Evaluation of Rubisco and PEP-carboxylase Levels as Affected by Salicylhydroxamic Acid within Developing Grains of Wheat

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Abstract: Effects of exogenous application of salicylhydroxamic acid (SHAM) on relative levels of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phosphoenolpyruvate carboxylase (PEPC) were studied at different grains growing in the same spikelet of wheat (*Triticum aestivum* L. var. *PBW-343*). A concentration of 10 ppm salicylhydroxamic acid was applied at anthesis stage in five replications with the help of cotton plugs, which remained on ears of mother shoots (MS) for 48 hours. The labeled spikes were sampled five times, seven-day intervals started from seventh day after anthesis (DAA) up to 28th DAA, and at maturity. The spikelets were divided into two grain types included basal (bold) and apical (small). The salient point emerging through the use of salicylhydroxamic acid was that both bold and small grains showed an increase in relative levels of Rubisco and PEP-carboxylase at 21st and 28th DAA stages. The behavior of Rubisco in two types of grains showed an increase in its levels upto 14th DAA in bold grains and 21st DAA for smaller grains followed by a gradual decrease towards maturity. The smaller grains possessed a lesser *per se* levels of Rubisco per unit basis with the highest gap at mid ripening stage. The only exception was at maturity which the smaller grains possessed relatively higher levels of Rubisco than the bolder grains. Analysis of data with regard to PEP-carboxylase activity revealed more or less the similar pattern as that of Rubisco activity.

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1. Introduction

An appraisal of parameters regulating cereals productivity divulges that their full potential to yield is still unrealized. One of the grey areas, which have 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 developing in the same ear (Asana 1968, Stoy 1969; Nautiyal *et al.* 1999; Yang *et al.* 2003; Gutam *et al.* 2008). It further discloses that the yield may be influenced by the availability of photosynthates to the developing sinks (Yin *et al.* 1998; Ravi *et al.* 2001; Sharma-Natu and Ghildiyal 2005; Foulkes *et al.* 2010). Various sugar responsive genes in plants potentially affect the partitioning (Geiger *et al.* 1996) and have been stressed to be key determinant of plant productivity (Gifford *et al.* 1984). Dry matter partitioning also plays a paramount role in growth rate of sink organs (Heuvelink and Bertin 1994). 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 (Dua and Sehgal 1981; Dua *et al.* 1990). A few biochemical components as advocated by Abrol *et al.* (1984), Hakaka (1998) and Hasan and Kamal (1998), might be of significance in determining sink efficiency and/or the grain yield. Since, the harvest index is the culmination of innumerable events, most of the view points on sink efficiency appears to be speculative and need a holistic approach in isolating obligatory events to produce the net assimilates. The revelation that the electron transport chain, in operation during biological oxidation, might find an alternate route without performing the target aim of creating proticity and may downgrade the overall impetus of meristems to grow by 10 to 25 percent (Siedow and Umbach 1995). Indeed, it has been reported that higher alternative respiration could be one of the reasons of lower growth of grains at distal

position in a spikelet (Sunita-Kumari and Ghildiyal 1997). It is, therefore, advocated that any attempt to interrupt this process may prove beneficial in improving productivity.

In the present study, it is proposed to analyze the relative levels of Rubisco and PEP-carboxylase as affected by specific inhibitor of salicylhydroxamic acid in different grains growing in the same spikelet of wheat.

2. Material and Methods

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 (Hoagland and Arnon, 1939) was supplied to the pots. The plants were grown in a screen covered hall under otherwise natural conditions. A concentration of 10 ppm salicylhydroxamic acid was applied at anthesis stage in five replications with the help of cotton plugs, which remained on ears of mother shoots (MS) for 48 hours. The labeled main spikes were sampled five times, seven-day intervals started from seventh day after anthesis (DAA) up to 28th 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.

PEPC was studied according to the method of Vance and Stade (1984) with some modifications as follows:

Extraction of the enzyme - Crude extracts were prepared by homogenizing 1 g fresh grain with acid-washed sand in a pre-chilled pestle and mortar in grinding medium (1 ml/1 g tissue) containing 50 mM tris-HCl (pH of 8.0), 50 mM $MgCl_2$, 5 mM, 2-mercaptoethanol and 1 mM EDTA. The homogenate was passed through four layers of cheese cloth, and filtrate centrifuged at 30,000 g for 30 minutes and the supernatant assayed for enzyme activities of PEPC. All the steps were carried out at 4°C.

