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Different Level of Tolerance to Herbicides is Displayed by *Triticum aestivum* L. Cultivars Depending on Herbicide Category and Mode of Application

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Abstract

Herbicides (HBs) are an integral part of modern agricultural practice globally as one of the most suitable methods for chemical weed control. Hence, they may inadvertently damage crops through similar mechanisms well-functioning at weeds. Tolerance to three categories of treatments (tribenuron-methyl, 2,4 D and their combination), and the impact of the mode of application (pre-treatment of seeds or spraying on plants) was assessed through morphometric analysis (total plant length, root length, stem length, lengths of the first three leaves, relative water content-RWC), and pigment content analysis for some wheat (*Triticum aestivum* L.) cultivars in use in Albania. Results speak of a cultivar specific tolerance to each HB category, discriminating "Dajti" as the most resistant cultivar, and suggesting that the stress imposed to plants by the combination of HBs can produce significant differences in pigment content. The mode of treatment, on the other side showed that glyphosate pre-treatment of seeds led to inhibition of germination, and plants germinated from seed pre-treatment with in 2,4-D displayed significant modification of plant morphology, and germination rate, and had limited impact on RWC. On contrary, few statistically significant changes in morphometric traits and pigments content were evidenced when plants were treated with 2,4- D or glyphosate after the germination. In conclusion, the combination of HBs produced significant changes in plant pigments content compared to the impact of each HB alone, and the pre-treatment of seeds lead either to inhibition or delayed germination. Based on the above we recommend avoiding these practices, and suggest further exploration of alternative HB combinations and modes of treatment before large scale applications.

Keywords: herbicide-HB, photosynthetic pigment, morphometric parameters, significant changes

Introduction

Weeds negatively affect crop growth and production [1], thus their management is essential for efficient and profitable cereal production. Today, HBs are an integral part of modern agricultural practice globally as one of the most suitable methods for chemical weed control [2], however, there are several factors affecting this process such as herbicide (HB) application rate, coverage, soil characteristics, plant growth stage, and environmental conditions before, during, and after HB application [3]. HBs with different modes of action [4] are in use worldwide [5-10], and depending on the cause, intensity, and degree of damage, wheat protection is done 1-2 times during wheat growth [11]. The damage to crops can be evidenced visually, or by the reduced yield, and to avoid it the selectivity of HBs (the application of HBs with minimal damage to the crop, minimizing the phytotoxicity [12]) is important. However, there is different selectivity between species, thus the genetic content of species or cultivars may determine the degree of tolerance or sensitivity to HBs. The selectivity is also related to the ability of the plant to recover after the application of the HB through the inactivation or metabolism of the HB molecules. Thus, the factors that affect the level of damage are the type of crop and the physiological state of the plant, the type of HB, the methods of HB application, and the dose used [13]. The negative effect caused by stress (HB treatment too) is often mediated by oxidative damage initiated by reactive oxygen species such as superoxide radicals (O_2^-), hydroxyl (OH^-), hydrogen peroxide (H_2O_2), and oxygen molecules (O_2) [1]. Reactive oxygen species re-

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act with lipids, proteins, pigments, and nucleic acids and cause lipid peroxidation, membrane damage, and enzyme inactivation. Plant cells have defence mechanisms to prevent oxidative damage, including antioxidant enzymes such as catalases and non-enzymatic antioxidant compounds such as carotenoids and phenols [8]. Numerous studies have been done on the effect of HBs on the photosynthetic pigments of cereals [1, 7-9, 14, 15]. To avoid malpractices, the investigation of the selectivity of HBs should precede taking in consideration that the degree of tolerance or sensitivity may be among other factors due to the genetic content of species or cultivars. Here, we discuss results on the tolerance of some wheat (*Triticum aestivum* L.) cultivars to treatment with tribenuron-methyl (a sulfonylurea herbicide, which inhibits the plant acetolactate synthase, resulting in impaired branch chain amino acid synthesis), 2,4-dichloro-phenoxyacetic acid (a synthetic auxin, which is absorbed through the leaves, is translocated to the meristems and triggers uncontrolled, unsustainable growth, causing stem curl-over, leaf withering, and eventual plant death), and their combination. Also, the impact of the mode of treatment on plant development was evaluated using herbicides 2,4-dichloro-phenoxyacetic acid and glyphosate. The last disrupts the shikimic acid pathway through inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, which leads to reductions in aromatic amino acids that are vital for protein synthesis and plant growth [16, 17].

Materials and Methods

Experiment 1. Tolerance of wheat cultivars to recommended doses of different HBs

2.1. Plant Material and Growing Conditions

Seeds of the foreign cultivar "Pobjeda" were purchased in the commercial market in Kukës, Albania, while seeds of local cultivars "Dajti" and "Adelajde" were obtained from the Public Seed Station, Korça, Albania. Wheat seeds were planted in plastic pots in soil with the following content: Basic materials, organic soil material (95%), limestone fertilizer, clay, perfit, nitrogen, phosphorus, and potassium (NPK) fertilizers, and other secondary materials 300 mg/l nitrogen in the form of N_{CaCl2}, 300 mg/l phosphate (P_2O_5), 300 mg/l potassium oxide (K_2O), 180 mg/l magnesium in the form of Mg_{CaCl2}, 180 mg/l sulfur in the form of S_{CaCl2}, pH value: 5.5, salt content 1.2 g/l potassium chloride (KCl). In each pot, 20-30 grains of wheat were planted. After planting, seeds for the three cultivars were allowed to germinate and grow under normal lighting conditions and temperatures ranging from 15°C to 22°C, while being periodically watered with tap water (20 ml for each pot).

2.2. Treatment with Herbicides

2.2.1. Treatment with Granstar® (tribenuron-methyl)

Twenty-one-day-old seedlings were sprayed with a 0.07 mg/ml aqueous solution of the HB Granstar® according to the manufacturer's instructions. Spectrophotometric measurements for chlorophylls a + b, and carotenoid content were made one week, two weeks, and three weeks after treatment according to [14]. Plots not treated with HBs served as control.

