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## Butachlor Tolerance in Wheat Seedlings

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### Abstract

Butachlor, though reported to adversely affect growth by interfering with amino acid and protein synthesis, is still recommended for weed control in wheat. The present study reports for the first time the butachlor dose-dependent activation of phenol metabolism in wheat. Application of herbicide leads to stress in plant and it is well known fact that plants growing under stressful condition biosynthesize more phenolic compounds in comparison to plants growing under normal conditions. All the butachlor treatments stimulated the mean guaiacol peroxidase (GPX) activity while all but one (double the recommended field dose, 2RFD) of the treatments enhanced phenylalanine ammonia lyase (PAL) activity. The mean phenol content, which is dependent on relative activities of PAL and GPX, increased significantly at the lowest application rate (half the recommended field dose, 0.5RFD) of butachlor. The mean PAL activity and mean phenol content increased significantly on 20 and 30 days after application while mean GPX activity declined progressively throughout the experimental period. Butachlor-induced PAL activity showed hyperbolic dependence on its substrate, phenylalanine concentration that conformed Michaelis-Menten kinetics. Measurement of kinetic parameters of PAL indicated that the maximum velocity ( $V_{max}$ ) remains unchanged ( $11.1 \mu\text{mol } t\text{-cinnamic acid produced h}^{-1} \text{ g}^{-1}$ ) while Michaelis constant ( $K_m$ ) varied between 2.24 and 5.34  $\mu\text{M}$  and increased with increasing butachlor treatments. Butachlor at its lower application rate with its role as a positive modulator of PAL enhances phenol accumulation, which forms a part of butachlor tolerance in wheat.

**Keywords:** Butachlor, herbicide, PAL enzyme, total phenol, wheat

### 1. Introduction

Butachlor (N-[butoxymethyl]-2-chloro-2,6'-diethylacetanilide), a chloroacetanilide herbicide, provides excellent control of many annual grasses and broad-leaf weeds in several major crops including wheat, corn, and soybean (Dwivedi et al., 2012). Chloroacetanilide herbicides in addition to their effects on physiological processes such as seed germination, cell division, spindle inhibition, membrane permeability (Scarponi et al., 1991; Felisbino et al., 2018) are known to interfere with several biochemical processes such as the synthesis of lipids (Boger et al., 2000), chlorophyll (Scarponi et al., 1992), carbohydrate (Scarponi et al., 2002), amino acids and protein (Nemat Alla et al., 2008). The application of herbicides is also affecting the secondary metabolism either by the accumulation of constituent acids or the formation of unwanted compounds (Rockenbach et al., 2018). Herbicides induces formation of reactive oxygen species (ROS) that eventually leads to oxidative stress (Hassan and Nemat Alla, 2005).

Tolerance of plants that determines herbicide selectivity between crop and weed, however, is related to differential

rates and routes of metabolism of herbicides via a three-phase reaction involving conversion (phase-I), conjugation (phase-II), and deposition (phase-III) that can detoxify these herbicides at sufficient rates to prevent their accumulation and persistence at phytotoxic levels. In addition, plants have evolved well-defined antioxidant machinery involving enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), glutathione reductase (GR), etc. and non-enzymatic like vitamin-C, vitamin-E, etc. that can overcome the herbicide-induced oxidative stress (Yuan et al., 2017; Wang et al., 2018; Jiang et al., 2016). Alachlor and metalochlor at lower concentrations are reported to stimulate activities of SOD and CAT in lettuce, and pea leaves (Stazner et al., 2003). Further, Burns et al., 2017 reported that in *Avena fatua* L. glutathione-related proteins are essential for herbicide resistance. Wang et al., 2018 have noticed a severe negative impact of nicosulfuron herbicide in the ascorbate-glutathione cycle in *Zea mays* L. Moreover, modulation of phenol content by the herbicide treatment is dependent on either PAL activity or relative activities (Garcia et al., 2001) of PAL and oxidative enzymes such as polyphenol oxidase (PPO) and peroxidases (GPX). Phenolic compounds provide higher tolerance to plants