Estimation of Enzyme - Phosphoenolpyruvate carboxylase was assayed spectrophotometrically at 30°C and 340 nm for at least 3 min in the assay media with a final volume of 1 ml. For the PEPC, the assay medium consisted of 10 mM $NaHCO_3$, 10 mM $MaCl_2$, 0.2 mM NADH and 4 mM PEP in 100 mM bicine-KOH buffer (PH of 8.5) optimized from Vance and Stade (1984).

Before placing samples in spectrophotometer 0.2 mM NADH was added as quickly as possible in to the test tube with a vigorous mixing well and the initial absorbance was recorded and thereafter every 30 sec for at least 3 minutes. Phosphoenolpyruvate carboxylase activity was expressed as µmole per min per gram fresh weight of sample. PEPC activity was calculated with decrease in absorbance for one minute and with the following formula:

µmoles per min 0.2 ml enzyme extract = Absorbance decrease/min × 0.1613 × 3 (volume of the reaction mixture in ml).

Rubisco was studied radiometrically according to the method of Bravdo and Pallas (1982) with some modifications, in terms of stopping the activity by glacial acetic acid. The enzyme is made to utilize labelled CO_2 as the substrate and the radioactivity in the products in counted as a measure of enzyme activity.

Extraction of the enzyme - Grains samples, weighing one gram were homogenized in chilled mortar and pestle. Enzyme was isolated in 8 ml medium of 100 mM tris-HCl buffer (pH of 8.0) containing 5 mM DDT, 20 mM $MgCl_2$, 0.5g PVP and 0.2 mM EDTA. The crude extract was filtered through four layers of muslin cloth and the filtrate was then centrifuged at 20,000 g for 30 minutes at 4°C. The supernatant was collected as the enzyme source.

Estimation of the enzyme - In a total volume of 250 µl, the assay mixture for Rubisco contained 98 mM tris HCl (pH of 7.8), 20 mM $MgCl_2$, 20 mM $NaH^{14}CO_3$ (specific activity 48.1 mci/m mole, activity 0.5 mci, obtained from BARC; Bombay). 20 µl of crude enzyme was incubated with all components except RuBP for 5 minutes at 30°C and the reaction was initiated by addition of RuBP. After 2 minutes the reaction was stopped by adding 250 µl of glacial acetic acid. Blank reaction mixture was prepared by adding all ingredients except RuBP. The samples were kept overnight in fume hood. Next day known volume was taken and added 10 ml of scintillation medium. Counting was done in liquid scintillation counter (Packard Tricarb, Liquid Scintillation Spectrometer).

Scintillation liquid was prepared by the method of Bray (1960). According to this 4 gm P.P.O., 0.2g PoPoP and 10 g nepthalene were dissolved in 1 litter of toluene. For dilution of $NaH^{14}CO_3$ solution, 1.0 ml of $NaH^{14}CO_3$ (0.5 mci) was diluted with cold $NaHCO_3$ solution so

as to give a final concentration of 100 mM. Rubisco activity was estimated as:

$$(CO_2 \text{ fixed}) \text{ dpm mg}^{-1} \text{ f.w. h}^{-1} = \frac{dPm \times V_1 \times 60}{V_2 \times t \times w}$$

Where net dpm is the disintegrations per minutes minus back ground counts, V_1 is the volume of assay system, V_2 is the amount loaded for counting, t is the reaction time and w is the weight of the sample.

3. Results

According to Figure 1, the inhibitor behaved in an enigmatic way and proved to be a promoter when being assessed under the criterion of dry matter accumulation in grains. During the earlier period of grain development, it had no significant effect thereby indicating that the underlying physiological process may not be in operation. Subsequently a significant increase in dry matter accumulation in both the types of grains at 21st DAA onwards up to maturity was noticed at both the concentrations of SHAM. The salient points emerging through the use of salicylhydroxamic acid were that (i) both bold and small grains showed a significant increase in dry matter from 21st DAA stage with its applications and (ii) in spite of the aforementioned increment gathered by grains, they continued to exhibit the disparity between them and at maturity the smaller grains still showed approximately 25 percent lower dry matter than the bolder grains.