2.2.2. Treatment with D-Amine® (2,4-dichloro-phenoxyacetic acid)

Thirty-day-old seedlings were sprayed with a 6.25 ml/l aqueous solution of the HB D-Amine® according to the manufacturer's instructions. Measurements for chlorophylls a+b, and carotenoids content were made one hour, 120 hours, and 170 hours after treatment according to [1]. Plots not treated with HBs served as control.

2.2.3. Treatment with the combination of both HBs (Granstar® and D-Amine®)

Wheat seedlings were left for about 30 days to grow and treated with the combination of Granstar® and D-Amine® with a dose of 0.07 mg/ml aqueous solution of the Granstar® and with a 6.25 ml/l aqueous solution of D-Amine® by diluting both HBs together in the same amount of water. They were evaluated for the content of chlorophylls and carotenoids one hour after treatment, 120 hours after treatment, and then every week until the third week after treatment of wheat plants. Plots not treated with HBs served as control.

2.3. Leaf Pigment Content

Chlorophylls and carotenoid contents were measured according to [18, 19].

Experiment 2. Impact of the mode of treatment with HBs of wheat plants in the third week of germination

2.4. Plant Material and Treatment with Herbicides

For the aim of this experiment, cultivars of wheat (*Triticum aestivum L.*) "Victoria", "Tomborzo", "Dajti", "Progres", "L-7", "Pobjeda", "Adelajde" were collected. They were donated by the Plant Genetic Bank of the Agricultural University of Tirana, Albania. Healthy selected seeds were hydrated in water for 30 min and planted in plastic pots with soil, which was maintained at room temperature (RT) 20-25°C and exposed to natural light equally. Three-week-old seedlings were subjected to treatment with a 0.5 ml/l aqueous solution of the herbicide D-Amine®, or 24 ml/l glyphosate according to the manufacturer's instructions.

M*orphometric traits* (total plant length, root length, stem length, and lengths of the first three leaves) were measured manually;

Relative Water Content (RWC) was measured following [20];

Leaf pigment content was evaluated according to [18, 19]; All measurements were performed simultaneously i.e. in the third and fourth week for plants sprayed with HB.

Experiment 3: Impact of the pre-treatment of seeds with HBs

2.5. Pretreatment of seeds

Healthy seeds selected based on their Germination Index

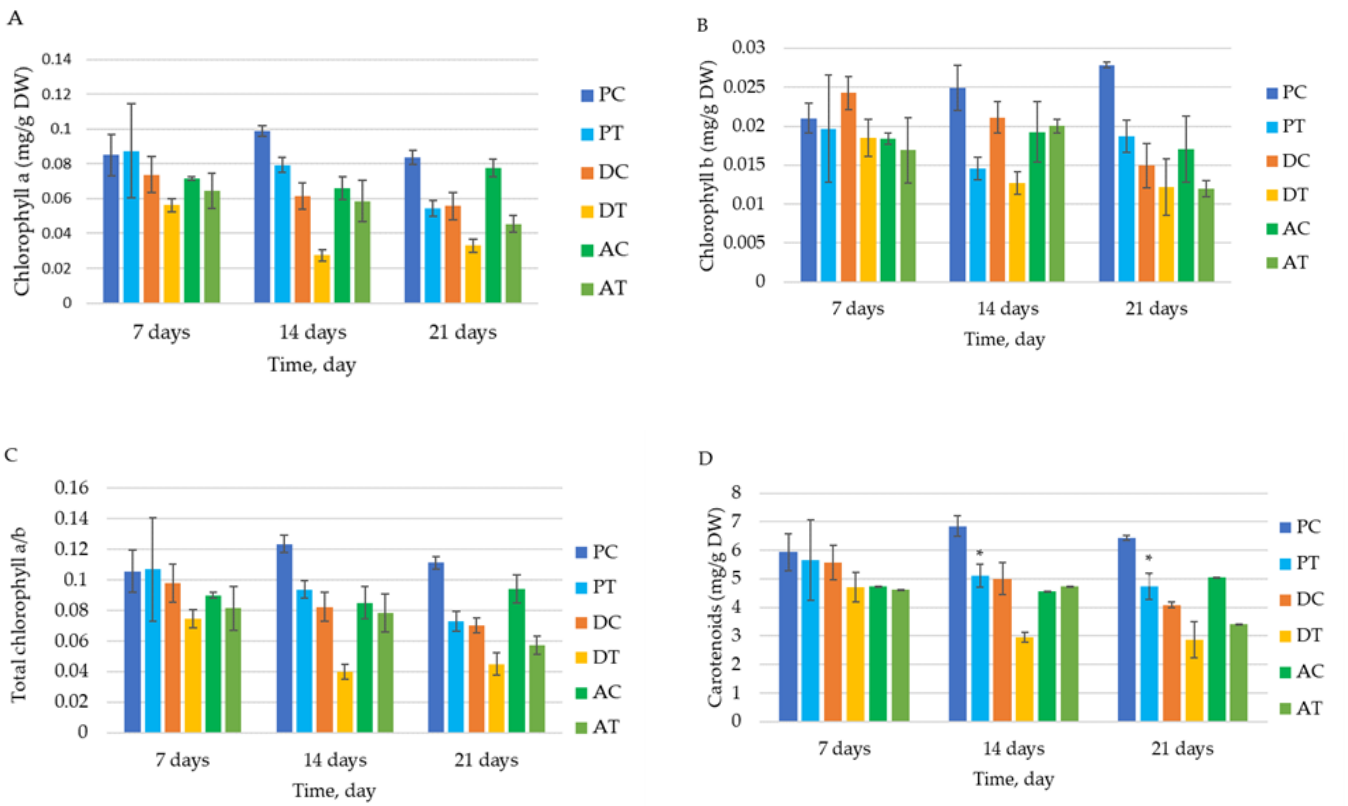


Figure 1. Chlorophyll a (A), chlorophyll b (B) content, total chlorophyll a+b (C), and carotenoids content (D) in wheat plants treated with HB Granstar®. Data are mean values ± standard deviation. The one-way analysis of variance (ANOVA) was applied using the Tukey test to determine significant differences between mean values (*$p < 0.05$). Legend: PC – Pobjeda control, PT – Pobjeda treated, DC – Dajti control, DT – Dajti treated, AC – Adelajde control, AT – Adelajde treated.

according to [20] were hydrated for two hours either in water (control seeds) or in HB solution (treatments). Dilutions were prepared consisting of a 0.5 ml/l aqueous solution of the D-Amine®, and a 24 ml/l glyphosate solution following the instructions of the manufacturers. Hydrated seeds were planted in plastic pots in the soil. Seeds that were not treated with HBs served as a control group.

