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POLYPHENOL OXIDASE ACTIVITIES IN WHEAT (*TRITICUM AESTIVUM* L.) GRAIN

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Abstract

Wheat (*Triticum* spp.) is one of the most popular cereals used in the world. An important problem for the flour and flour related products is their darkening and discoloration which is believed to result from polyphenol oxidase (PPO) activities. Pakistani wheat grain collection from NARC gene bank and some other cultivars were investigated for their PPO activities for the first time. Different PPO substrates were employed which included caffeic acid, catechol, tyrosine, L-DOPA and phenol. Among these substrates, L-DOPA at pH 6.5 produced the highest enzyme activity. Among the genotypes tested, Chalsi showed the highest (150 units/min/g) sum of activities PPO activity while the minimum activity was exhibited by Wafaq-2001 (34units/min/g). All the genotypes and the substrates tested, differed in a highly significant manner in variance analysis; and our results are also supported by correlation analysis. These results showed presence of genotypes both very high and very low in PPO activities and also supported the hypothesis that more than one PPO activities may be present in wheat grains.

Introduction

Grown all over the world wheat covers more of the earth's surface than any other cereal crop (Kayani *et al.,* 2010). Wheat is the major food for the people of Pakistan and is used in the form of chapatti, bread, porridge and many bakery items. In other East Asian nations it is mostly consumed in the form of noodles. On storage noodles and flour develop brown coloration while consumer preference requires noodles to have a bright and creamy white color (Kruger *et al.,* 1992). PPO is thought to be playing the foremost role in darkening of noodles, after which this product becomes undesirable to purchasers (Morris & Rose, 1996).

The durum wheat quality is also affected by polyphenol oxidase (PPO) activity (Watanabe *et al.,* 2006). The compounds of phenol are mainly present in bran tissues of wheat, which after milling react with endogenous PPO and cause change in color of wheat products (Beta *et al.,* 2005). Therefore, it has become one of the goals of many wheat-breeding programs to develop wheat varieties with reasonably low-PPO levels in mature grains (Ransom *et al.,* 2006). Browning of vegetables, fruits and other cereal products are also caused by polyphenol oxidases (Feillet *et al.,* 2000). Conversely, Plant PPOs are also considered protective enzymes against various stresses such as toxicity stress due to heavy metals (Lei *et al.,* 2011) and pests and pathogens stress (Rai *et al.,* 2011).

Plant PPOs are nuclear coded enzymes with spin coiled copper pair each having three histidine residues in the active site. PPOs are totally oxygen dependent and catalyze two types of reactions at a time. In first reaction they hydrolyze the monophenols (L-tyrosine and phenol) into *o*-diphenols and in second reaction they oxidize these *o*-diphenols (Catechol, Caffeic acid and L-DOPA) into *o*-quinones. Thus, PPO shows two types of activities namely monophenolase activity and diphenolase activity respectively (Gao *et al.,* 2009). These resulting quinines further react non-enzymatically with other phenolic compounds, amino acids and proteins, and result in formation of brown complexes called melanins (Anderson & Morris, 2001). Polyphenol oxidase exists in a latent form. The activation of PPO can be done by removal of an inhibiting component from the latent enzyme or it can be activated by a conformational change (Okot-Kotber *et al.,* 2002).

Polyphenol oxidase owes many names due to its ability to catalyze various substrates most important of which are L-DOPA, catechin, pyrocatechol, chlorogenic acid, L-tyrosine, methylcatechol, and epicatechin (Kihara *et al.,* 2005), out of all L-DOPA and catechol are the best substrates (Anderson & Morris 2001). PPO is reportedly resistant to heat up to 90^{o}C ((Soysal & Soylemez, 2004; Kayani *et al.,* 2011). The optimum pH range was found to be 4.5-8.2 for plant PPO's (Ziyan & Turk, 2004).

In immature kernels PPO proteins are more abundant, but PPO activity was greater in mature kernels (Anderson *et al.,* 2006). Main emphasis of this study was on evaluating PPO activities by enzymatic assays in grains of different Pakistani wheat genotypes and to group them on the basis of low, medium and high PPO activity, using different PPO substrates.

Materials and Methods

Grains of 101 different wheat genotypes were obtained from National Agriculture Research Center (NARC) and Department of Plant Breeding and Genetics (PMAS Arid Agriculture University Rawalpindi) and their polyphenol oxidase activity was measured using five phenolic substrates includingare 3, 4-dihydroxycinnamic acid (caffeic acid) 3, 4-dihydroxybenzene (catechol), L-tyrosine L-DOPA and phenol.

