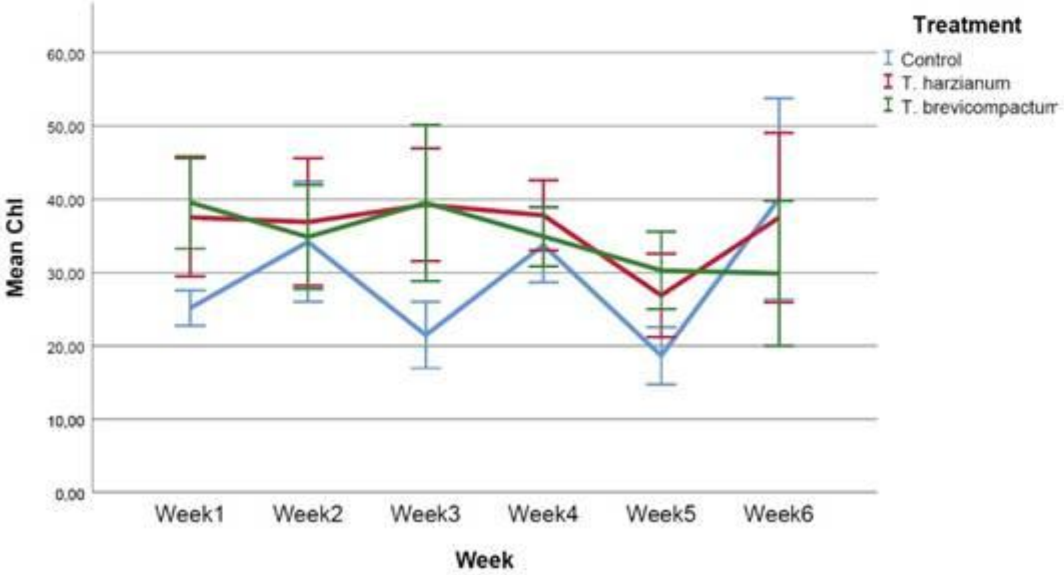

Figure 2. Mean values and standard deviations of chlorophyll content as affected by treatments in Union across all measurement periods

In the second studied hybrid, Union, the most significant increase in chlorophyll content due to applied treatments was observed in the third week of measurement. The increase reached 82.67% with the application of *T. harzianum*, while the application of *T. brevicompactum* resulted in an increase of 83.90%, both compared to the control group.

3.2. Content of epidermal flavonols (Flav)

Flavonols showed the most pronounced variability during seasonal development (F = 413.95; $p < 0.001$). The main effects of hybrid and treatment were significant ($p < 0.001$), with pronounced interactions Week×Treatment and Week×Hybrid×Treatment (Table 1).

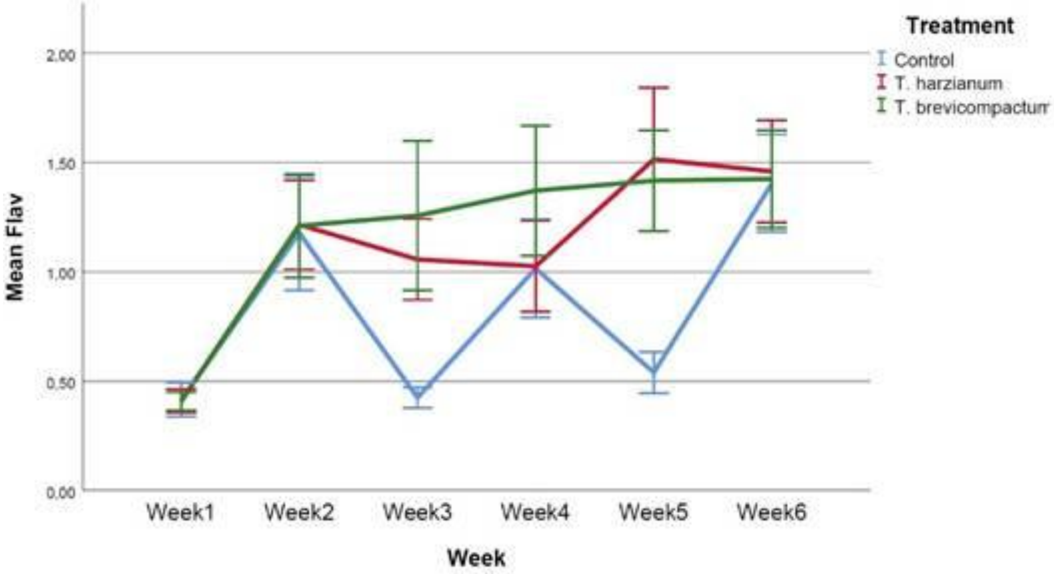

Figure 3. Mean values and standard deviations of the content of epidermal flavonols as affected by treatments in Bonanca across all measurement periods.

