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The Allelopathic Effects of Sunflower and Wheat Root Exudates on *Sinapis arvensis* and *Sinapis alba*

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Abstract: In this study, we aimed to investigate the allelopathic effects of sunflower and wheat root exudates on the common weeds such as wild mustard and white mustard in our region. The root exudates which were obtained by soaking 8 weeks old sunflower and wheat seedlings (20 or 40 seedlings) in 100 mL of distilled water for 3 days were applied to the leaves of wild mustard and white mustard. In order to compare the allelopathic effect, the recommended dose (1 $g.da^{-1}$) and twice the recommended dose (2 $g.da^{-1}$) of Gromstor (Tribenuron-methyl), a herbicide preferred by farmers for the chemical control of these weeds was also applied. The allelopathy was performed for wild mustard and white mustard seedlings by the measurement of different physiological and biochemical parameters, such as chlorophyll a, chlorophyll b, total chlorophyll, carotenoid, proline, total protein amounts and superoxide dismutase enzyme activity. The amounts of total chl and carotenoid in wild mustard leaves decreased in all treatment groups compared to control. The highest decrease in total chl (50.93%) and carotenoid (46.69%) was oberved in the treatment of 40 wheat seedlings. 100 mL^{-1} distilled water. In the white mustard leaves, the amount of total chl in all treatment groups except the treatment group of Gromstor 2 $g.da^{-1}$ and carotenoid in all treatment groups increased compared to the control. The highest increases again were observed in 40 wheat seedlings. 100 mL^{-1} distilled water treatment. The proline amounts in wild mustard and white mustard increased in all treatment groups. The highest increase was observed for the treatment of 20 wheat seedlings. 100 mL^{-1} distilled water in wild mustard (459.69%) and 40 sunflower seedlings. 100 mL^{-1} distilled water in white mustard plant (104.70%). In superoxide dismutase enzyme activities, treatments decreased activity except treatment of 40 sunflower seedling root exudate in wild mustard, while increased activity outside commercial herbicide treatment in white mustard. The results showed that sunflower and wheat root exudates have allelopathic effects on wild mustard and white mustard weeds. It is thought that the study will be a reference for new studies that will enable the use of plant root exudates as bioherbicides or foliar fertilizers and will contribute to the fight against weeds in organic agriculture.

Keywords: Allelopathy; antioxidant enzymes; bioherbicide; photosynthetic pigments; white mustard; wild mustard

1 Introduction

Approximately 1800 weed species exist in Turkey [1]. Weeds are a major problem in agricultural production. If they are not controlled, product losses can reach 100% [2].

Herbicides, which became a part of the agricultural production with the green revolution, play an important role in protecting the crop plants against weeds and maintaining the crop production. About 50% of the chemicals used in weed control are herbicides in the world while this ratio 26% in Turkey [3-5].

However, their misuse causes environmental pollution and health problems and their use is a questionable topic. In recent years, there are increasing number of studies on different herbicide application techniques, changing the herbicide dose and application time and using allelopathic plant extracts or exudates.

The importance of allelopathy increases day by day due to their significant effect on weed control and their environment friendly content [6]. The chemical interactions between living things such as plants, insects and microorganisms etc., which causes direct or indirect harmful or beneficial effects, are called as allelopathy [7]. This interaction can be achieved by evaporation of allelochemicals from plants, leakage from roots, release, leakage from leaves and deterioration of dead plant parts [8].

The species of *Sinapis alba* and *Sinapis arvensis* are in the genus of *Sinapis* belonging to the Cruciferae family; they are among the weeds in agricultural fields and common in the flora of Turkey [9]. Sunflower and wheat are plant species that have an important role in Turkey's economy and have strong alellopatic activity [10,11].

Although there are many studies examining allelopathic effects of plant root exudates on weeds germination and seedling growth [12-15], very few studies investigating the physiological and biochemical parameters. However, these studies are very important for understanding the mechanism of influence.

In this study, it was aimed to determine the allelopathic effects of the sunflower and wheat root exudates (20 seedlings. 100 mL^{-1} distilled water and 40 seedlings. 100 mL^{-1} distilled water) on the biochemical and physiological parameters of certain common weeds in our region and especially in grain fields as *S. arvensis* (wild mustard) and *S. alba* (white mustard) and their potential as bioherbicides or foliar fertilizers.

