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*Original Research*

# The Effect of Haloxyfop-Ethoxyethyl on Antioxidant Enzyme Activities and Growth of Wheat Leaves (*Triticum vulgare* L.)

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## Abstract

Haloxyfop-ethoxyethyl (HE) is a herbicide active against most grasses, whereas dicotyledonous plants are rather resistant to it. The effects of HE on the growth of wheat seedling leaves (cv. Almari) as well as the activity of several enzymes involved in the plant defense system against stress such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POX), ascorbate peroxidase (APX), were studied.

The growth of wheat leaves was inhibited by 50% 12 h after the HE treatment. The changes observed in the enzyme activities suggest that the herbicidal effect on enzyme activity depends on the distance between the place where the HE was applied and the section of the leaf analyzed as well as on the duration of the plant with the HE. The changes in SOD, POX, and CAT activities observed in the leaves of the wheat treated with the compound might mean that HE induces oxidative stress in the species studied. These results may confirm the hypothesis related to the mechanism of graminicides action.

**Keywords:** haloxyfop-ethoxyethyl, wheat, superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase

## Introduction

Haloxyfop-ethoxyethyl (HE) is a selective post-emergence herbicide which is active against many annual and perennial grasses in broad-leaf crops [1, 2]. The herbicide is a competitive inhibitor of the acyl-coenzyme A carboxylase (ACCase) enzyme in susceptible grass plants [3], thereby inhibiting the biosynthesis of fatty acid necessary for membrane growth and function [4]. In spite of many hypotheses formed since haloxyfop was introduced to agricultural use, there are still many open questions on how graminicides kill plants [5].

Oxidative stress is known to play an important role in a plant's rapid death, which is connected with the generation of different reactive oxygen species (ROS) in response to various biotic and abiotic signals [6]. Banaś et al. [7, 8] have indicated that the mode of haloxyfop action is related to overproduction of free radicals.

Reactive oxygen species (ROS) are produced in plants as part of normal metabolism and in response to stress conditions, such as drought [9], chilling and herbicide action [4, 5]. Reactive oxygen species such as superoxide $O_2^{\cdot-}$, singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($OH^-$) are highly reactive and can cause DNA mutation, protein denaturation, chlorophyll bleaching and lipid peroxidation, leading to the loss of membrane integrity and to cell death [10]. Several functionally interrelated antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POX), and catalase (CAT) [11], are involved in ROS metabolism.

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Superoxidase dismutase (SOD) catalyses the disproportionation of $O^{\cdot-}_2$ to $H_2O_2$ and $O_2$, and plays a major role in the defense against superoxide-derived oxidative stress in the plant cells. In addition, detoxification of cellular $H_2O_2$ through the action of the ascorbate-glutathione scavenging cycle has been found in chloroplasts, and the oxidation and re-reduction of ascorbate and glutathione by the APX and glutathione reductase (GR) takes place in cytosol. While APX catalyses the reaction of ascorbate with $H_2O_2$, the GR catalyses the regeneration of ascorbate [12]. Catalase is not shown as it is mainly a housekeeping enzyme and it is typically inactivated by the levels of peroxide, which occur during acute oxidative stress [13].

From our previous study it is evident that haloxyfop affects the activity of antioxidant enzymes in wheat and maize roots. Mioduszewska et al. [14] and Wiloch et al. [15] observed an increased peroxidase activity in the roots of wheat and maize, whereas Kielak et al. [16] noted an increased catalase activity in the roots of wheat following treatment with HE.

The aim of the present study was to investigate the effect of haloxyfop-ethoxyethyl on the growth of wheat leaves and to examine the activity of main antioxidant enzymes in the leaves of wheat cv. Almari.

## Material and Methods

### Plant Growth and Haloxyfop-Ethyl Treatment

The seeds of wheat cv. Almari were germinated on moistened filter papers (in darkness at 25ºC) for 3 days. The uniform seedlings thus obtained were transferred to a complete nutrient solution [17] to be grown in a cabinet under 16h light/ 8h dark regime, with a moderate light intensity ($60W \cdot m^{-2}$) and day/night temperature of 24/20ºC.

The HE was applied on the leaves when the wheat seedlings were about 17 cm high and 7 days old. The HE, as a 10 µl drop of 50 µM solution in 52% ethanol, was placed on the upper part of the first leaf. The control plants were treated with 10 µl of a 52% ethanol solution.

In order to examine the effect of HE on leaf growth, the leaf section where the herbicide had been applied was cut off at the upper end of the coleoptiles sheath and the plant was placed in a growth chamber in the conditions described in Fig. 1. Leave growth was measured 12, 24 and 48 hours later. The cut-off leaf was further divided into a 1cm section above the treatment spot and a 1cm section with the treated spot. The remaining part of the leaf was divided in half (section 3 and section 4), and tissues of all sections were homogenized and analyzed separately.