In the early weeks (week 1), the differences between treatments were minimal, while later (weeks 3–6), treatments with *Trichoderma* significantly increased the values of flavonol content. In both studied hybrids, statistically significant differences were found between the content of flavonols in the control variant and, due to the application of treatment, in the second, third, fourth, fifth, and sixth measurement periods. The biggest differences between the control and applied treatments were established in the third and fifth week of measurement in both hybrids (Figures 3 and 4).

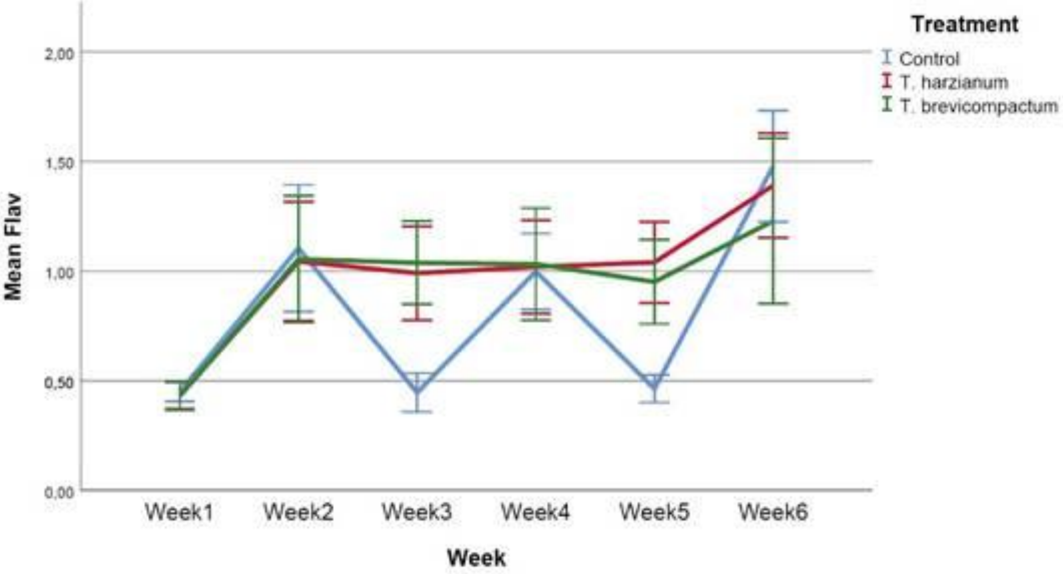

Figure 4. Mean values and standard deviations of the content of epidermal flavonols as affected by treatments in Union across all measurement periods.

3.3. Anthocyanin content (Anth)

Although the main effects of hybrid and treatment were not significant ($p > 0.05$), all interactions (including Week×Hybrid×Treatment) were statistically significant ($p \le 0.001$). This means that the response of the plants to the treatments manifested in certain phenophases and depended on the hybrid.