2 Materials and Methods

2.1 Materials

The wild mustard (*Sinapis arvensis* L.) seeds collected from the fields in Imamoglu district of Adana province were assessed according to the Flora of Turkey and the East Aegean Islands [16]. The white mustard (*Sinapis alba* L.) seeds were purchased from Mersin Kemal Cuce Tarim Transport Food Construction Trade Industry Limited Company. The seeds of Sems sunflower (*Helianthus annuus* L.) and Sarıbasak wheat (*Triticum aestivum* L.) were obtained from the Eastern Mediterranean Agricultural Research Institute. All chemicals used in the study were purchased from Sigma-Aldrich and Merck companies.

2.2 Obtaining the Root Exudates

The sunflower and wheat root exudates were obtained according to the method of Kroschel [17]. According to this method, 20 and 40 seedlings of 8 weeks old sunflower and wheat plants were pulled up and soil remains on their roots were cleaned. In a beaker, 20 or 40 seedlings of each plant were placed in 100 mL of distilled water for 3 days. The resulting exudates were filtered through Whatman no:2 filter paper, applied to the leaves as foliar without waiting.

At the same time, the recommended dose (1 $g.da^{-1}$) and twice the recommended dose (2 $g.da^{-1}$) of Gromstor which is a herbicide with 75% Tribenuron-methyl as an active ingredient, commonly used in chemical control of white mustard and wild mustard weeds, were applied to compare the degree of influence and to investigate their bioherbicide potential.

2.3 The Growing Conditions, Trial Pattern and Sampling of the Weeds as S. arvensis and S. alba

The experiment was designed as a random trial pattern with 3 replicates. In the 3 seeds were sowed in each drill in 28 × 15 cm pots, watered day by day with constant humidity (50 ± 5%), 16:8 photoperiod and 23 ± 2°C. When the seedlings were 20 days old, sunflower and wheat exudates (20 seedlings. 100 mL^{-1} distilled water and 40 seedlings. 100 mL^{-1}) and herbicide (1 $g.da^{-1}$ and 2 $g.da^{-1}$) were applied as

foliar to the leaves of the plants. The seedlings were collected for biochemical and physiological analyzes when they were 30 days old.

2.4 Determination of the Photosynthetic Pigments

The improved method of Witham et al. [18] was applied to determine the amount of photosynthetic pigments in the *S. arvensis* and *S. alba* leaves. The absorbance values for 450 nm, 645 nm and 663 nm wavelengths were measured with VIS Spectrophotometer (Boeco). The measured absorbance values were used in the formulas given below, thus chlorophyll a, chlorophyll b, total chlorophyll and carotenoid amounts in 1 gram leaf tissue were calculated.

mg chlorophyll a.g^{-1} tissue = [12,7 × (D663) - 2,69 (D645)] × (V/1000.W)

mg chlorophyll b.g^{-1} tissue = [22,9 × (D645) - 4,68 (D663)] × (V/1000. W)

mg total chlorophyll.g^{-1} tissue = [20,2 × (D645) + 8,02 (D663)] × (V/1000. W)

mg total carotenoid.g^{-1} tissue = 4,07 × D450 - (0,0435 × amount of Chl a + 0,367 × amount of Chl b)

In the formulas: D shows the optical density (absorbance value) of the chlorophyll extract at the related wavelength; V shows the final volume of 80% acetone; W shows the fresh weight of the extracted tissue in grams.

2.5 Determination of the Total Amount of Proline

According to the method of Bates et al. [19], 1 g of leave samples were collected and homogenized in mortar with 10 mL of 3 % sulfosalicylic acid solution, the homogenate were filtered through filter paper to determine the amount of proline in the wild mustard and white mustard leaves. The resulting filtrate was allowed to stand for 24 hours in a dark and cool room, 2 mL of acid ninhydrin and 2 mL of glacial acetic acid were added to 2 mL of the filtrate and incubated at 100°C for 1 hour. After the reaction was stopped in an ice bath, 4 mL of cold toluene was added to the tubes and mixed with the stirrer. The fraction containing toluene was aspirated from the liquid phase and it's absorbance was measured at 520 nm by using the VIS Spectrophotometer (Boeco). The amount of proline was determined by using the calibration curve which was drawn for 0.1, 0.2, 0.3, 0.4, 0.5 µmol proline containing standards and was expressed as µmol proline. g fresh $weight^{-1}$.