Measurement of PPO activity

Polyphenol oxidase activity assay with tyrosine: The tyrosine PPO activity was observed according to the method of Anderson & Morris (2001). Initially, 3 grains

of a genotype were placed in test tubes containing 1.5 mL of 10 m*M* phenolic substrate (Tyrosine) made up in 50 mM Tris [tris (hydroxyethyl) aminomethane]. Because of solubility constraints, tyrosine assays were conducted at pH 8.5. Then tubes were incubated at 20°C with rotary shaker at a speed of 160 rpm for 2 hours. Following incubation, solutions were removed from the tubes and the change in absorbance at 475nm was compared with a substrate only control. One unit of PPO activity was defined as a change of 10^{-3} absorbance unit/min/g seed.

PPO activity assay with caffeic acid, catechol, L-DOPA and phenol: The experiments were performed at least in triplicate for each genotype and substrate by following same method with a little change. Three grains of a genotype were placed in a test tubes containing 1.5 mL of phenolic substrate (Caffeic Acid, Catechol, L-DOPA or Phenol) made up in 50 m*M*3-(*N*-morpholino) propane sulfonic acid(MOPS) buffer at pH 6.5. All the substrates were adjusted to 10 m*M* with the exception of phenol, which was diluted to a 2 g/lit solution. For phenol, the tubes were incubated at 20°C with rotation of 160 rpm for 2 hours. For caffeic acid, catechol and L-DOPA the tubes were incubated at 25°C with rotation of 250 rpm for 2 hours. Following incubation solutions part was removed from the tubes and the change in absorbance was compared with a control containing only the respective substrate. For phenol and catechol reactions, changes in absorbance wererecorded at 410 nm, while for caffeic acid and L-DOPA reactions, changes in absorbance were recorded at 475 nm. One unit of PPO activity was defined as a change of 10^{-3} absorbance unit/min/gm.

The PPO activity with all the substrates was calculated as under:

$$\text{Enzyme activity} = \Delta \text{ OD.min}^{\uparrow}(-1).\ [\text{grain weight in gm}]^{\uparrow}(-1)$$

Analysis of variance (ANOVA) was done by Factorial Design using MSTAT-C data analysis package. PPO activities of wheat genotypes were analyzed by cluster analysis through Minitab v15 software to determine extent of correlation. All possible combinations i.e. caffeic acid and tyrosine, caffeic acid and phenol, caffeic acid and catechol, caffeic acid and L-DOPA, tyrosine and phenol, tyrosine and catechol, tyrosine and L-DOPA, phenol and catechol, phenol and L-DOPA and catechol and L-DOPA, were grouped together.

Results and Discussion

Wheat is the staple food for a large proportion of world population. The wheat flour and other wheat products undergo time dependent darkening/browning in appearance, which is undesirable for consumers (Morris & Rose, 1996). The changes in color are considered due to polyphenol oxidase (PPO) enzymatic activity. The scope of studies conducted on wheat PPO have either been limited due to use of few genotypes or by using one or a few of the available substrates, making comparison or in depth analysis fairly difficult. In this study a relatively large number of wheat genotypes were assayed for PPO activity with five substrates separately, which gives a better idea about the diversity of PPO activities. These substrates included caffeic acid, catechol, L-DOPA, phenol, and tyrosine. The sum of PPO activities of these substrates fell in a range of 34.15-150.27 units/min/g (Table 1) with the lowest and highest sum of activities shown by wheat genotypesWafaq-2001 and Chalsi respectively.

The activity observed by using different substrates showed different pattern of high and low activity for different wheat genotypes. The lowest activity range was observed when caffeic acid was used as substrate while the highest activity was observed with L-DOPA. The activities were different not only for substrates but also for the wheat genotypes used. Wheat genotypes with highest and lowest PPO activities are shown in Table 1.Tyrosine is a stable relatively cheaper substrate which shows a well detectable activity range however it is less sensitive as it is inefficiently solubilized even at pH 8.5. On the other hand catechol and L-DOPA did not offer any problem in solubility at pH 6.5, and may be used as a good indicator of total PPO activity.

Analysis of PPO activities in response to different substrates was initially performed by ANOVA on all the five activities which revealed that wheat genotypes and activities differ in a highly significant manner. Interaction between genotypes and substrates was also highly significant. The value of Coefficient of Variation (10.30%) as determined by ANOVA verifies the reliability of the analysis as nearly 90% variation is accounted for in the difference in genotypes and substrates used.