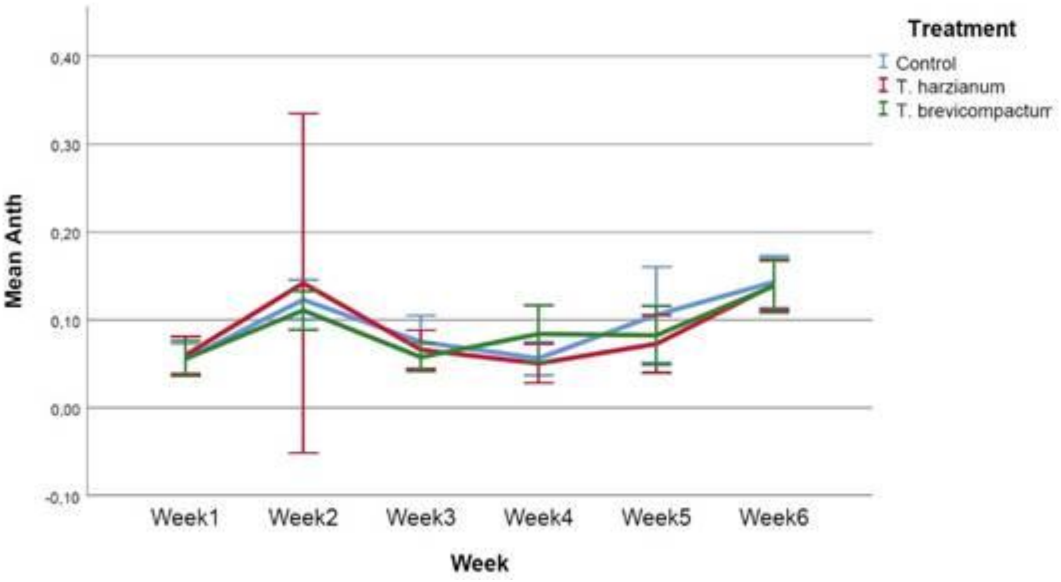

Figure 5. Mean values and standard deviations of anthocyanin content as affected by treatments in Bonanca across all measurement periods.

In the first stages of development (week 1-2), in Bonanca, the first applied treatment (*T. harzianum*) led to an increase in the anthocyanin content. Across the remaining measurement periods, a decrease in this parameter was observed when *T. harzianum* was applied (Figure 5). In the second studied hybrid, the application of *T. harzianum* also led to an increase in anthocyanin content in the first two weeks, and this trend continued until the end of the growing season (Figure 6). The second applied treatment, *T. brevicompactum*, mainly led to an increase in anthocyanin content in both studied hybrids. The highest increase was recorded in Bonanca during the fourth week of measurement, and was 50% compared to the control.

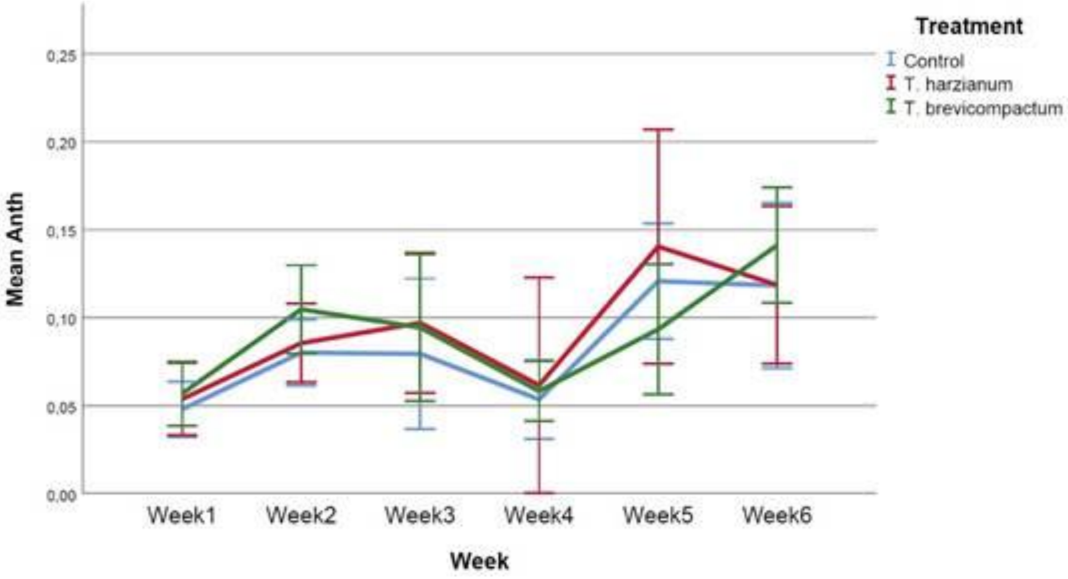

Figure 6. Mean values and standard deviations of anthocyanin content as affected by treatments in Bonanca across all measurement periods.