2.6 Determination of the Total Protein Amount

The improved method of Bradford [20] was used. 1 g fresh wild mustard and white mustard leaf samples of the control and treatment groups were weighed 3 times and extracted in 5 mL pH 7.8, 0.05 M sodium phosphate buffer in ice bath. This extract was centrifuged at 13000 rpm for 20 minutes in a cooled centrifuge as globally descibed in [21] with minor modifications.

After centrifugation, 100 µL of supernatants were added to a 1 mL reaction mix containing a protein dye. The absorbance values of the samples which were kept at room temperature for 10 minutes were measured at 595 nm using a Boeco VIS Spectrophotometer. The measurements were applied to the calibration curve generated by BSA standards (0.02-0.2 mg.mL^{-1}); the total amount of soluble protein was determined as mg.g fresh $weight^{-1}$.

2.7 Determination of Superoxide Dismutase Enzyme Activity

2.7.1 Preparation of the Enzyme Extract

The method of extraction which was used to determine total protein content was also performed to use the extract in the measurement of superoxide dismutase (SOD) activity [21].

2.7.2 Determination of Superoxide Dismutase Enzyme Activity

SOD enzyme activity was determined by using the method of Beauchamp et al. [22]. The method is based on the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm by the SOD

enzyme in the sample. The reaction was waited to occur after adding 3 mL reaction mixture containing 50 mM sodium phosphate buffer, 33 µM NBT, 10 mM L^{-1} Methionine, 0.66 mM EDTA and 0.0033 mM Riboflavin to the supernatants for 10 minutes at 300 μmol^{-1} m^{-1} s^{-1} light intensity and room temperature. The absorbance values of the samples were measured at 560 nm wih the VIS spectrophotometer. Enzyme activity, SOD amount required for 50% inhibition of NBT was calculated as 1 enzyme unit; EU was determined as mg $protein^{-1}$.g $fresh\ weight^{-1}$.

2.8 Statistical Analysis of the Data

The data were analyzed with Tukey test (Variance analysis in SPSS 16.0 program) and $p < 0.05$ was accepted as statistically significant Tukey [23]. Standard deviations and errors were calculated in the same program.

3 Results

3.1 Determination of Photosynthetic Pigment Amount

The amounts of chl a, chl b, total chl and chl a/b ratios and carotenoid amounts in wild mustard and white mustard leaves are given in Figs. 1 and 2, respectively.

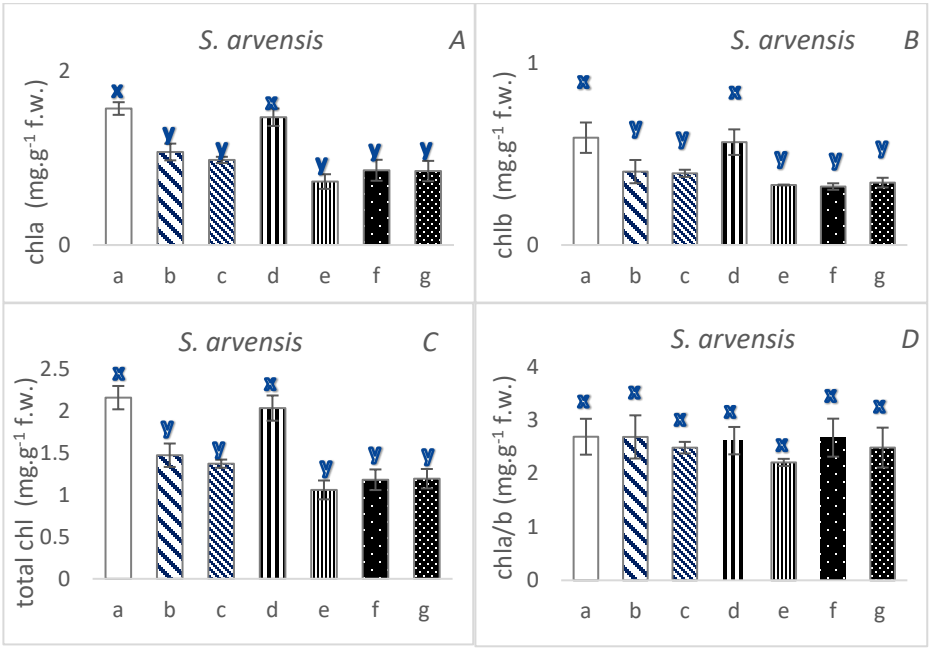

Figure 1: The amounts of chlorophyll a (A), chlorophyll b (B), total chlorophyll (C) and chlorophyll a/b (D) ratio in wild mustard leaves

a: Control (distilled water), b: 20 Sunflower seedlings. 100 mL^{-1} distilled water, c: 40 Sunflower seedlings. 100 mL^{-1} distilled water, d: 20 Wheat seedlings. 100 mL^{-1} distilled water, e: 40 Wheat seedlings. 100 mL^{-1} distilled water, f: Gromstor 1 $g.da^{-1}$, g: Gromstor 2 $g.da^{-1}$ (n:3). x, y shows the significant difference ($p < 0.05$).