against various stress conditions like pesticides, drought, heavy metals, salinity, and temperature (Naikoo et al., 2019, Handa et al., 2019, Sharma et al., 2016, Wang et al., 2019, Shahzad et al., 2018). These compounds have antioxidative properties and are capable of scavenging free radicals, hence protecting plant cells from the ill effects of oxidative stress (Noctor et al., 2018; Tanase et al., 2019). Salicylic acid is a phenolic compound playing a key role in regulating the enzymatic antioxidant system and thus controlling ROS production in plants under biotic or abiotic stresses (Fatma et al., 2018). Furthermore, Jasmonic acid a plant hormone, triggers the biosynthesis of ascorbate, glutathione, tocopherol, and phenolics, under stress conditions and plays an important role in scavenging ROS (Malekpoor et al., 2016; Mendoza et al., 2018). Thus, it seems reasonable to assume that plant defense against herbicide-induced oxidative stress in part is related to phenol metabolism which on the other hand may benefit human health with its antioxidant potential when the product with high phenol content forms a part of our diet. Furthermore, the phenol metabolism varies depending on plant species, type, and concentration of herbicides. However, phenol metabolism in relation to the activities of PAL and GPX with consequent phenol accumulation in response to a varying application rate of butachlor is hardly understood in wheat. We, therefore, extended our study to include the measurement of kinetic properties of PAL such as Michaelis constant ( $K_m$ ) and maximum velocity ( $V_{max}$ ) attainable and their modulation by different application rates of butachlor.

## 2. Materials and Methods

Grains of wheat (*Triticum aestivum* L., PBW-343) after soaking with water for 8 hours were raised in sandy-clay soil in earthen pots (30 cm height×30 cm diameter) in the University greenhouse at Mohanpur, Nadia, West Bengal, India during 2016. 10-days-old seedlings were treated with five treatment of butachlor at 0, 0.5, 1.0, 1.5, 2.0 fold of recommended field dose (RFD, 1.0 kg ai ha<sup>-1</sup>). The pot culture experiment was conducted in a completely randomized design with three replications of each treatment. Wheat leaves were collected before treatment of these herbicides and subsequently at 3, 6, 10, 20, and 30 days after treatment (DAT) from each treatment replication. A portion of leaf samples was immediately used for enzyme extraction and assay while another portion after drying at 40°C was ground and stored in a desiccator for phenol analysis.

### 2.1. Analysis of enzyme activity

All extraction steps were carried out at 4°C. PAL (EC 4.3.1.5) and GPX (EC 1.11.1.7) were extracted in 100 mM phosphate buffer (pH 7.5) containing 2% PVP and 0.25% Triton-X according to the method described by (Garcia et al., 2001). The PAL activity was examined spectrophotometrically by the formation of *t*-cinnamic acid at 270 nm in 100 mM Tris-HCl buffer (pH 8.8) and 10  $\mu$ M phenylalanine. PAL activity was

expressed as  $\mu$ mol *trans*-cinnamic acid formed h<sup>-1</sup>g<sup>-1</sup> of the leaf. GPX activity was assayed in 100 mM phosphate buffer (pH 7.5), 30 mM H<sub>2</sub>O<sub>2</sub>, and 2 mM guaiacol. The oxidation of guaiacol was monitored at 470 nm. The activity was calculated using the extinction coefficient of 26.6 mM<sup>-1</sup>cm<sup>-1</sup> and expressed as  $\mu$ mol of guaiacol oxidized min<sup>-1</sup>g<sup>-1</sup> of leaf sample.

### 2.2. Analysis of total phenol and antioxidant activity

The total phenol was extracted after boiling at 90°C for 2 hours with 1.2 M HCl in 50% aqueous methanol (Vinson et al., 1998) and determined using Folin-Ciocalteu Reagent (FCR). The phenol content was expressed as  $\mu$ mol gallic acid equivalent per gram fresh weight ( $\mu$ mol GAEg<sup>-1</sup> FW).

The antioxidant activity of the phenol extract was determined according to the earlier reported protocol (Thaipong et al., 2006). The antioxidant activity is expressed as  $\mu$ mol gallic acid equivalent per gram fresh weight ( $\mu$ mol GAEg<sup>-1</sup> FW).