The morphometric traits, RWC, and leaf pigment content were measured as described in Experiment 2. All measurements were carried out in the third week for plantlets germinated from seeds that had been pretreated (with 10 leaves selected randomly per each category and processed).

Statistical Analysis of Data

All experiments were carried out in triplicate. For experiment 1, one-way analysis of variance (ANOVA) with post hoc Tukey's test ($p < 0.05$) was performed to estimate the significant differences between the control and treated variants of cultivars. For Experiments 2 and 3, to visualize the differences between the treated plants, statistical analysis was conducted utilizing R-4.2.1 software involving the Kruskal-Wallis test and Student's t-test to determine if there were significant differences in the morphometric parameters (total plant length, root length, stem length, lengths of the first three leaves, relative water content, and leaf pigment content (chlorophyll a, chlorophyll b, total chlorophyll, carotenoids and xanthophylls), among the treatments for each cultivar. The data are presented as mean value ± standard deviation (SD).

Results

3.1. Chlorophylls and carotenoids content in wheat cultivars treated with Granstar®

HB Granstar® is one of the post-emergence HBs used to control broad-leaved weeds in cereals and other crops [21]. Figure 1 shows the effect of the Granstar® HB on the photosynthetic pigments of wheat cultivars under study.

For all wheat cultivars, the changes in chlorophyll content were nonsignificant when the effect of the HB was active. For the content of the carotenoids for wheat cultivar "Pobjeda", the changes were significant showing a decrease with *$p < 0.05$. This shows that even though photosynthesis pigment content is reduced during the effect of the HB on wheat, the effect is not significant. An exception is the case of the cultivar "Pobjeda", where carotenoid content is higher compared to the controls.

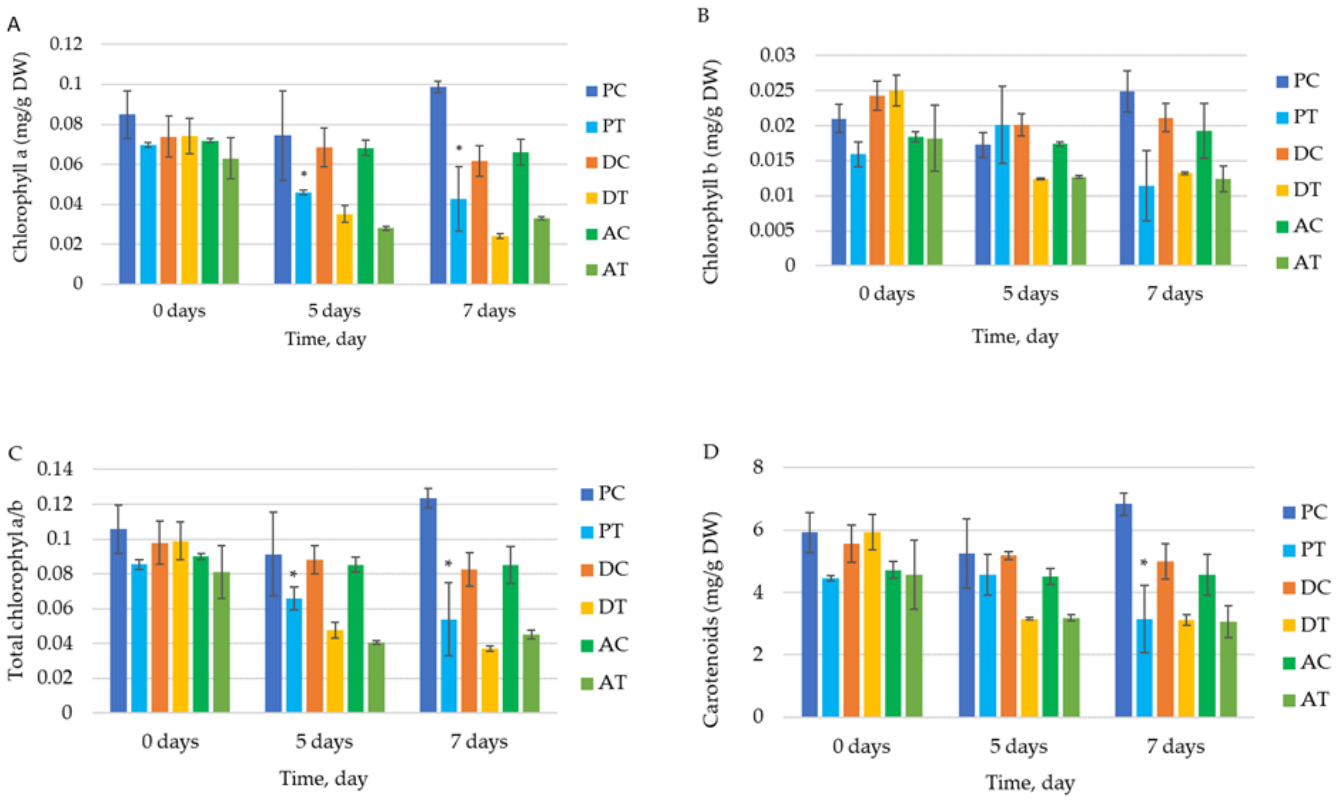


Figure 2. Chlorophyll a (A), chlorophyll b (B) content, total chlorophyll a+b (C), and carotenoids content (D) in wheat plants treated with HB D-Amine®. Data are mean values ± standard deviation. The one-way analysis of variance (ANOVA) was applied using the Tukey test to determine significant differences between mean values (*$p < 0.05$). Legend: PC – Pobjeda control, PT – Pobjeda treated, DC – Dajti control, DT – Dajti treated, AC – Adelajde control, AT – Adelajde treated

3.2. Pigments content in wheat cultivars treated with D-Amine®

Figure 2 shows the results of the chlorophylls and carotenoids content of wheat plants subjected to the effect of HB D-Amine®. It can be evidenced by a reduction in the levels of chlorophylls and carotenoids for "Pobjeda", which starts the day after the treatment. For two other cultivars, the effect is observed 5 days after the treatment, reducing the level of all photosynthetic pigments. A significant difference was evidenced for treatments of "Pobjeda" for chlorophyll a, total chlorophyll in five and seven days after treatment, and carotenoids in seven days after treatment.