The amounts chl a, chl b and total chl in wild mustard leaves decreased in all treatment groups compared to control. The highest decrease for chl a was observed in the treatment of 40 wheat seedlings. 100 mL^{-1} distilled water as 53.50% ($p < 0.05$) (Fig. 1(A)), 45.52% for the treatment of Gromstor 1 $g.da^{-1}$ in terms of chl b ($p < 0.05$) (Fig. 1(B)). The highest decrease in total chl was oberved in the treatment of 40 wheat seedlings. 100 mL^{-1} distilled water as 50.93% ($p < 0.05$) (Fig. 1(C)). The chl a/b ratios showed reductions in all treatment groups which were not significant (Fig. 1(D)).

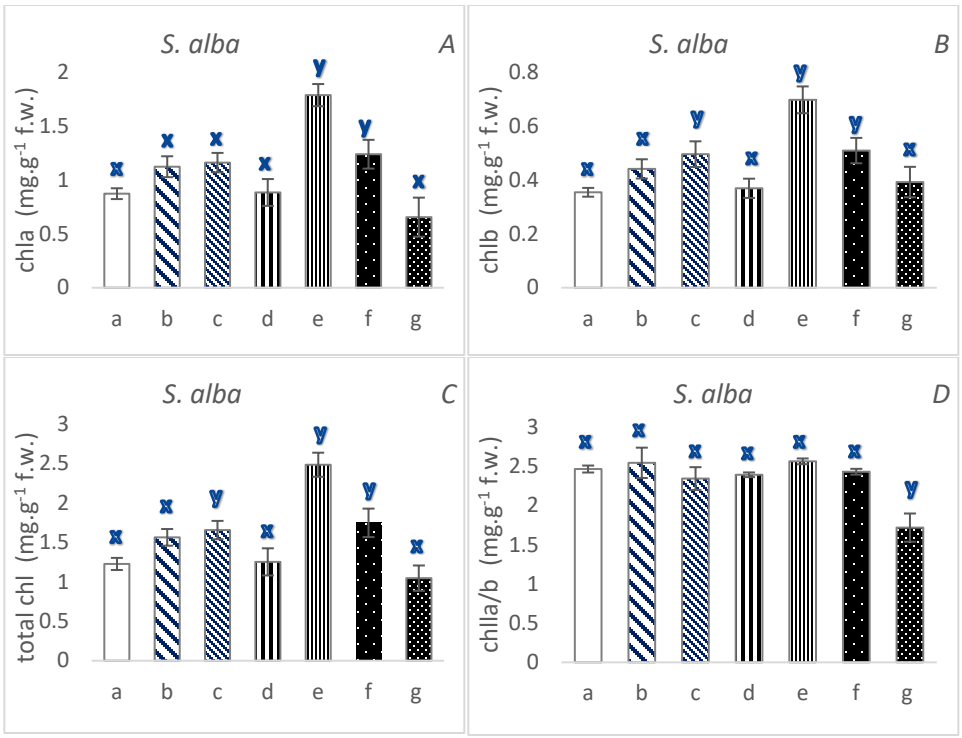

Figure 2: The amounts of chlorophyll a (A), chlorophyll b (B), total chlorophyll (C) and the ratio of chlorophyll a/b (D)

a: Control (distilled water), b: 20 Sunflower seedlings. 100 mL^{-1} distilled water, c: 40 Sunflower seedlings. 100 mL^{-1} distilled water, d: 20 Wheat seedlings. 100 mL^{-1} distilled water, e: 40 Wheat seedlings. 100 mL^{-1} distilled water, f: Gromstor 1 $g.da^{-1}$, g: Gromstor 2 $g.da^{-1}$ (n:3). x, y shows the significant difference ($p < 0.05$).