### 2.3. Determination of kinetic parameters

Enzyme extracts that were used for the determination of  $K_m$  and  $V_{max}$ , were obtained from leaves of butachlor treated and untreated seedlings after 30 days following treatments. PAL activity was measured at varying substrate concentrations ( $[S]$ ) ranging between 2 and 10  $\mu$ M of phenylalanine in the reaction mixture. Michaelis-Menten plot was prepared by plotting velocity ( $v$ ) against  $[S]$ . The Michaelis-Menten equation was transformed algebraically by taking the reciprocal of both sides of the equation to form Lineweaver- Burk equation. The kinetic properties of PAL such as  $K_m$  and  $V_{max}$  were calculated from Lineweaver- Burk plot, which was made by plotting  $1/v$  against  $1/[S]$ .

## 3. Results and Discussion

### 3.1. Effect of butachlor on phenol content and antioxidant activity in wheat

The phenol content in different doses of butachlor treated and untreated leaves on different sampling days are presented in Table 1. The results indicated that the mean phenol content over sampling days was significantly higher over control in wheat leaf treated with 0.5 RFD of butachlor, while other treatments did not produce any significant differences in mean phenol content, but declined below control. While comparing means of days over treatments, showed a significant increase in phenol content throughout the experimental period beyond 10 DAT. Similar to phenol content, there were no significant differences in mean antioxidant activity across different treatments except 0.5 RFD, and the mean phenol over treatments was significantly increased beyond 10 DAT, before that there were no significant differences (Table 2). The interaction between treatment and sampling days was non-significant for both phenol content and antioxidant activity. Phenol, a ubiquitous plant secondary compound, accumulates in varying amounts



Table 1: Leaf Phenol content ( $\mu\text{mol GAE g}^{-1}$  FW) in wheat at different butachlor treatments

Treatment	Days after treatment					Mean
	3	6	10	20	30	
0 RFD	18.16	17.52	17.36	22.93	26.15	20.42
0.5RFD	20.77	21.30	22.51	27.11	27.84	23.90
1 RFD	20.03	19.79	20.34	22.39	25.61	21.63
1.5 RFD	19.37	19.01	19.27	22.34	25.37	21.07
2 RFD	18.93	18.68	18.88	22.05	25.30	20.76
Mean		19.41	19.26	19.67	23.36	26.05
		T	S	T×S		
	SEm±	0.651	0.651	1.455		
	CD ( $p=0.05$ )	1.854	1.854			

Table 2: Antioxidant activity ( $\mu\text{mol GAE g}^{-1}$  FW) of total phenol extract of wheat leaves

Treatment	Days after treatment					Mean
	3	6	10	20	30	
0 RFD	30.45	29.85	31.72	33.29	35.49	32.16
0.5RFD	32.78	33.58	35.02	37.11	37.84	35.27
1 RFD	31.74	30.06	32.64	34.08	35.98	32.90
1.5 RFD	30.68	29.94	30.18	33.62	35.12	31.91
2 RFD	28.74	28.10	29.68	32.88	34.72	30.82
Mean		30.88	30.31	31.85	34.20	35.83
		T	S	T×S		
	SEm±	0.319	0.319	0.713		
	CD ( $p=0.05$ )	1.21	1.21			

depending on plant species, type, and concentration of herbicides. The present study documented significant differences in mean leaf phenol content at 0.5 RFD over control, while means of sampling days were significantly different on 20 and 30 days after treatment. Thus, butachlor stimulated phenol accumulation in wheat, which is more pronounced at a lower dose (0.5 RFD). Similar enhanced phenol content is also reported with alachlor in maize and soybean (Nemat Alla and Younis, 1995), in rice with insecticide Diazion (Mahdavi et al., 2015), in mustard with imidacloprid (Sharma et al, 2018 and Sharma et al, 2019), in sorghum (Molin et al., 1986) glyphosate in oat seedling (Falco et al., 1989), and aciflourfen in spinach (Komives and Casida, 1982). Pistachio plants treated with pesticides imidacloprid and phosalone displayed elevated levels of total phenols and total proteins coupled with significant increases in proline and total soluble carbohydrate contents in treated plants in comparison to untreated plants during 14 days after treatment (Homayoonzadeh et al., 2021). Sharma et al., 2018 also reported that the application of thiacloprid at their RFD and even at double the RFD triggers greater