3.3. Pigment content in wheat cultivars treated with the combination of both HBs

The combination of HBs is one of the forms used for weed management in cereals. This method is used to expand the spectrum of weeds that will be killed by HBs. Figure 3 shows the results of the photosynthesis pigments content of wheat cultivars after the treatment with a combination of two HBs in doses according to manufacturers' instructions. The results have shown that the content of photosynthetic pigments started to decrease on the day of treatment, but the difference was insignificant. Their reduction is greater on the fifth day and beyond. The immediate reduction of the content of pigments may be a consequence of the quick effect of the HB D-Amine® and the later effect may be a consequence of the effect of the HB Granstar®. The changes with significant differences in the content of chlorophyll-a were found for all three cultivars on the fifth day after treatment, and after. For chlorophyll b, there was a significant difference only for the "Pobjeda" after the fifth day after the treatment, and onward. For the total content of chlorophylls, the most significant difference was for all three cultivars on the fifth day after treatment, and thereafter. For the content of carotenoids, there was a significant difference only for "Pobjeda" and "Adelajde" on the fifth day after treatment and thereafter, while for "Dajti", the difference was nonsignificant.

3.4. Impact of the HB leaf spray on the third week following germination

3.4.1. Impact of the Mode of HB treatment on morphometric traits

The results indicate that when the plants were subjected to HB treatment during the third week after germination, the changes that occurred in the observed morphometric traits from that point onward were not of statistical significance.

In our study the herbicidal application of 2,4-D and glyphosate at plantlets at the third week after sprouting did not in-

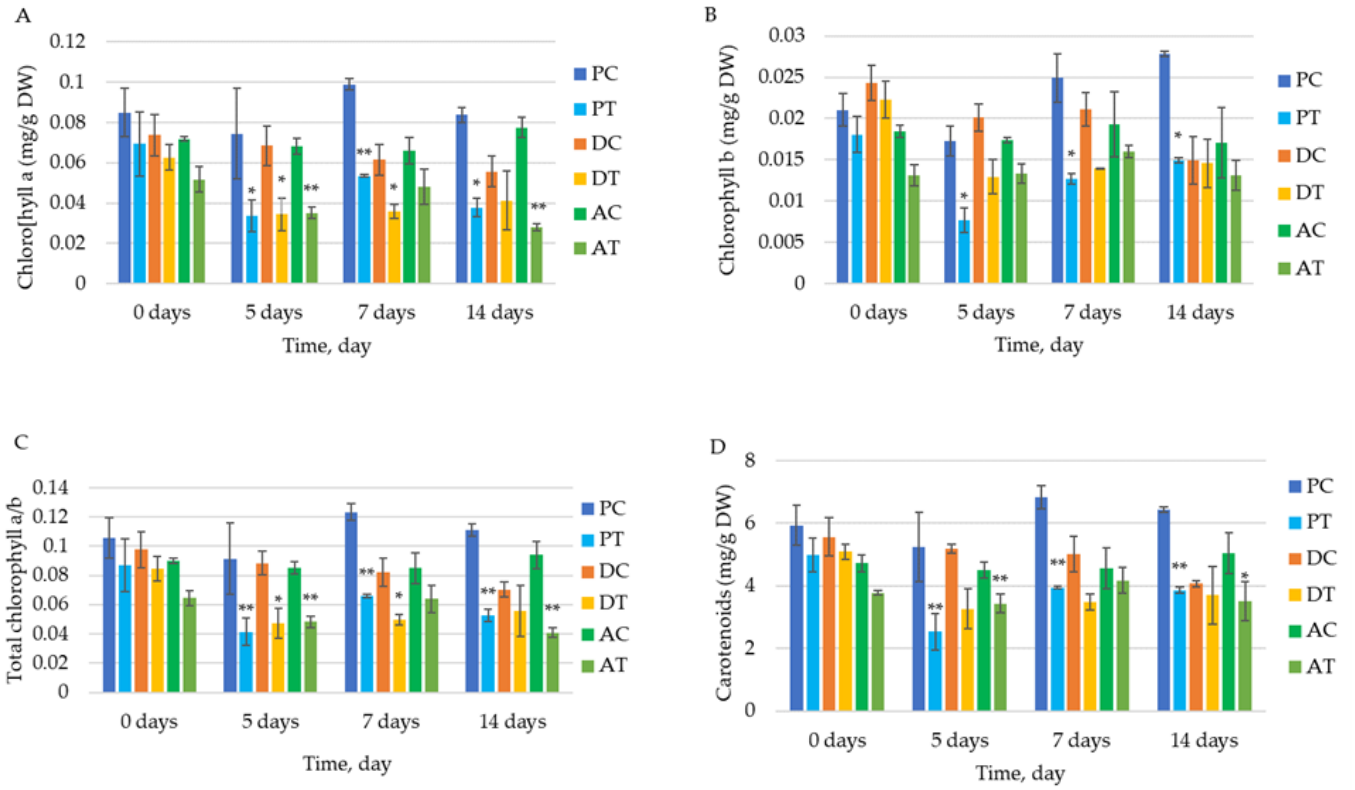


Figure 3. Chlorophyll a (A), chlorophyll b (B) content, total chlorophyll a+b (C), and carotenoids content (D) in wheat plants treated with a combination of HBs Granstar® and D-Amine®. Data are mean values ± standard deviation. The one-way analysis of variance (ANOVA) was applied using the Tukey test to determine significant differences between mean values (**p < 0.01; *p < 0.05). Legend: PC – Pobjeda control, PT – Pobjeda treated, DC – Dajti control, DT – Dajti treated, AC – Adelajde control, AT – Adelajde treated.

duce noticeable alterations in the total length of the plant, root length, stem length, average length of leaves or RWC.