In the white mustard leaves, the amount of chl a and total chl in all treatment groups increased compared to the control except the treatment group of Gromstor 2 $g.da^{-1}$. The highest increase in chl a (104.79%) (Fig. 2(A)) and total chl (102.51%) (Fig. 2(C)) were observed in 40 wheat seedlings. 100 mL^{-1} distilled water treatment ($p < 0.05$). The amount of chl b increased in all treatment groups compared to the control group. The highest increase was observed in 40 wheat seedlings. 100 mL^{-1} distilled water treatment (96. 89%) ($p < 0.05$) (Fig. 2(B)). The ratio of chl a/b decreased in all groups except the groups of 20 sunflower seedlings. 100 mL^{-1} distilled water and 40 wheat seedlings. 100 mL^{-1} distilled water (Fig. 2(D)).

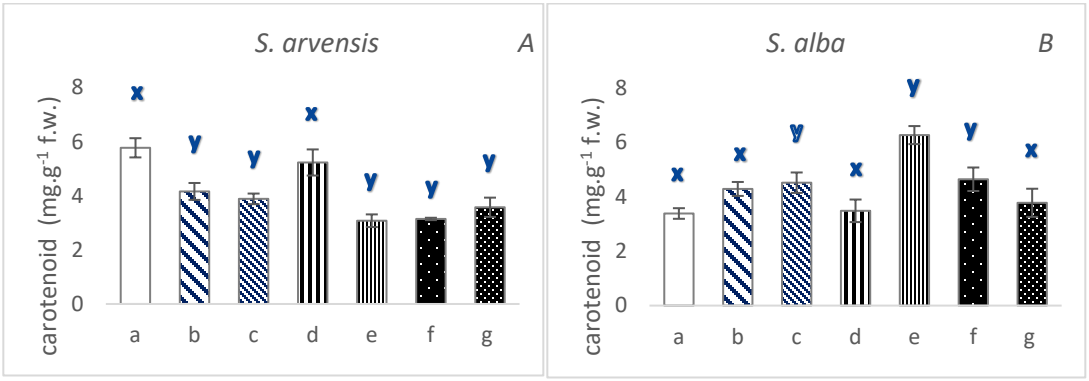

Figure 3: Carotenoid amounts in the leaves of wild mustard (A) and white mustard (B)

a: Control (distilled water), b: 20 Sunflower seedlings. 100 mL^{-1} distilled water, c: 40 Sunflower seedlings. 100 mL^{-1} distilled water, d: 20 Wheat seedlings. 100 mL^{-1} distilled water, e: 40 Wheat seedlings. 100 mL^{-1} distilled water, f: Gromstor 1 $g.da^{-1}$, g: Gromstor 2 $g.da^{-1}$ (n:3). x, y shows the significant difference ($p < 0.05$).

Carotenoid amounts of wild mustard leaves were also decreased in all treatment groups as in chlorophyll amounts compared to control. The most important decrease was observed in 40 wheat seedlings. 100 mL^{-1} distilled water treatment (46.69%) ($p < 0.05$) (Fig. 3(A)). The carotenoid amount in white mustard leaves increased in all treatment groups compared to the control group. The most important increase was observed in the treatment of 40 wheat seedlings. 100 mL^{-1} distilled water (85.13%) (Fig. 3(B)).

3.2 Determination of Proline Concentration

The proline amounts in wild mustard and white mustard weeds increased in all treatment groups compared to the control group. The highest increase was observed for the treatment of 20 wheat seedlings. 100 mL^{-1} distilled water in wild mustard plant (459.69%) while the highest increase was observed for the treatment of 40 sunflower seedlings. 100 mL^{-1} distilled water in white mustard plant (104.70%) ($p < 0.05$) (Figs. 4(A), 4(B)).

