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**ORIGINAL ARTICLE**

# Ascorbic Acid Oxidase and Amylases Levels in Different Grains Growing in the Same Spikelet of Wheat

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## ABSTRACT

Relative levels of ascorbic acid oxidase, a- and b- amylases were studied at different types of grain (bold and small) growing in the same spikelet of wheat (*Triticum aestivum* L. var. *PBW-343*). Ten labeled spikes were sampled five times, seven-day intervals started from seventh day after anthesis (DAA) up to 28$^{th}$ DAA, and at maturity. The level of ascorbic acid oxidase in two different types of grains increased in the first 14 days post-anthesis stages followed by a gradual decrease which continued until maturity. A more or less similar pattern was apparent in smaller grains, which despite the fact being endowed with higher levels of ascorbic acid oxidase showed a declension right from 14$^{th}$ DAA onwards, reaching to a minimum level at maturity. The overall deduction in the absolute levels of ascorbic acid oxidase was higher in smaller grains as compared to bolder grains. Furthermore, the distribution of the enzyme was unequivocally higher in smaller grains than bolder grains at all the stages of investigations. According to amylases, the data reveal that as the grains progressed towards maturity the levels of these hydrolytic enzymes increased correspondingly. A comparative look into the levels of a- and b-amylase and their distribution in bold and small grains, disclosed that smaller grains were endowed with their relatively higher levels at all stages of investigations. The analysis of data revealed that the higher quantum of distribution in smaller grains was maximum at 7$^{th}$ DAA. At maturity smaller grains possessed relatively more of amylases than their counterpart bolder grains. The results bring forth, in no uncertain terms, the findings that the ear of wheat is a developing place for a definite number of grains which intern are separate biological entities endowed with their inherent potentials to grow.

***Key words:*** Hydrolytic enzymes; developing grains; *Triticum aestivum* L.

## Introduction

An appraisal of parameters regulating cereals productivity divulges that their full potential to yield is still unrealized. One of the grey areas, which has remained untapped is the host of physiological and genetical barriers of developing kernels to grow to an optima and their manipulation by desirable traits and methodologies. The potential up gradation of components constituting the total yield in wheat (number of productive tillers m$^{-2}$, grains per spike and 1000-grain weight), would help to raise the production substantially. Though, significant milestones have been achieved in the first two parameters the last component, the individual grain weight has eluded scientific investigations and rather paradoxically has declined with the advent of high yielding varieties. A study into the physiology of grain yield shows the existence of variation among different varieties or genotypes or even the grains

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developing in the same ear [3,21,17,23,11]. It further discloses that the yield may be influenced by the availability of photosynthates to the developing sinks [24,19,20,7]. Various sugar responsive genes in plants potentially affect the partitioning [9] and have been stressed to be key determinant of plant productivity [10]. Dry matter partitioning also plays a paramount role in growth rate of sink organs [14]. Working on the grain growth in wheat and buckwheat variation among varieties was traceable to endogenous hormone production in variety vis-à-vis that in the ear [6,5]. A few biochemical components as advocated by Abrol *et al.* [2], Hakaka [12] and Hasan and Kamal [13], might be of significance in determining sink efficiency and/or the grain yield. In the present study, it is proposed to analyse the relative levels of a few enzymes belonging to the hydrolytic class namely, ascorbic acid oxidase, a- and b- amylases in different grains growing in the same spikelet and to assess whether they are variable amongst the differentially participating grains or not.

## Materials and methods

*Crop management and sampling:*

The investigation was conducted with a common bread wheat (*Triticum aestivum* L. var. *PBW-343*), which was sown in circular earthenware pots (50x30x30 cm) containing 35 kg of soil mixed with farmyard manure (4:1). Eight seeds per pot were sown and after 15 days, seedlings were thinned to two. Hoagland's nutrient solution [15] was supplied to the pots. The plants were grown in a screen covered hall under otherwise natural conditions. Ten labeled main spikes were sampled five times, seven-day intervals started from seventh day after anthesis (DAA) up to 28$^{th}$ DAA, and at maturity. Grains were usually taken from three different segments in the ear. The labeled samples of grains were brought to laboratory and separated to two types of grains (small and bold) and the following biochemical analysis was carried out in the above aged grains.