3.4.2. Impact of the mode of HB treatment on pigment content

The groups of plants that were treated with 2,4-D and glyphosate 3 weeks after sprouting displayed nonsignificant variations within the same cultivar concerning photosynthetic pigments.

3.5. Impact of the HB pre-treatment of seeds

3.5.1. Morphometric traits

The results highlight noticeable differences between the control group and cultivars treated with 2,4-D as seeds. While there was no inhibition in germination, they did exhibit delayed germination by seven days. Conversely, no glyphosate-pretreated seed was able to germinate and a specific cultivar, "Progres" did not germinate when pretreated with 2,4-D. In terms of total plant length, 2,4-D pretreated cultivars, namely, "Adelajde" (p=0.002), "Dajti" (p=0.003), "Progres" (p<0.001), and "Tomborzo" (p=0.03) displayed major differences compared to the control group. Thus, the use of this herbicide contributed to wheat plant elongation. When considering root length, "Victoria" (p=0.01) exhibited longer roots than the control group. Regarding stem length, the stems of the cultivars "Adelajde" (p<0.001), "Progres" (p<0.001) and "Tomborzo" (p=0.001) resulted more elongated compared to the rest. The same cultivars displayed significant changes in the length of the leaf. These findings highlight significant modification of plant morphology and germination rate and show limited impact of HB pretreatment on RWC.

3.5.2. Photosynthetic pigments

The graphs below illustrate the variations of plants germinated from pretreated seeds and control groups in terms of photosynthetic pigments. Among all cultivars, there is a nonsignificant change to the amount of chlorophyll a, except "Tomborzo" (p=0.01) and "Victoria" (p=0.04), which had elevated levels of it. Similar to the results of chlorophyll a, most cultivars except "Victoria" (p=0.04) which exhibited significantly higher chlorophyll b levels, had little to no change among them. "Tomborzo" (p=0.03) and "Victoria" (p=0.04) displayed higher levels of total chlorophyll compared to the control group.

Discussion

4.1. Impact of different categories of HB and their combination on wheat pigment content

The use of HBs can have notable effects on the synthesis of chlorophyll a, b, and carotenoids [1, 5-7, 15], in many cases by inhibiting them [1, 15, 22]. Across all wheat cultivars, the great-

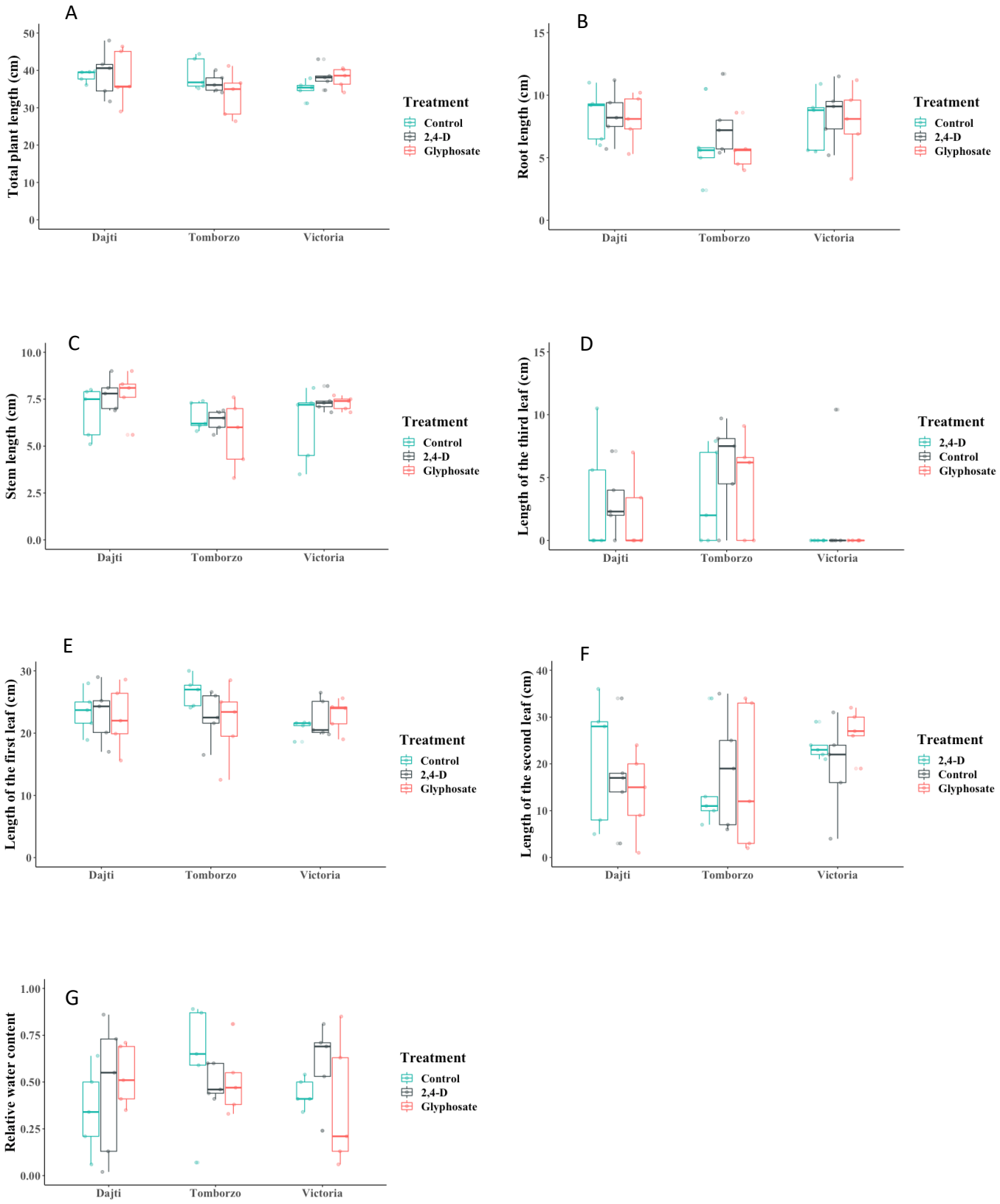


Figure 4. Effect of 2,4-D and glyphosate application on the following wheat plant growth parameters: A) Total plant length, B) root length, C) stem length, D) length of first leaf, E) second leaf, F) third leaf and G) RWC. These parameters were observed within the timeframe of a week after the application of herbicides on wheat plants. The Kruskal-Wallis test did not find any significant overall differences.