phenol accumulation whereas deltamethrin is more effective in producing good quality phenol. Moreover, stimulation of phenol accumulation at a lower dose is also observed with Derby, a mixture of florasulan and flumetsulan in wheat (El-Rokiek Kowthar et al., 2012) and fungicide carbendazim in tobacco (Garcia et al., 2001). Butachlor thus forms a representative member in the growing list of herbicides that can positively modulate the phenol accumulation in plants. Phenols, in addition to their diverse physiological and ecological role, are also involved in resistance to different types of stress including herbicides (Nemat Alla and Younis, 1995). The biological function of phenols results from their participation in redox reactions (Narwal et al., 2014). Available literature indicates that herbicides impose enhanced formation of ROS (Jiang and Yang, 2009) and phenols can detoxify these species either via non-enzymatic reaction or being a substrate of peroxidase reaction, which in turn can provide precursors of lignin synthesis. In a study, (Schmidt et al., 1986) showed that tolerance of different weed species to aciflourfen is related to the ratio of vitamin-C and vitamin-E. The regulatory role of phenol on seedling growth induced

by alachlor in maize and soybean (Nemat Alla and Younis, 1995) is also reported. Butachlor, though retard growth by inhibiting amino acids and protein formation, nevertheless, triggers enhanced phenol accumulation particularly with lower doses at the seeding stage. Thus, butachlor-dose dependent phenol accumulation forms a part of the defense mechanism, which eventually overcomes herbicide-induced oxidative stress.

### 3.2. Effect of butachlor on PAL and GPX activity

The results relating to PAL and GPX activity in leaves on different sampling days in response to different butachlor treatments are summarized in Table 3 and Table 4 respectively. The mean PAL activity (Table 3) oversampling days increased significantly over control with all the treatments but without any differences between control and 2 RFD. Moreover, the mean PAL activity increased with decreasing rates of butachlor application. On the other hand, mean PAL activity of sampling days over treatments though declined progressively till 10<sup>th</sup> day but increased significantly on 20 and 30 days after treatment with greater stimulation on 30<sup>th</sup> day. However, there were no significant differences

in PAL activity while comparing the interaction between treatment and sampling days.

The mean GPX activity (Table 4), in contrast to PAL activity, increased significantly over control with increasing rates of butachlor treatment, while the means of sampling days decreased significantly. The interaction between treatment and sampling day, though observed non-significant, but in all the treatments but 0.5RFD, GPX activity decreased progressively with time. In the present study, significant increases in mean PAL activity with decreasing rates of butachlor application and with the advancement of growth stage are noteworthy. Of interest in this connection, PAL activity in soybean seedlings increased by several herbicides such as DPX-4189 (Suttle and Schreiner, 1982), acifluorfen (Hoagland, 1989), alachlor (Scarponi et al., 1991), (Nemat Alla and Younis 1995) and metalochlor (Scarponi et al., 1992), in mustard with imidacloprid stress (Sharma et al., 2019), in cucumber with spirotetramat application (Homayoonzadeh et al., 2021). Pistachio plants treated with pesticides imidacloprid and phosalone showed greater specific activities of superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase, phenylalanine ammonia-

Table 3: PAL activity ( $\mu\text{mol t-cinnamic acid produced h}^{-1} \text{g}^{-1}$ ) in wheat at different butachlor

Treatment	Days after treatment					Mean
	3	6	10	20	30	
0 RFD	3.98	3.84	3.74	4.52	5.12	4.24
0.5RFD	4.58	4.78	5.02	6.11	6.78	5.45
1 RFD	4.38	4.16	4.04	5.74	6.32	4.93
1.5 RFD	4.16	4.08	3.88	5.32	5.84	4.66
2 RFD	4.07	3.96	3.90	4.78	5.36	4.41
Mean		4.23	4.16	4.12	5.39	5.88
		T	S	T×S		
	SEm±	0.086	0.086	0.193		
	CD ( $p=0.05$ )	0.246	0.246			