3.4. Nitrogen balans index (NBI)

NBI was highly significantly affected by week ($p < 0.001$) and treatment ($p < 0.001$), while the hybrid effect did not show statistical significance. However, all interactions were significant, which confirms that the reaction to *Trichoderma* depends on the combination of hybrid and phenophase.

In the early weeks, the differences were moderate, but subsequently they became more pronounced. In Bonanca, in the 6th week of application of both products, the NBI significantly increased (36.94% *T. harzianum* and 37.04% *T. brevicompactum*) compared to the control (Figure 7). In Union, the differences were less pronounced. The highest value of NBI was observed in the 5th week of measurement when applying the *T. brevicompactum* treatment (44.4) (Figure 8).

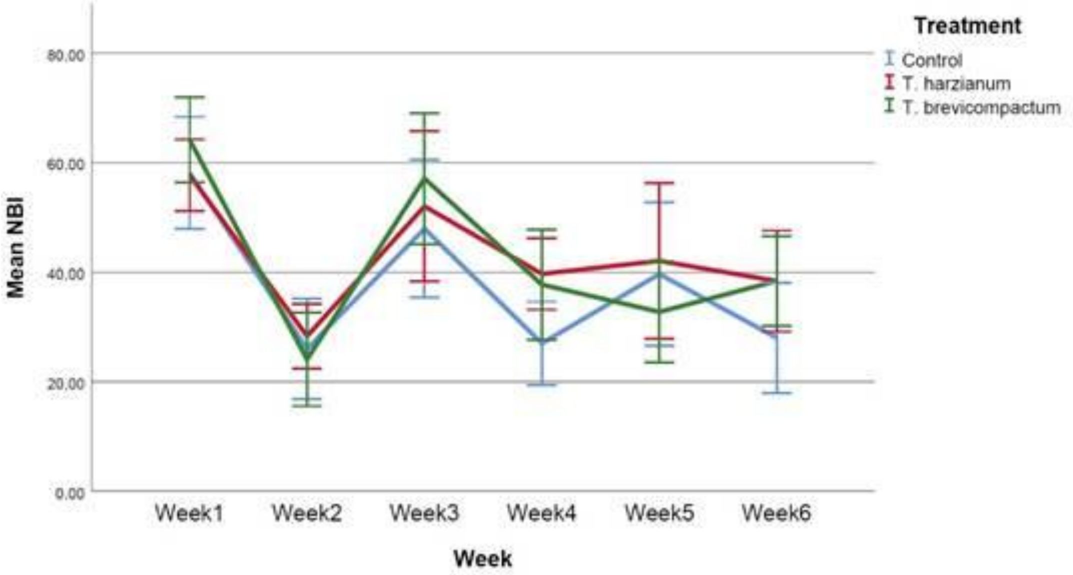

Figure 7. Mean values and standard deviations of the nitrogen balance index as affected by treatments in Bonanca across all measurement periods.