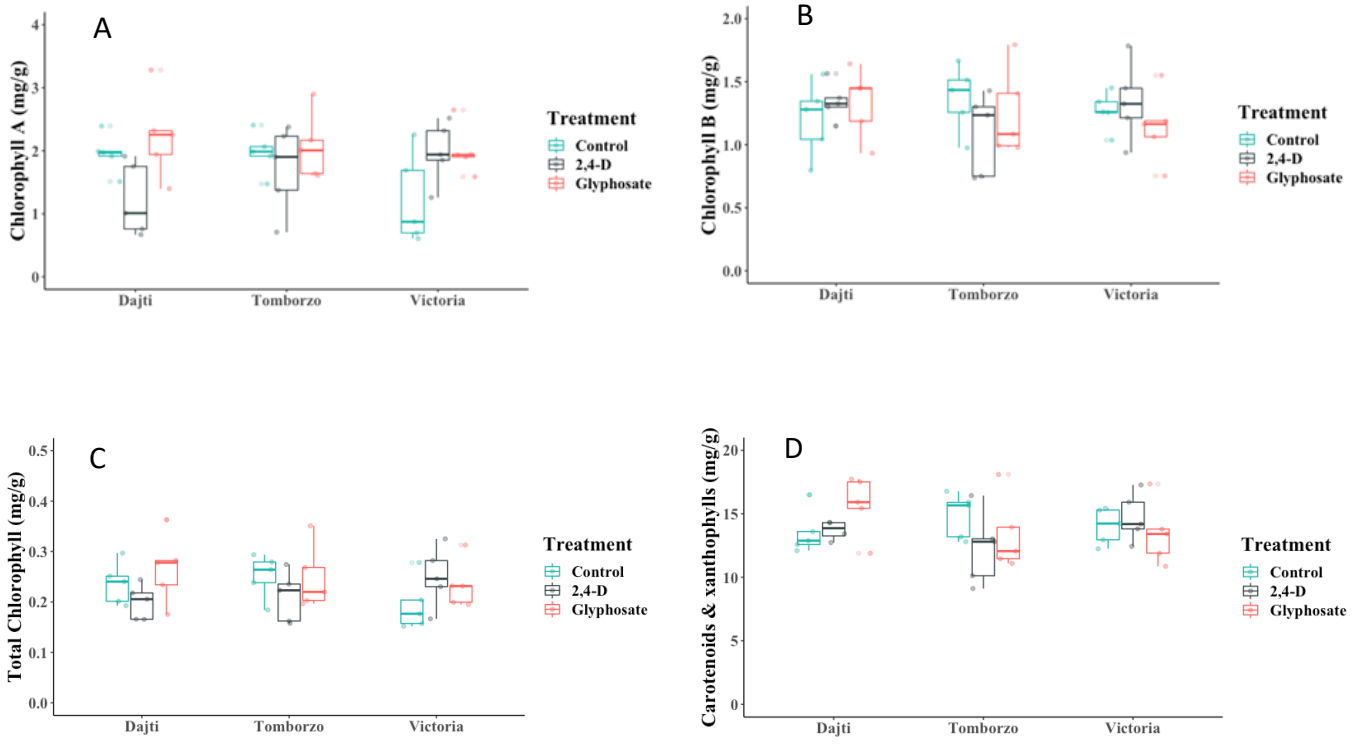


Figure 5. Stability of: A) Chlorophyll a; B) chlorophyll b; C) total chlorophyll and D) carotenoids and xanthophylls levels, in wheat plants following 2,4-D and glyphosate application. The Kruskal-Wallis test did not find any significant overall differences.

er effect of the HB on the synthesis of pigments is observed during the second and third weeks after treatment. Soliman *et al* [14] reported that all HB treatments slightly decreased chlorophyll a, b, and total chlorophyll and increased carotene content. Hana *et al* [5] reported that the levels of chlorophyll b in plants subjected to the effect of two HBs were lower and the levels of chlorophyll a were higher compared with their content in plants not treated with herbicides. Based on our results for all wheat cultivars in local use, the changes in chlorophyll content were nonsignificant when the effect of this HB was active. Figure 2 shows the results of the chlorophylls and carotenoids content of wheat plants subjected to the effect of HB D-2,4-D. The evidenced reduction of pigments is in accordance to other reports [1, 7, 8, 23]. Meanwhile, the combination of HBs is one of the forms used for weed management in cereals to expand the spectrum of killed weeds. Different combinations of HBs have been reported in cereals [9, 10, 13, 24]. Our results have shown that the content of photosynthetic pigments started to decrease on the day of treatment with the combination of HBs, displaying cultivar-specific significant or non-significant changes.

4.2 Impact of the Mode of HB treatment on Morphometric traits and pigment content at the third week after germination and at pretreated seeds

The results indicate that when plants were subjected to HB treatment in the third week after germination, changes in the observed morphometric traits from that point onward were not of statistical significance. The two herbicides that lead to the reduction of aromatic amino acids that are vital for protein synthesis and plant growth are 2,4-dichloro-phenoxyacetic acid, a synthetic auxin, and glyphosate, which acts by disrupting the shikimic acid pathway through inhibition of the enzyme 5-enolpyruvylshikimate- 3-phosphate (EPSP) synthase. From previous reports [16, 17] we know that wheat plants treated with sulfonylurea HBs experience a highly significant decrease in protein content while by contrast, there is evidence of a significant variation in APX activity. Also, they were found to inhibit branched chain amino acids [25]. Depending on the mode of action of each HB the protein and carbohydrate content in the seedlings of the wheat was found to be affected by the dose rate of HBs, suggesting that cell metabolism, nucleic acid synthesis, enzymes, and other functional proteins are one of the target sites of HB action [25, 26]. Thus, depending on the above the treatment might lead to the alteration in the protein metabolism of the seedlings, which might be reflected in the morphometric traits of growth. In our study the herbicidal application of 2,4-D and glyphosate at plantlets in the third week after sprouting did not induce noticeable alterations in the total length of the plant, root length, stem length, average length of leaves or RWC, which suggests that the recommended dosage and mode of treatment are suitable for the early development of