Table 1. The influence of haloxyfop-ethoxyethyl on growth of wheat leaves, cv. Almari.

| Time after treatment (hours) | Analyzed sample | Increase of leave length (mm) | Percentage in comparison to control |
|---|---|---|---|
| 12 | Control | 13.2 ± 1.6 | - |
| | HE | 7.1 ± 1.6$^e$ | 54 |
| 24 | Control | 22.4 ± 2.1 | - |
| | HE | 15.1 ± 4.1$^e$ | 67 |
| 48 | Control | 50.0 ± 4.6 | - |
| | HE | 23.7 ± 10.0$^e$ | 47 |

(±) standard deviation, S.D.
($^e$) significantly differs from control $p \leq 0.001$
HE- haloxyfop-ethoxyethyl

### Preparation of the Enzyme Extract

The spots where the leaf had been treated with HE and alcohol were carefully washed with 80% ethanol before the enzymes were extracted. In order to determine the activity of the all enzymes, the wheat leaves were homogenized in a 50 mM sodium phosphate buffer (pH 7.0). The homogenates thus obtained were centrifuged at 10,000 g for 10 minutes at 4ºC. The supernatants were stored at -84ºC.

### Enzymes Assay

The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolinum (NTB ), using the Beauchamp and Fridovich method [18]. The 3 ml of the reaction mixture contained a 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 70 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 50 to 100 µl of the plant extract. Riboflavin was added as the final ingredient and the vials with the reaction mixture were placed under two 15 W fluorescent lamps. The reaction was started by switching on the light for 10 min. The reaction was stopped by switching off the light and the vials were covered with black cloth. The reaction mixture without illumination served as a control mixture. Absorbance at 560 nm was measured. The volume of the enzyme extract corresponding to 50% inhibition of the reaction was considered enzyme unit.

Catalase (CAT) activity was determined by monitoring the decomposition of $H_2O_2$ at 240 nm following the method of Chance and Maehly [19]. The reaction mixture contained a 50 mM sodium phosphate buffer (pH 7.0), 15 mM $H_2O_2$ and 50 µl plant extract in a 3 ml volume. One unit of CAT is defined as the oxidation of 1 µmol $H_2O_2$ per minute.

Guaiacol peroxidase activity (POX) was measured spectrophotometrically with guaiacol as a substrate [14]. The reaction mixture contained 0.5 ml 50 mM acetate buffer (pH 5.6), 0.5ml 6 mM $H_2O_2$, 0.5ml 2 mM guaiacol and 0.5ml enzyme extract. The increase in the absorbance at 480 nm was monitored for 4 min at 30ºC. It was assumed that the increase in absorbance of 1.0 in 1 min. incubation was one activity unit.

Ascorbate peroxidase (APX) activity was measured at 430 nm following the method of Nakano and Asada [20]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM pirogallol , 0.1 mM $H_2O_2$ and the suitable volume of enzyme extract. One unit of APX activity was defined as the oxidation 1 μmol of pirogallol per minute.

All reagents used in analyses were purchased from Sigma (Poland), except the pure standard of haloxyfop--ethyl (HE) obtained from Dow Chemical Co., UK.

### Protein Analysis

The protein concentration was determined spectrophotometrically at 595 nm using the Bio-Rad Protein Assay Dye Reagent Concentrate in a method based on Bradford [21] using bovine albumine serum as a standard.

### Statistical Analysis

The data presented in the table and figure attached are arithmetical means and standard deviations (S.D.) from three independent experiments (three independent replications with 6 plants in each replication). The statistical significance for all tests was determined at $p \leq 0.05$ ($^a$), $p \leq 0.04$ ($^b$), $p \leq 0.03$ ($^c$), $p \leq 0.02$ ($^d$) and $p \leq 0.01$ ($^e$) according to t-test.

## Results and Discussion

The growth of the wheat seedling leaves was inhibited by haloxyfop-ethoxyethyl (HE) placed in the form of a drop as a 50 μM solution (Table 1). The decrease ranged from 33 to 53%. The effect of the herbicide on the growth of the wheat leaves was similar 48 and 12 h after the treatment. The experiment results suggest that HE has inhibited the growth of the wheat leaves and probably stimulated of their senescence. A similar effect was observed earlier by Banaś et al. [22]. The same authors also noted that leaf growth was inhibited 12 hrs after the HE application and continued up to 48 hours.