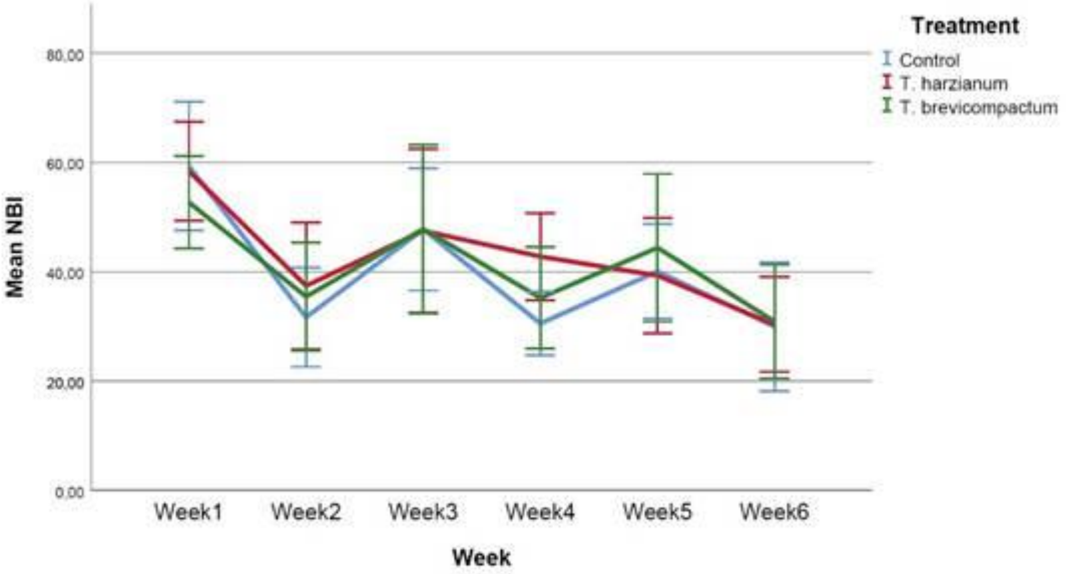

Figure 8. Mean values and standard deviations of the nitrogen balance index as affected by treatments in Union across all measurement periods.

4. Discussion

The present study showed that physiological parameters of sweet corn were strongly influenced by phenological stage and *Trichoderma* spp. application, while hybrid effects were parameter specific. Week as a factor was the dominant for all examined parameters, indicating that phenological stage is important to be followed during plant growth. Literature shows that physiological parameters relate to Trichoderma–plant interactions, although the size and expression of these effects depend on genotype, *Trichoderma* applied dose, and measured parameter. Wahyurini et al. [18] reported that *Trichoderma* sp. application did not significantly affect overall growth and yield of sweet corn across genotypes, but a specific genotype-applied dose of Trichoderma combination resulted in increased cob weight, indicating specific plant physiological response. Similarly, Apzani and Wardhana [19] observed that fungal bioformulators containing *Trichoderma* spp. influenced physiological parameters such as leaf greenness and biomass accumulation under dryland conditions, concluding that microbial application affects chlorophyll indices, as measured by SPAD meter. Studies using mixed microbial formulations further support these findings, as Meng et al. [20] showed that *Trichoderma*-based microbial fertilizers enhanced biomass production and protective enzyme activity in sweet waxy corn. Together, these results indicate that *Trichoderma* effects on sweet corn physiology are often investigated parameter-specific and genotype-dependent, which is consistent with the differential responses observed in the present study.

In our study, both *Trichoderma harzianum* and *Trichoderma brevicompactum* increased chlorophyll content compared with the control in both hybrids. Maximum chlorophyll increases were observed in the third week of measurements in both Bonanca and Union. Positive effects of *Trichoderma* on chlorophyll status were observed also by Csótó et al. [21] who demonstrated that foliar application of *Trichoderma* stimulated chlorophyll accumulation in sweet corn, measured non-destructively using a SPAD 502 Plus meter. Their results showed that *Trichoderma* treatment increased SPAD values by up to 19.1% relative to the untreated control, indicating enhanced pigment formation following microbial application. Similarly, Bojović et al. [22] examined the effects of *Trichoderma* application on two tomato cultivars during 84 days (12 weeks) of the plants' growth (12 measurement points in total). They concluded that combinations of *Trichoderma* strains significantly enhanced the chlorophyll content in the 'Narvik' tomato cultivar during 3rd, 11th, and 12th measurement points, respectively, as measured non-destructively by the Dualex sensor. These observations confirm the results of our study showing that the response of Chl relative indices to *Trichoderma* application was similar in both hybrids, although Union showed a slight decrease in chlorophyll at the final measurement.

Epidermal flavonol indices showed high variability across the growing season. Differences between treatments were minimal in the first week but became pronounced from the second week onward. Both *Trichoderma* treatments resulted in significantly higher flavonol values compared with the control in later measurement points. The strongest differences were detected in weeks three and five in both examined hybrids. To our knowledge, no previous study has evaluated epidermal flavonol indices in sweet corn under *Trichoderma* spp. treatments using non-destructive optical sensors (such as Dualex or SPAD). Existing papers either evaluated *Trichoderma* effects on sweet corn growth and chlorophyll content using analytical methods [23], or applied Dualex-based flavonol measurements without *Trichoderma* in other crops [9]. Vukelić et al. [16] examined the effect of *Trichoderma* application on photosynthetic characteristics and fruit quality of tomato plants, including changes in epidermal flavonols. They found out that *Trichoderma* treatment can increase epidermal flavonols in one tomato cultivar and decrease them in another.