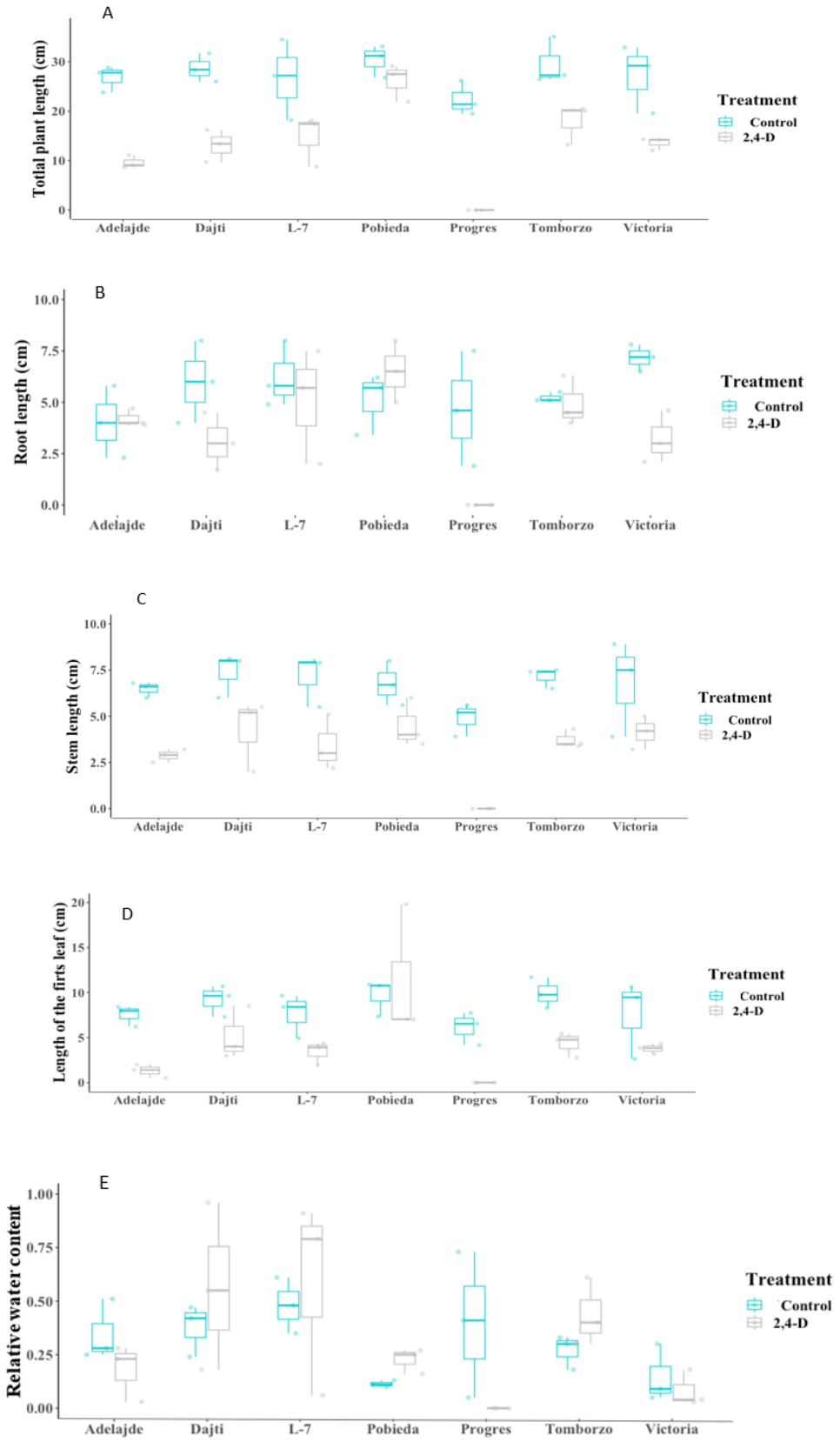


Figure 6. The impact of 2,4-D and glyphosate on: A) Total plant length; B) root length; C) stem length; D) length of first leaf, and E) RWC was observed within the timeframe of a week after the application of the herbicides on wheat plants. Asterisks denote significant differences after Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