The growth inhibition of the wheat leaves may be caused by the ethylene induced by HE, similar to other graminicides. The induction of ethylene by HE has a rapid and significant effect which appears to be related to the lethal mechanism of aryloxyphenoxypropionate (AOPP) herbicides in susceptible plants [7]. Ethylene is one of the most common products which cause cell death similarly to other stress factors which induce the production of ROS. It is quite likely that ethylene is not a phytotoxic agent which causes cell death but, on the other hand, it is a collateral product whose role in the lethal process is still uncertain. Ethylene may serve as a signal whose transduction may be required in the activation of various genetically programmed processes that lead to rapid cell death [23].

HE inhibited the growth of the leaves probably by stimulating the production of the reactive oxygen species (ROS), which is characteristic of the plant metabolism under stress conditions, such as drought [9], chilling and herbicide treatment [4, 5]. Removing ROS from the plant tissue is dependent on several functionally interrelated antioxidant enzymes such as superoxide dismutase, glutathione reductase, ascorbate peroxidase, peroxidase and catalase [12].

Our results show that HE affected the SOD, POX, CAT and APX activity in the leaves of wheat. It has been observed 48 h after treatment that the SOD activity increased in the fourth part (the youngest part of the leaves) and decreased in the first part (the oldest). The decline of SOD was noted in the third part of the wheat leaves already 12 h after HE treatment (as presented in Fig. 1). SOD is an important enzyme in protecting the cell from oxidative damage. The increase in total SOD activity in the leaves has been attributed to enhanced $O_2^{-\cdot}$ production [12]. Therefore, total SOD activity in the leaves of wheat subjected to HE may induce the overproduction of $H_2O_2$ necessary for $O_2^{-\cdot}$ to be destroyed. The results obtained may suggest that the increase in total SOD activity results in a higher tolerance of oxidative stress.

APX has been found to play an essential protective role in the scavenging process when coordinated with SOD activity [24]. It is the main enzyme responsible for the detoxification mechanism of cellular $H_2O_2$ which is a part of the ascorbate-glutathione scavenging cycle. APX catalyses the reduction of $H_2O_2$ to water and has high specificity and affinity to ascorbate as a reductant [25]. Our results indicate that HE stress caused enhancement of total APX activities. A significant increase in APX activity was observed 12 h after the HE treatment (part 1, 2 and 3). Two days (48 h) after treatment, the enzyme activity increased in all the analyzed parts of the wheat leaves (Fig. 1).

When paraquat was applied in *Conyza bonariensis*, the SOD and APX activity increased as early as 6 h after treatment [13], whereas in the case of wild wheat [12] the same effect was observed 24 h after treatment. The effect of HE on enzyme activity depended on the leaf part analyzed and the time after treatment. The changes observed in the activity of the antioxidant enzymes suggest that herbicidal activity of HE depends on the time following its application and the speed of translocation in the leaf.

Banaś et al. [26] has shown that the HE is transported both upwards and downwards to all the parts of the plant. The speed of the herbicide translocation decreased in a function of the distance from the place of treatment.

Catalase is an enzyme responsible for the levels of peroxide occurring during acute oxidative stress [13]. It was noted that in the case of CAT its activity increased in the third part (below the spot treated with the HE) on 12 and 24 h after treatment. The same was also observed in the second (the place of the HE treatment) and the fourth part (the youngest), but only after 48 h (Fig. 1). The increase in catalase activity in the wheat leaves as a result of HE treatment is the plant's reaction to the synthesis of hydrogen peroxide and its further conversion into water and molecular oxygen.