*Ascorbic Acid Oxidase Analysis:*

The method used for estimation of ascorbic acid oxidase was according to Oberbacher and Vines [18] and it's briefly described as follows:

(i)Extraction of the enzyme - 100 mg of grain tissue was homogenated in a clean and pre-cooled mortar and pestle with 5ml of 0.1 M phosphate buffer (pH of 6.5). The homogenate was centrifuged at 15,000g for 10 minutes at 4°C. All procedures were carried out at 0-4°C and the supernatant was used as a source for enzyme estimation.

(ii)Estimation of the enzyme - In order to quantify ascorbic acid oxidase, 3 ml of substrates solution (prepared by making 8.8 mg ascorbic acid dissolved in 300 ml phosphate buffer at pH of 5.6) was added to reference cuvette of a spectrophotometer. 0.1 ml of enzyme extract was added to the cuvette (enzyme was added so as to get a positive increase in absorbance values). The absorbance change was measured at 265 nm in 30 sec intervals time for 5 minutes. For calculation of the enzyme from the linear phase of reaction, the change in optical density per minute was computed. Enzyme activity was expressed as micromole oxygen per minute according to above method.

*a-amylase Analysis:*

The method of Fuma [8] with certain modifications was used. The method is outlined below:

(i) Extraction of the enzyme - 1 g of grain was homogenized in 7 ml of phosphate buffer (pH of 7.5) containing 0.001 M cysteine and 0.8 M KCl. The extract was centrifuged at 15,000 g for 30 minutes. The residue was re-extracted with 3 ml phosphate buffer and supernatant was pooled together and kept at 0-4°C for estimation.

(ii) Estimation of the enzyme - The following reagents were used for estimation of this enzyme.

1) KI solution, 25.4 mg of $I_2$ and 0.4 g KI were dissolved in 100 ml of distilled water.

2) 1 percent starch solution, a fresh solution by dissolving 1 g starch in 100 ml acetate buffer was prepared.

3) Phosphate buffer (0.05 M pH of 7.5)

A - Monobasic sodium phosphate (1.39 g/200 ml of distilled water); B - Dibasic sodium phosphate (1.78 g/200 ml of distilled water); For desired pH of 7.5, 16 ml of A and 84 ml of B were pooled together and diluted to 200 ml with distilled water.

4) Cysteine 0.242 g/200 ml buffer.

5) KCl 11.92 g/200 ml buffer.

4 ml of the 1 percent starch solution and 1 ml acetate buffer at pH of 5.2 were added to 0.5 ml of enzyme extract and the reaction mixture was incubated at 30°C for 2 hours. The reaction was stopped by adding 1.0 ml of 1 N HCl and the amount of starch left unhydrolysed was determined by adding 3 ml of KI solution. The resultant solution was determined at OD 520 nm. The enzyme activity was expressed as mg glucose released per unit gram fresh weight of tissue and was calculated from the standard curve prepared by using different glucose concentrations.

*β-Amylase Analysis:*

The b-amylase was estimated according to the method of Bernfield [4] with slight modifications given as below:

(i) Extraction of the enzyme – 1 g of acetone defatted grains was homogenized in 66 mM phosphate buffer (pH of 7.0) containing 0.5 M NaCl. The extract was centrifuged at 20,000g for 15 minutes. The residue re-extracted with 3 ml phosphate buffer and supernatant was pooled together and kept in refrigerator at 4°C for estimation. All operations were carried out at 4°C.

(ii) Estimation of the enzyme - 1 ml of starch solution mixed with 1 ml of properly diluted enzyme extract was incubated at 27°C for 15 minutes. After 15 minutes 2 ml of dinitro salicylic acid was added to stop the reaction. The solution was then heated in boiling water bath for 5 minutes and allowed to cool. When the tubes were warm, 1 ml of potassium sodium tartrate solution was added. The tubes were cooled in running tap water and the volume was made upto 10 ml by addition of 6 ml water. The OD of blue coloured solution was measured with spectrophotometer at 560 nm. The enzyme activity was expressed as mg of maltose produced during 5 minutes incubation with 1 percent starch and was calculated from the standard curve prepared by using different maltose (0-100 mg) concentrations.