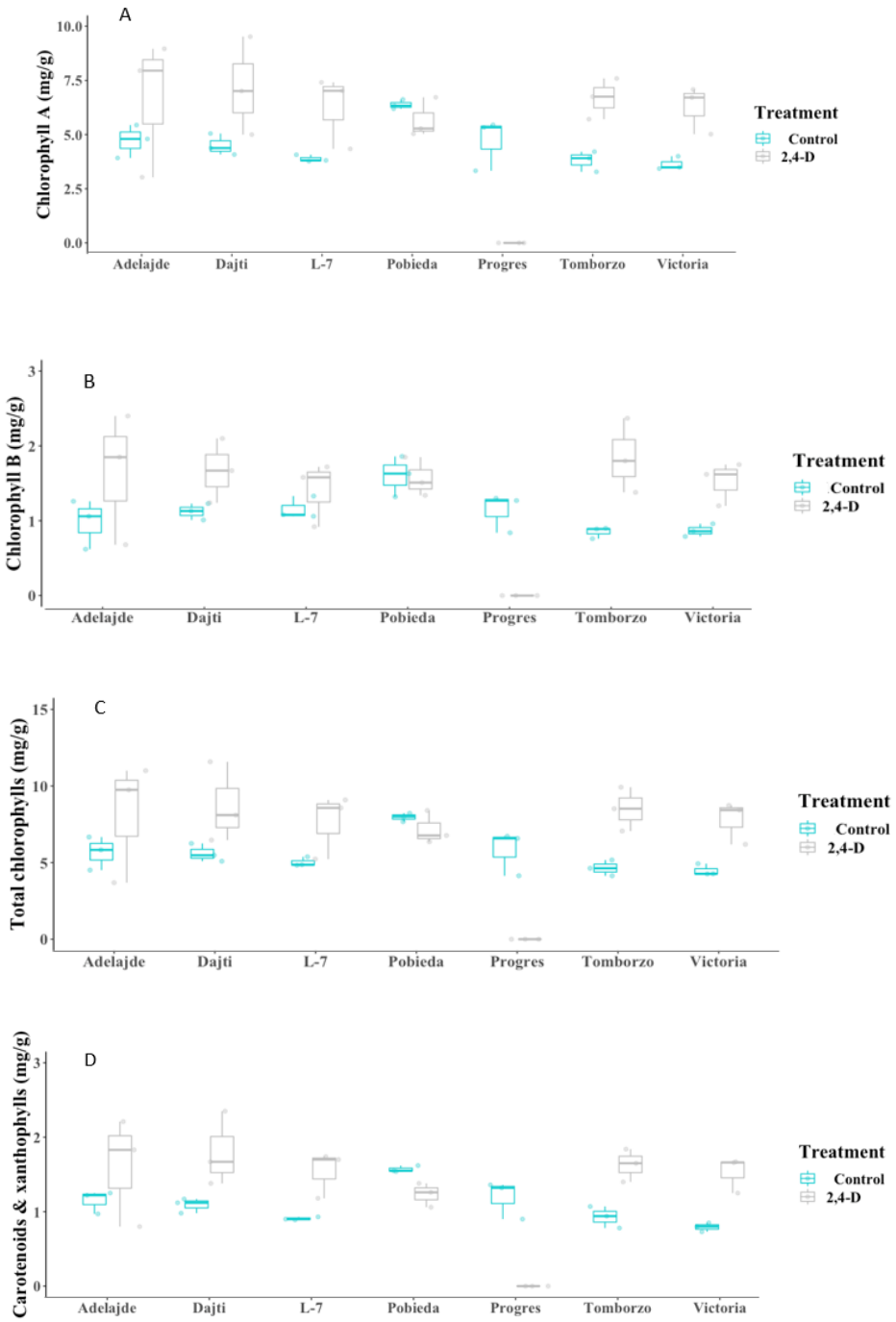


Figure 7. Graphs detailing: A) chlorophyll a; B) chlorophyll b; C) total chlorophyll and D) carotenoids and xanthophylls variations in in HB pretreated plants vs. control. Asterisks denote significant differences after Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

the specific local wheat cultivars. The same group of treatments displayed nonsignificant variations within the same cultivar concerning photosynthetic pigments. These findings show the resistance of chosen local cultivars of wheat towards HB treatment and suggest that the genetic makeup of these cultivars may allow them to withstand the HB application.

The results highlight noticeable differences between the control group and cultivars treated with 2,4-D as seeds. While there was no inhibition in germination, they did exhibit delayed germination. Conversely, no glyphosate-pretreated seed was able to germinate. These results are in accordance with several previous reports [24-27] that highlight the impact of HB treatments on cell metabolism, nucleic acid synthesis, enzymes, and other functional proteins, which as a consequence can stop or delay the seed development by preventing germination. The investigation of the leaf pigment content showed that there is nonsignificant change to the amount of chlorophyll a and b, except "Tomborzo" and "Victoria", which, had elevated levels of both. Similar results on the cultivar-specific impact of HB treatments on wheat photosynthetic pigments (chl_a, chl_b) are

previously reported [1, 27]. Also, in cultivars "Tomborzo" and "Victoria", the HB treatment significantly increased the level of carotenoids and xanthophylls. This is in accordance with their function as antioxidants in chloroplasts, reducing the ROS produced during intense reactions in these organelles, and HBs are proven to be potential inducers of ROS production [1, 27]. It should be also noted in this interpretation of results that the significance of "Progres" cultivar is exclusively attributed to the lack of germination of the HB treated seeds. Others have also described the cultivar-specific response to HB treatments, reporting the malfunctioning of the detoxifying machine up to the stop of seed development by preventing germination. It seems that the seed reserves cannot ensure sufficient nutrients for further development of the plant because of the inhibition of the synthesis of amino acids and proteins, and/or the impaired photosynthesis mechanism. Categories of stress other than HB are also known to impact the synthesis of photosynthetic pigments hence the mode of variation remains cultivar-specific [28-30]. Following [28, 30] the salt-induced alterations of leaf Chl content could be due to impaired biosynthesis or accelerated pigment degradation, Chl_b may be converted into Chl_a, thus resulting in the increased content of Chl_a for some cultivars.

Conclusions

The presence of weeds within the crop may adversely affect production in several ways since they compete with crop species for water, nutrients, and light, and ultimately reduce crop yield. To avoid malpractices, the investigation of the selectivity of herbicides (the ability of plants to metabolize herbicides to form non-phytotoxic compounds) should precede taking into consideration that the degree of tolerance or sensitivity may be among other factors due to the genetic content of species or cultivars.

Results of treatment with Granstar®, D-Amine®, and their combination showed a cultivar-specific impact on the leaf photosynthetic pigments content. However, the combination of the two HBs had a greater effect. Significant differences were observed for all three cultivars "Adelajde". "Pobjeda", and "Dajti" for chlorophyll a and total chlorophyll content. For carotenoid content significant differences were observed in cultivars "Pobjeda" and "Adelajde", while for chlorophyll b only for cultivar "Pobjeda".

The impact of the mode of HB treatment was also investigated. The plants from pre-treated seeds have delayed germination, increased total plant length, and elongated stems and leaves. No glyphosate pre-treated seed was able to germinate, and only one cultivar out of five did not germinate when pre-treated with 2,4-D, which highlights the specific sensitivity of cultivars to HB treatment at the seed stage. These findings underscore the intricate responses of wheat plants to HBs, emphasizing the critical role of timing, HB type, mode of treatment and genomic makeup of cultivars in plant germination and growth. Based on the above, we recommend further exploration of alternative HB combinations on wheat cultivars before large scale applications

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