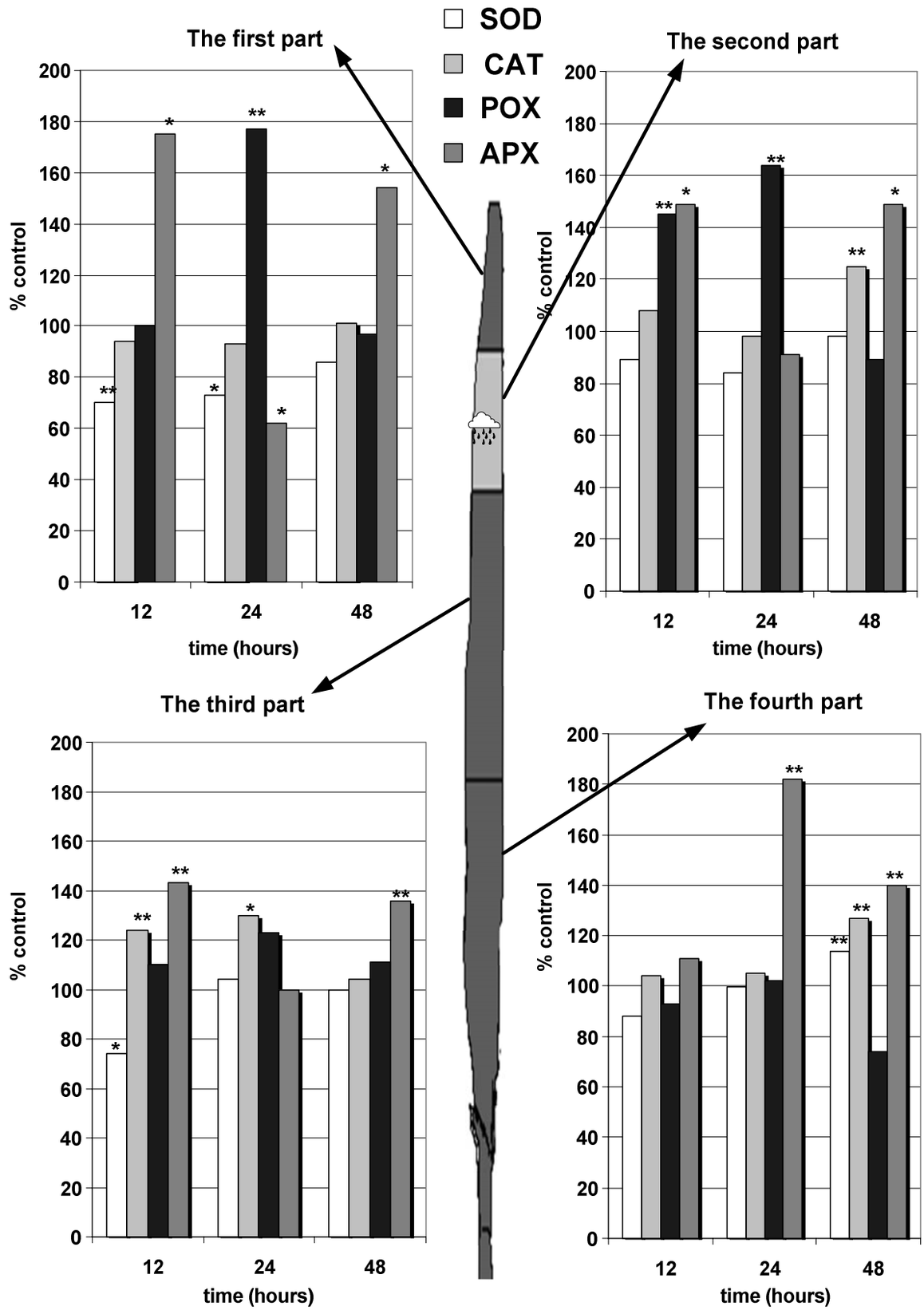


Fig. 1. Influence of HE on superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) activities in different parts (1-4) of wheat leaves.

- the place of applied of HE. * mean significantly differs from control, $p \leq 0.005$, ** mean significantly differs from control, $p \leq 0.001$

Peroxidases are known to utilize different substrates to metabolize $H_2O_2$. POD activity reflects changes in the mechanical properties of the cell wall and membrane integrity in plant leaves under stress conditions [13].

In the case of guaiacol used as a peroxidase substrate, an increase in the enzyme activity was noted in the first part (the oldest) 24 h after HE treatment, while in the second part (place of the HE treatment) after 12 and 24 h (Fig. 1). The increase in the POX activity in wheat leaves as a result of the HE treatment is probably due to the peroxidation of the membrane lipids, as observed earlier by De Prado et al. [23]. Many authors claim that haloxyfop effectively depolarize membrane potential [23, 27, 28]. The HE effectively depolarizes membrane potentials and causes rapid inhibition of growth and other physiological and biochemical effects described above in susceptible plans [7], including a substantial increase in ethylene production.

However, the changes observed in the activity of basic antioxidant enzymes in wheat leaves treated with haloxyfop-ethoxyethyl indicate that the HE induces oxidative stress. As compared to catalase, the induction of the APX activity may have an even more dramatic effect on the protection of plants against stress induced by the HE. In reaction to stress plants generate hydrogen peroxide, which is accumulated in intercellular space. It can be transferred into the cytosol in which the cytosolic APX is present, and then it is further transported to peroxisome, where catalase is a typical enzyme. The cytosolic APX has a higher affinity to $H_2O_2$ than the catalase does [29]. The scavenging of $H_2O_2$ by APX is the first step in the ascorbate-glutathione cycle [20, 30]. Our results suggest that the APX in wheat leaves may be a key enzyme in the decomposition of hydrogen peroxide under HE treatment.

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