Table 1. Wheat genotypes with low (Sr. No.1-5) and high sum of PPO activities (Sr. No.6-10) in response to different substrates.

Sr. No.	Wheat genotypes	Substrates					
		Caffeic acid	Catechol	L-DOPA	Phenol	Tyrosine	Sum of activities
1.	Wafaq-2001	0.953 ± 0.183*	4.614 ± 0.138	16.593 ± 2.460	1.317 ± 0.045	10.673 ± 0.418	34.150
2.	Damote (Red)	1.397 ± 0.109	7.865 ± 0.703	14.354 ± 3.734	2.692 ± 0.442	11.336 ± 1.053	37.645
3.	Sara	1.652 ± 0.177	7.416 ± 0.861	15.512 ± 0.589	3.327 ± 0.408	10.561 ± 0.681	38.468
4.	Sarhad-82	1.194 ± 0.141	5.582 ± 0.374	17.24 ± 3.775	1.843 ± 0.327	14.231 ± 2.088	40.089
5.	Local	1.811 ± 0.344	6.502 ± 0.835	21.361 ± 1.283	2.188 ± 0.425	8.835 ± 0.977	40.697
6.	Lishi	10.52 ± 1.133	15.65 ± 1.534	79.606 ± 4.445	8.939 ± 0.457	12.473 ± 1.750	127.19
7.	Sharoti	5.488 ± 0.458	28.55 ± 1.797	56.533 ± 3.665	9.027 ± 1.067	30.023 ± 1.137	129.62
8.	Karoti	2.945 ± 0.574	21.772 ± 1.305	80.125 ± 2.304	4.474 ± 0.481	27.452 ± 2.803	136.77
9.	Gor	6.063 ± 0.533	14.58 ± 1.403	81.887 ± 6.987	8.832 ± 1.068	26.768 ± 1.539	138.13
10.	Chalsi	10.25 ± 1.51	22.93 ± 2.831	78.861 ± 4.011	13.49 ± 0.662	24.74 ± 2.980	150.27

*units.min^{-1}.g^{-1} seed

It was also interesting to note that the ranking based on one activity against one substrate was not valid for the activity against other substrates, which would have been the case if there was only one dominant or only one PPO activity/enzyme. To accurately estimate this apparent lack of correlation in various activities, Pearson correlation was calculated by using Minitab v15. A highly significant relationship among the wheat genotypes using all possible pairs was evident from the outcome of genotype-substrate interaction (Table 2).

Substrate specific PPO activities were also plotted in pairs for all the ten possible substrate pairs (results shown for only two pairs) to get a clear view of the scatter (Figs. 1 and 2). It was interesting to note that although most of the activity pairs fell in one cluster along the correlation line, there were some outliers in each plot. Figure 1 shows five outliers which exhibited comparatively higher phenol PPO activity as compared to tyrosine PPO activity. The accession #31 is of special interest which has a quite high phenol PPO activity while quite low tyrosine PPO activity. In Fig. 2 accessions 4, 46, 27, and 71 show a higher activity for catechol PPO vs. caffeic acid PPO while inversely a very low activity for the former and very high for the latter. These deviations indicated probability of governing of at least some activities by different forms of PPO. PPO enzyme is reported to exist in soluble and insoluble fractions (Fuerst *et al.,* 2006). Two different classes of PPO from wheat (Anderson *et al.,* 2006) and three distinct PPOs from the genome of *T. monococcum* have already been reported (Massa *et al.,* 2007).