**Result and discussion**

In order to establish whether the bold and small grains were separate biological entities produced as a consequence of the specific biochemical process or were simple chance manifestations of development, the relative levels of a few enzymes belonging to the hydrolytic class namely, ascorbic acid oxidase, a- and b- amylases were studied in them at different stages of growth and development. The salient features of results are given below.

*Ascorbic Acid Oxidase:*

The representation of the data in Figure 1 indicates the level of ascorbic acid oxidase in two different types of grains. As apparent its levels increased in first 14 days post-anthesis stages followed by a gradual decrease which continued until maturity. The fall was to the tune of 32.0 percent at 21$^{st}$ DAA which further fell by another 46.3 percent at 28$^{th}$ DAA followed by another decline of 25.8 percent at maturity, thereby showing an overall declension to the tune of 66.9 percent in bold grains. A more or less similar pattern was apparent in smaller grains, which despite the fact being endowed with higher levels of ascorbic acid oxidase showed a declension right from 14$^{th}$ DAA onwards, reaching to a minimum level at maturity. Interestingly, the overall deduction in the absolute levels of ascorbic acid oxidase was approximately 75 percent in smaller grains as compared to 67 percent in bolder grains. The distribution of the enzyme was unequivocally higher in smaller grains than bolder grains at all the stages of investigations e.g., at 7$^{th}$ DAA its level was higher by 110.8 percent and by 92.8, 72.3 and 62.1 percents more at 14$^{th}$, 21$^{st}$ and 28$^{th}$ DAA stages respectively with a final figure of 57 percent more in smaller grains at harvest (Figure 4).

*a- and b-amylases:*

The data on the levels of amylases reveal that as the grains progressed towards maturity the levels of these hydrolytic enzymes increased correspondingly (Figures 2 and 3). A comparative look into the levels of a-amylase and its distribution in bold and small grains, disclosed that smaller grains were endowed with its relatively higher levels at all stages of investigations. The analysis of data revealed that the higher quantum of distribution in smaller grains was maximum at 7$^{th}$ DAA (108.0 percent more than bolder grains) and subsequent to that the differences were to the tune of 36.5, 14.4 and 29.7 percents more in smaller grains at 14$^{th}$, 21$^{st}$ and 28$^{th}$ DAA (Figure 4). At maturity smaller grains possessed relatively more (72.0 percent) of a-amylase than their counterpart bolder grains. With regard to the levels of b-amylase a similar pattern as that of a-amylase was recordable. As evident it showed a significant disparity with respect to its distribution in the two types of grains. The disparity was sustainable throughout the ontogeny of grains' development with a maximum gap at 7 days after anthesis (133.3 percent higher in smaller grains) and the values were 42.8, 21.9, 30.6 and 75.0 percents more at 14$^{th}$, 21$^{st}$ and 28$^{th}$ DAA and at maturity respectively (Figure 4).

The results bring forth, in no uncertain terms, the findings that the ear of wheat is a developing place for a definite number of grains which intern are separate biological entities endowed with their inherent potentials to grow and accumulate dry matter. This axiom was advocated by Abolina [1] and is in line with the observations of innumerable workers [21,16,22,17,23].

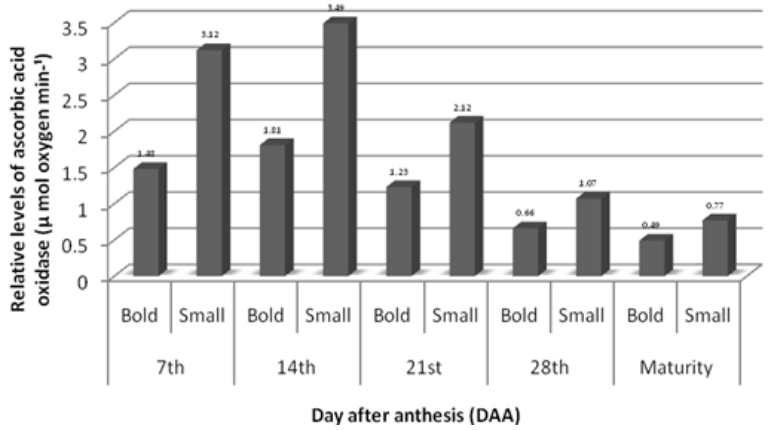


**Fig. 1:** Relative levels of ascorbic acid oxidase (µ mol oxygen $min^{-1}$) within developing grains (bold and small) of wheat (*Triticum aestivum* var. *PBW-343*)