Table 4: GPX activity ( $\mu\text{mol guaiacol oxidized g}^{-1} \text{min}^{-1}$ ) in wheat at different butachlor treatments

Treatment	Days after treatment					Mean
	3	6	10	20	30	
0 RFD	12.72	10.99	10.48	9.64	8.58	10.48
0.5RFD	13.38	13.97	11.38	10.85	9.14	11.74
1 RFD	15.62	14.47	13.04	11.76	9.78	12.93
1.5 RFD	17.78	16.64	14.42	12.42	10.48	14.35
2 RFD	18.98	17.58	15.84	13.21	10.96	15.31
Mean		15.70	14.73	13.03	11.58	9.79
		T	S	T×S		
	SEm±	0.341	0.341	0.763		
	CD ( $p=0.05$ )	0.972	0.972			



lyase, glutathione reductase, and glutathione S-transferase compared with untreated plants during 14 days after treatment, whereas a significant decrease in polyphenol oxidase activity (Homayoonzadeh et al., 2021). Therefore, the changes in PAL activity in the present investigation could be considered a consequence of herbicide-induced stress that conforms with the report of Scarponi et al. (1992) and (Homayoonzadeh et al., 2021). In contrast to PAL activity, the mean GPX activity (Table 3), increased significantly over control in a dose-dependent manner. On the other hand, mean GPX activity, in contrast to PAL activity and phenol content, decreased progressively with time in all the treatments. Rajabi et al. (2012) also reported that herbicide metribuzin significantly increased the activities of guaiacol peroxidase, ascorbate peroxidase, and polyphenol oxidase. However, Islam et al. (2016) reported that the lower doses of butachlor enhanced the activity of GPX in cultivar ZJ 88 of rice, but under the higher concentration, its activity was down-regulated, whereas Alves et al. (2018) reported *Vicia sativa* showed an enhanced in the GPX activity when increased the herbicide concentration. Our results thus differed with the report of Islam et al. (2016), which probably explains the considerable heterogeneity between crop species. The PAL activity and phenol content in response to varying butachlor treatments showed a similar trend. Unlike phenol content, which declined below control at later stages of growth with higher doses of butachlor, PAL activity increased over control during that period. The inverse relation between PAL and GPX activity as observed by different treatments and sampling periods appears to suggest that phenol accumulation is not solely controlled by PAL activity. Thus, key enzymes of phenol synthesis (PAL) and phenol oxidation (GPX) appear to regulate phenol accumulation in wheat at a lower dose is similar to the observation of carbendazim in tobacco at 0.5RFD (Garcia et al., 2001). The growth reduction in wheat with butachlor treatment is reported to result from malfunction of nitrogen metabolism involving significant accumulation of ammonia and soluble-N accompanied by a diminution in total-N and protein content. Protein synthesis inhibition, though likely to be associated with diminution of enzyme concentrations, nevertheless, enhanced PAL activity with the corresponding increase in hydroxyl-phenolic acids is reported with alachlor in maize and soybean seedlings (Nemat Alla and Younis, 1995).

### 3.3. Effect of butachlor on kinetic properties of PAL

Figure 1 shows the PAL reaction rate as a function of its substrate phenylalanine concentration at different butachlor treatments. The results showed that PAL exhibits hyperbolic dependence of reaction rate on phenylalanine concentration conforming Michaelis-Menten kinetics. The Michaelis-Menten plot when transformed to Lineweaver-Burk plot (Figure 2), indicated that  $V_{max}$ , a descriptor of enzyme concentration ( $11.1 \mu\text{mol } t\text{-cinnamic acid produced h}^{-1}\text{g}^{-1}$ ) remain unchanged in response to varying butachlor treatments, while  $K_m$ , an