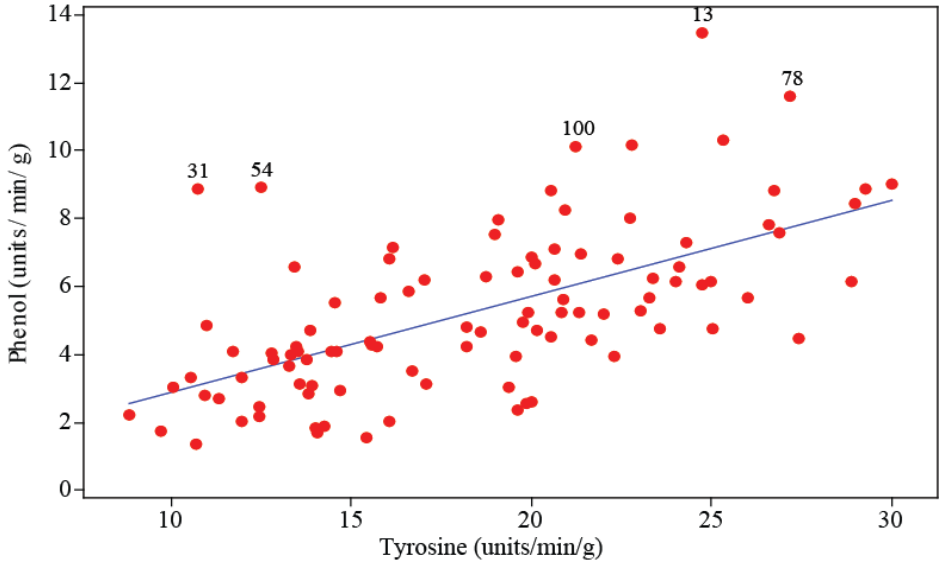

Fig. 1. Scatter plot of PPO activity using phenol as substratevs. PPO activity using tyrosine as substrate showing Pearson correlation, where numbers indicate outlier genotypes.

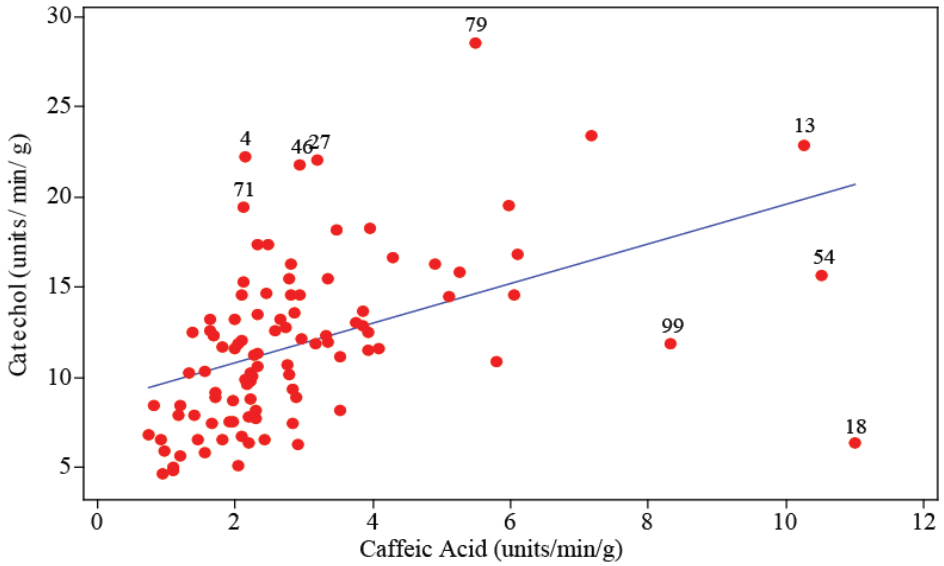

Fig. 2. Scatter plot of PPO activity using catecholas substrate vs. PPO activity using caffeic acid as substrate showing Pearson correlation, where numbers indicate outlier genotypes.

Table 2. Correlation of activities displayed by wheat grain PPO in response to pairs of substrates.

	Caffeic acid	Catechol	L-DOPA	Phenol	Tyrosine
Caffeic acid	1				
Catechol	0.457**	1			
L-DOPA	0.653***	0.499**	1		
Phenol	0.488**	0.505**	0.328*	1	
Tyrosine	0.331*	0.357*	0.415**	0.616***	1

*=Significant, **=Highly significant, ***=Very highly significant

The analysis of data by ANOVA showed that all the wheat genotypes and the PPO activities differed in a highly significant manner. Similar results were seen in correlation studies except for a very small number of genotypes. Combined results of ANOVA and correlation indicate that the hypothesis "There is a strong correlation between the results if there is a single PPO" although true for most of the genotypes, the outliers in correlation clearly indicated existence of multiple PPO isozymes. On the basis of these deviations/outliers, about 4-5 different types of PPO isozymes in different wheat genotypes under study may be well expected.

Conclusively, these results show that there are more than one PPO activities present in wheat grain, which show different preference for different substrates. This research work will provide a baseline for further research on wheat PPO. The information thus generated may be very useful for breeding low PPO varieties to reduce the losses due to browning of wheat flour and flour-based products. It may also be useful for molecular biologists to determine isozymes pattern in these genotypes to locate/characterize genes for different isozymes and to develop universal markers to aid in marker assisted selection. Further, characterization of the reaction centers of these PPO isozymes may be another interesting study resulting from the outcome of this investigation.

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