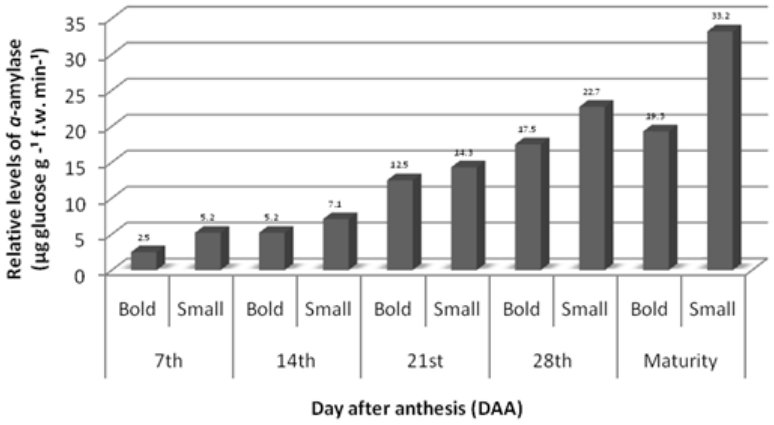


**Fig. 2:** Relative levels of a-amylases (µg glucose $g^{-1}$ fresh weight $min^{-1}$) within developing grains (bold and small) of wheat (*Triticum aestivum* var. *PBW-343*)

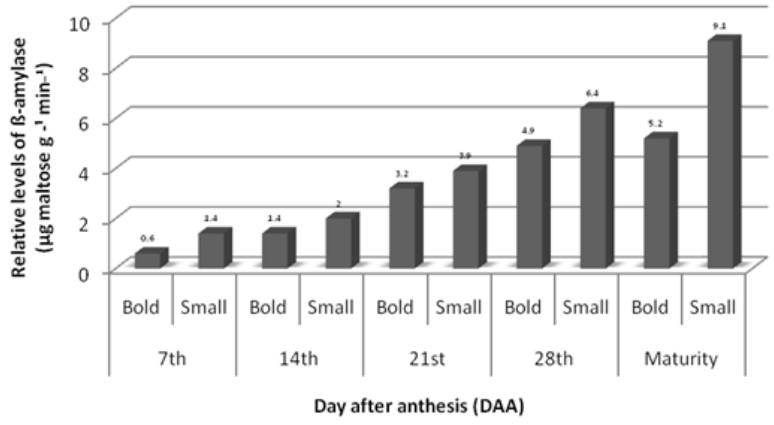


**Fig. 3:** Relative levels of b-amylases (µg maltose $g^{-1}$ $min^{-1}$) within developing grains (bold and small) of wheat (*Triticum aestivum* var. *PBW-343*)

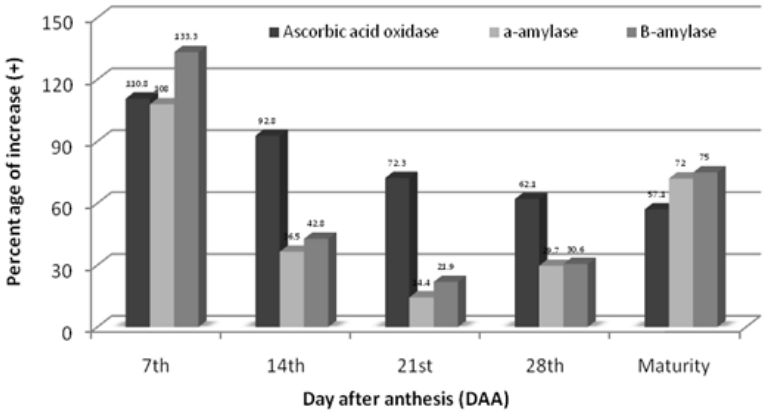


**Fig. 4:** Percentage increase (+) in relative levels of ascorbic acid oxidase, a- and b-amylases in small grains over their counterparts bold grains

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