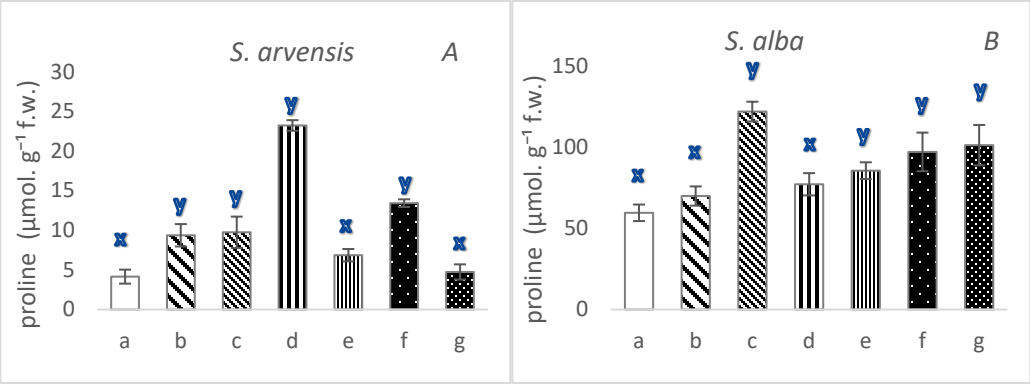

Figure 4: Proline amounts in wild mustard (A) and white mustard (B) leaves

a: Control (distilled water), b: 20 Sunflower seedlings. 100 mL^{-1} distilled water, c: 40 Sunflower seedlings. 100 mL^{-1} distilled water, d: 20 Wheat seedlings. 100 mL^{-1} distilled water, e: 40 Wheat seedlings. 100 mL^{-1} distilled water, f: Gromstor 1 $g.da^{-1}$, g: Gromstor 2 $g.da^{-1}$ (n:3). x, y shows the significant difference ($p < 0.05$).

3.3 Determination of Total Protein Amount

The amount of protein in wild mustard (Fig. 5(A)) and white mustard leaves (Fig. 5(B)) are as follows.

While the total amount of protein in wild mustard leaves decreased in sunflower root exudate treatments, it increased in wheat root exudate and herbicide treatments. The decreases were not significant. The highest increase (90.68%) was found in the treatment of 40 wheat seedlings. 100 mL^{-1} distilled water ($p < 0.05$) (Fig. 5(A)). The amount of protein in white mustard decreased in all treatments compared to control except gromstor treatments. While decreases were not significant, the increases were significant. The highest increase was in Gromstor 2 $g.da^{-1}$ (91.29%) (Fig. 5(B)).

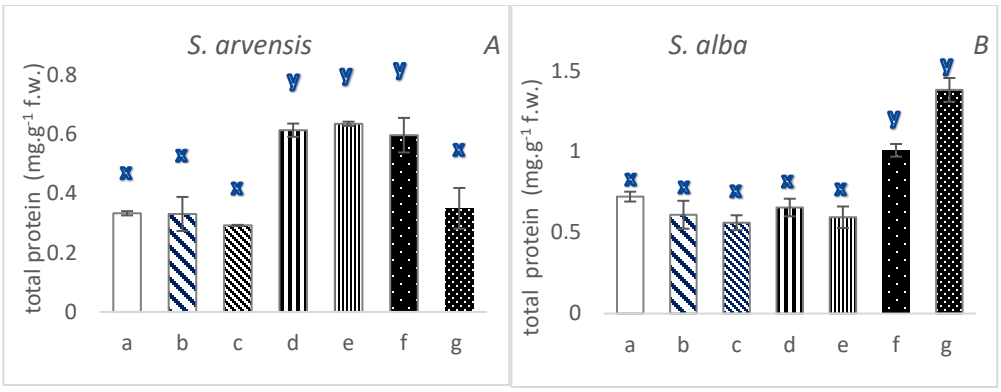

Figure 5: Total amount of protein in wild mustard (A) and white mustard (B) leaves

a: Control (distilled water), b: 20 Sunflower seedlings. 100 mL^{-1} distilled water, c: 40 Sunflower seedlings. 100 mL^{-1} distilled water, d: 20 Wheat seedlings. 100 mL^{-1} distilled water, e: 40 Wheat seedlings. 100 mL^{-1} distilled water, f: Gromstor 1 $g.da^{-1}$, g: Gromstor 2 $g.da^{-1}$ (n:3). x, y shows the significant difference ($p < 0.05$).

3.4 Determination of Superoxide Dismutase Activity

Figs. 6(A) and 6(B) show superoxide dismutase enzyme activities of wild mustard and white mustard seedling leaves, respectively.