The nitrogen balance index (NBI), calculated as the Chl/Flav ratio, is considered less sensitive to phenological stages than its individual components and provides a more reliable indication of nitrogen availability [24]. As noted by Agati et al. [25] the Chl-to-Flav ratio is a more reliable indicator of plant N status than chlorophyll alone because it is not affected by leaf mass per area and because chlorophyll and flavonols respond in opposite directions to nitrogen supply. Earlier studies have consistently shown that phenolic compounds tend to accumulate under nitrogen deficiency, while chlorophyll content decreases under the same conditions [26].

Values of NBI followed phenological stages, similar to chlorophyll. In Bonanca, both *Trichoderma* strains significantly increased NBI in the sixth week. In Union, the highest NBI value occurred in the fifth week following *T. brevicompactum* application. These findings show that *Trichoderma* application improved the nitrogen status of sweet corn plants. Similar results were reported by Soares et al. [9], who emphasized that the obtained NBI values were determined by the combination of maize genotype and phenological stage, rather than by the isolated effect of a single factor. Opposite observations of NBI variation have been reported by Andrzejak and Janowska [27] noted that in *Solanum lycopersicum* grown in presence of *Trichoderma*, NBI decreased due to increased epidermal flavonol accumulation combined with reduced chlorophyll content, indicating a shift in pigment concentrations. Their study also showed that *Trichoderma* spp. enhanced the nutritional status of *Gladiolus hybridus* L., improving the uptake of macro- (P, K, Ca) and micronutrients (Zn, Fe, B). In addition, several optical-sensor studies (Dualex or similar) in different crops (cabbage, muskmelon, cereals) show that N regime, stress level, and growth stage very strongly affect Flav and NBI, often more clearly than cultivar alone, or with cultivar effects mainly visible through interactions [28, 29].

Anthocyanin indices were not significantly affected by hybrid or treatment as main factors. However, all interaction effects were significant. This demonstrates that anthocyanin responses were phase-dependent and differed between hybrids. In Bonanca, *T. harzianum* increased anthocyanins only in early stages, followed by a reduction later. In Union, the same treatment caused a sustained increase throughout the season. *T. brevicompactum* generally increased anthocyanin content in both hybrids. The highest increase was recorded in Bonanca during the fourth week. These results indicate that anthocyanin responses to *Trichoderma* are strain-specific and show different responses through phenological stages. Findings from the raspberry study by Giovanelli et al. [30] align closely with our results. In their study, carried out using Dualex, demonstrated that anthocyanins are sensitive indicators under plant biostimulants application. Importantly, they reported that anthocyanins showed significant reactions to the interaction between Cultivar × Treatment × Year, rather than single effects. The same authors suggest that although differences between treatments and control were not significant, biostimulants' addition might have a positive effect on anthocyanins' biosynthesis and impacting plants' antioxidative potential. Anthocyanin increase in corn was reported by do Rêgo Meneses et al. [31] who examined the responses of aluminum-stressed plants inoculated with *T. asperelloides*. They concluded that certain isolates of *Trichoderma* had the ability to induce metabolic plant responses which are associated with alleviating the adverse effects of aluminum stress on corn plants.

5. Conclusions

This study provides one of the first integrated, non-destructive evaluations of *Trichoderma harzianum* and T. *brevicompactum* effects on sweet corn physiological parameters using Dualex measurements under field conditions. Both strains consistently increased chlorophyll indices in Bonanca and Union, with the most significant effects recorded in the third week of measurements, where chlorophyll increased by up to 68% in Bonanca and 84% in Union compared with the control. Flavonol content was minimally affected in early weeks, but significant differences were higher under both treatments in weeks three and five, indicating enhanced activation of secondary metabolism. Anthocyanin responses were hybrid-specific: *T. harzianum* increased anthocyanins only in the early phenological stages of Bonanca, whereas in Union the increase was recorded throughout all stages. *T. brevicompactum* induced increases in both hybrids, with the highest increase of 50% in Bonanca during week four. Nitrogen balance index values were also improved, especially in the sixth week in Bonanca and the fifth week in Union. Our results, based on non-destructive optical measurements, support the targeted use of *Trichoderma* formulations to enhance sweet corn physiological performance within sustainable agricultural practices.

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Conflicts of Interest: The authors declare no conflict of interest.

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