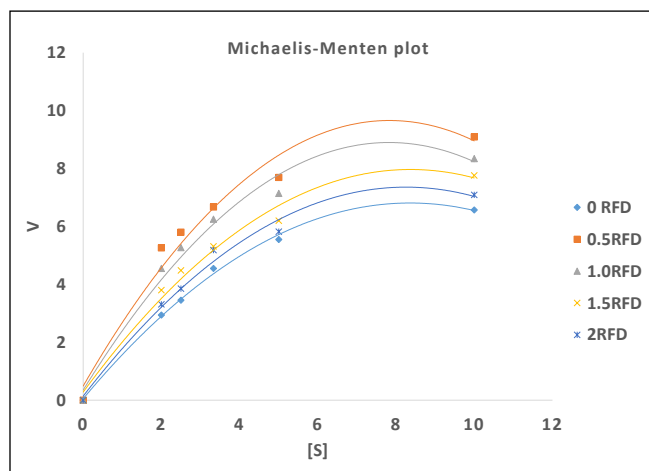


Figure 1: Michaelis-Menten plot

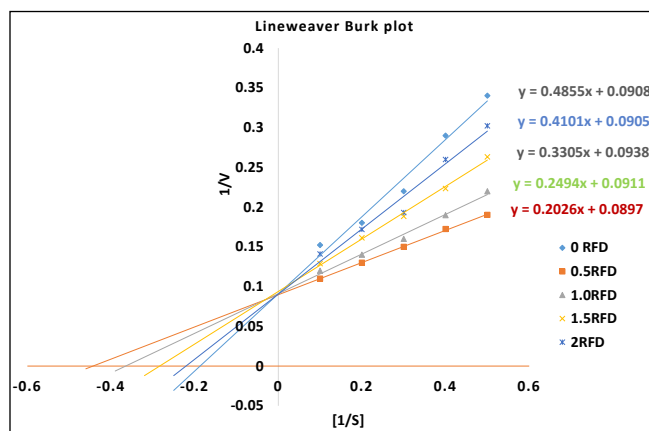


Figure 2: Lineweaver Burk plot

indicator of affinity of PAL towards phenylalanine, varied between 2.24 and 5.34  $\mu\text{M}$  and that increased with increasing rates of butachlor application. Activation of PAL by butachlor and that increases with decreasing herbicide concentration seem to result from an increase in PAL concentration, its isoenzyme distribution, or modification of its structural integrity. The results indicated that  $V_{max}$  ( $11.1 \mu\text{mol } t\text{-cinnamic acid produced h}^{-1}\text{g}^{-1}$ ) remains unchanged in response to varying butachlor treatments. Results of an earlier report suggested that the induction of PAL activity is dependent on the supply and availability of the substrate phenylalanine and PAL enzyme concentration. Therefore, butachlor is unlikely to modulate PAL activity as it is limited by PAL and phenylalanine concentration because of the inhibition of amino acid and protein synthesis in wheat by butachlor treatment (Nemat Alla et al., 2008). Nevertheless, activation of PAL supports the observation of (Nemat Alla and Younis, 1995).  $K_m$ , a descriptor of affinity of the enzyme towards its substrate, increased in the order 0.5 RFD < 1.0 RFD < 1.5 RFD < 2.0 RFD < 0 RFD, which indicated a striking increase in affinity of PAL towards its substrate phenylalanine with decreasing rates of butachlor application. In general, the  $K_m$  value of PAL for phenylalanine is observed to be 10  $\mu\text{M}$ . A lower  $K_m$

value obtained in the present study is related to the low phenylalanine concentration used in the assay. Thus, it may be stated that lower  $K_m$  at a lower butachlor application rate is associated with conformation modification of the active site of PAL, which subsequently enhanced PAL activity. Therefore, butachlor at lower application rate functions as a positive modulator of PAL, and consequently enhanced phenol content reverses the growth reduction caused by inhibition of protein synthesis.

#### 4. Conclusion

Butachlor at a lower application rate positively modulated the activity of PAL by decreasing the  $K_m$  value, and thereby stimulated phenol accumulation. Thus, it can be concluded that butachlor-induced activation of PAL enzyme with a simultaneous increase in phenol content formed a part of the defense mechanism of wheat to butachlor stress.

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