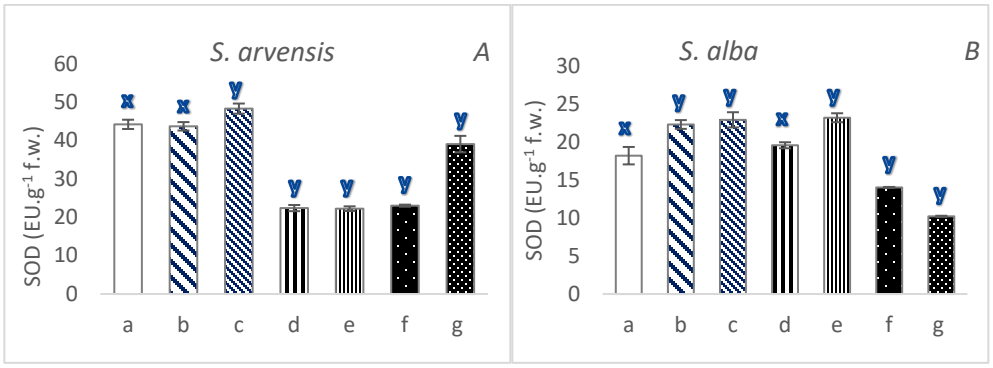

Figure 6: Superoxide dismutase enzyme activities of wild mustard (A) and white mustard (B) seedling leaves

a: Control (distilled water), b: 20 Sunflower seedlings.100 mL^{-1} distilled water, c: 40 Sunflower seedlings. 100 mL^{-1} distilled water, d: 20 Wheat seedlings. 100 mL^{-1} distilled water, e: 40 Wheat seedlings. 100 mL^{-1} distilled water, f: Gromstor 1 $g.da^{-1}$, g: Gromstor 2 $g.da^{-1}$ (n:3). x, y shows the significant difference ($p < 0.05$).

When the superoxide dismutase enzyme activities in wild mustard leaves were analyzed, it was understood that the treatments decreased activity except the treatment of 40 sunflower seedlings root exudate. The increases and decreases in the treatment were significant except the treatment of 20 sunflower seedlings. 100 mL^{-1} distilled water ($p < 0.05$). The highest value of 48,371 $EU.g^{-1}f.w.$ was obtained with 40 sunflower seedlings. 100 mL^{-1} distilled water treatment while the lowest value of 22,264 $EU.g^{-1}f.w.$ was obtained with 40 wheat seedlings. 100 mL^{-1} distilled water treatment (Fig. 6(A)). In white mustard, the activity incresed in all groups except the group of commercial herbicide treatment. The lowest activity of 10.241 $EU.g^{-1}f.w.$ was obtained with the treatment of Gromstor 2 $g.da^{-1}$ while the highest activity of 23.240 $EU.g^{-1}f.w.$ was obtained with the treatment of 40 wheat seedlings. 100 mL^{-1} distilled water. ($p < 0.05$) (Fig. 6(B)).

4 Discussion

The most effective method to kill weeds in agricultural production is to use herbicides. Since many of the herbicides commonly applied in our country are synthetic, they remain in nature for long years without degradation and cause soil and water pollution. Allelochemicals are natural compounds synthesized in plants and are easily degraded. Since they do not accumulate in nature, they do not harm the environment [15,24-26]. So, Therefore, Allelochemicals are very important for new and eco-friendly pesticide discovery efforts [27].

In the study, the chlorophyll and carotenoid amounts in wild mustard leaves decreased in all treatment groups. The most significant decreases ($p < 0.05$) for all pigments was found in the treatment of 40 wheat seedlings. 100 mL^{-1} distilled water except chl b (Figs. 1 and 3(A)). Similarly, the total chlorophyll content of rice and mustard seedlings treated with different *Amaranthus spinosus* extract concentrations was reduced [28]. The amount of photosynthetic pigments in the white mustard leaves was generally increased in the treatment groups (Figs. 2 and 3(B)). It has been thought that allelopathic substances greatly affect the photosystem efficiency [29,30]. It has been stated that the decrease in chlorophyll amount caused by allelochemicals is due to blockage in the biosynthesis pathway or the stimulation of chlorophyll degradation mechanism [31]. Kamal [32] reported that sunflower extracts which are highly used, cause wheat seed chlorosis which may be due to the Mg-chellatase activity of allelochemicals in sunflower seeds. The decrease in the amount of chlorophyll and carotenoid in wild mustard may be due to this situation. The chlorophyll and carotenoid amounts in the white mustard leaves were high; the sunflower and wheat root exudates did not much affect the pigment mechanism in the white mustard leaves. Also, chl a/b ratios showed a decrease in all treatment groups in wild mustard (Fig. 1(D)). It is known that the decrease in chlorophyll content under stress conditions is caused by oxidative stress [33,34]. It has been thought that the decrease in chlorophyll a/b ratio may be due to a faster degradation of chl a than chlb, or a decrease in chl a synthesis in oxidative stress [35].

Proline is an amino acid; it is amount increases in plants and it protects the plant in abiotic and biotic stress conditions. The increasing amount of proline is a widely used parameter to determine the degree of stress [36]. The proline amounts in wild mustard and white mustard seedlings increased in all treatment groups compared to the control group. The highest increase in wild mustard plant was observed in the treatments of 20 wheat seedlings. 100 mL^{-1} distilled water, while the highest increase in white mustard plant was observed in 40 sunflower seedlings. 100 mL^{-1} distilled water (Figs. 4(A), 4(B)). In consistent with our findings, allelochemicals in sunflower extracts significantly increased the proline amount of the wheat varieties such as Margalla 99 and Chakwall 97 [37]. In addition to it's osmoregulator role, proline can act as osmo-preservatives like other soluble organic compounds [38]. It has been thought that the increase in proline amount in our results is due to the biotic stress caused by allelochemicals.

The sunflower root exudates reduced the protein levels of both wild mustard and white mustard, while wheat root exudates increased the amount of protein of wild mustard and decreased the amount of protein of white mustard. Gromstor herbicide caused protein increase in both species (Figs. 5(A), 5(B)). Plants also have special proteins that provide resistance or tolerance to stress conditions as well as normal cell proteins such as enzyme inhibitors, phenol biosynthesis enzymes, hydrolases, molecular chaperones and structural proteins [39]. While some of the structural proteins can be destroyed under the conditions of biotic and abiotic stress, special stress proteins can be synthesized. Guerrero et al. [40], and Schmitz et al. [41] suggested that the protein synthesis in developing seeds is promoted by ABA, while Zhang et al. [42] stated that protein phosphorylation increased with the increase of ABA concentration. Bartels et al. [43] and Ingram et al. [44] emphasized the increase in LEA proteins which involved in the prevention of cellular damage during dehydration. In this study, it is thought that the amount of protein decrease was probably caused by structural protein degradation, while protein increase may be caused by the production of stress proteins.

The SOD enzyme activity in wild mustard increased after the treatment of 40 sunflower seedlings root exudate while it was decreased after other treatments (Fig. 6(A)). In white mustard, activity increases were determined in all treatments except commercial herbicide treatment (Fig. 6(B)). It is a known fact that

antioxidant enzyme production is increased in order to eliminate the reactive oxygen species caused by biotic and abiotic stress in plants [45-47]. Allelochemicals which cause an increase in enzyme activity at low concentrations, can negatively affect enxyme activity at high concentrations. In the studies, it was found that superoxide dismutase and peroxidase activity of many plants were affected by allelochemicals; the degree of damage caused by allelochemicals was negatively correlated with the increase of SOD and PO activities [48,49]. Increased SOD and PO activities under allelochemical stress were found by Ding et al. [50], Kamal et al. [37] and Wang et al. [51] too.

Narwal [52] stated that the reactions against to allelochemicals vary depending on the concentration; allelochemicals that inhibit the growth of certain species at certain concentrations show the same or different types of stimulating effect at different concentrations. If allelopathic interaction is to be used in weed management, it is important to know the concentration at which the specific response occurs. Furthermore, various plant parts may differ in terms of their alellopathic potential [53,54].

In this study, it was revealed that the sunflower and wheat root exudates had allelopathic effects on wild mustard and white mustard weeds; effect level was close to the commercial herbicide (the commercial herbicide was more effective in the amount of photosynthetic pigments in wild mustard and the amount of protein and SOD enzyme activity in white mustard compared to root exudates, in others vice versa) and weeds developed different tolerance mechanisms against biotic stresses caused by these plants. In addition, it can be said that wheat exudates have an effect on photosynthetic pigment system and sunflower exudates have more effect on antioxidant defense system. The root exudates have potential for use as bioherbicides according to the results of measurements and analyzes. While there are many studies which analyzed the allelopathic effects of sunflower and wheat root exudates on weeds based on morphological measurements, the study of physiological and biochemical parameters is a new area of study. Further studies are needed in order to understad the effects of allelopathic plants on target plants. We think that our findings will be the main source of information for future studies. We also think that the studies using different concentrations, different plant species, plant parts, purified active substances will reveal more about allelopathic interactions.

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