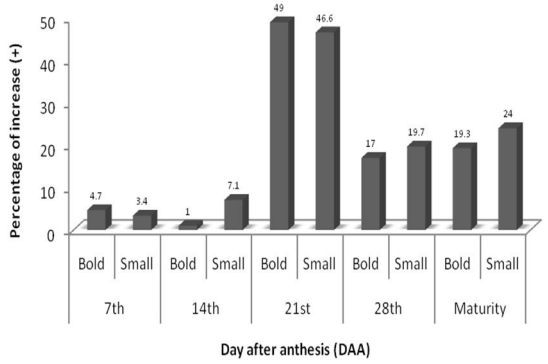

Figure 1. Percentage of increase (+) in dry weight of grains at different location within developing grains of wheat (*Triticum aestivum* L. var. *PBW-343*) as influenced by salicylhydroxamic acid

The scrutiny of the data with regard to the effect of salicylhydroxamic acid on relative levels of Rubisco, the key enzyme regulating C fixation, presented a few interesting correlations. A look into the Figure 2 depicts the behavior of Rubisco in two types of grains which showed an increase in levels of Rubisco upto 14 days in bold grains and 21 days for smaller grains post-anthesis stages followed by a gradual decrease toward maturity. Following by the application of salicylhydroxamic acid, both bold and small grains showed an increase in relative levels of Rubisco at 21st and 28th DAA stages (Figure 3). The two types of grains significantly possessed its differential levels. Analysis of the data revealed that the smaller grains possessed a lesser *per se* levels of Rubisco per unit basis with the highest gap at mid ripening stage. The exception was at maturity which the smaller grains possessed relatively higher levels of Rubisco than the bolder grains (43.3 percent higher in smaller grains) (Figure 6).

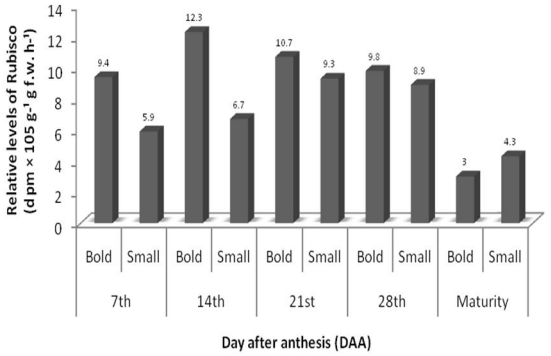

Figure 2. Relative levels of Rubisco (d pm × 105 g^{-1} fresh weight h^{-1}) at different location within developing grains of wheat (*Triticum aestivum* L. var. *PBW-343*) as influenced by salicylhydroxamic acid

Figure 3. Percentage of increase (+) or decrease (-) in the level of Rubisco over control at different location within developing grains of wheat (*Triticum aestivum* L. var. *PBW-343*) as influenced by salicylhydroxamic acid

Analysis of data with regard to PEP-carboxylase activity revealed more or less the similar pattern as that of Rubisco activity

(Figures 4, 5 and 6). Its levels increased as the grains progressed upto mid ripening stage followed by a gradual decrease towards maturity (Figure 4). Following by the application of salicylhydroxamic acid, both bold and small grains showed an increase in relative levels of PEP-carboxylase at 21st and 28th DAA stages (Figure 5). With regard to its distribution in bold and small grains, was apparent that the bolder grains possessed relatively higher levels of PEP-carboxylase at 7th, 14th, 21st and 28th DAA stages of investigation. The higher quantum of distribution in bold grains was maximum at 14th DAA (44.2 percent lesser in smaller grains) and subsequent differences were to the tune of 11.3 and 8.3 percents short in smaller grains at 21 and 28 days post-anthesis stages respectively. At maturity the smaller grains possessed relatively higher levels of PEP-carboxylase than the bolder grains (20.8 percent higher in smaller grains) (Figure 6).

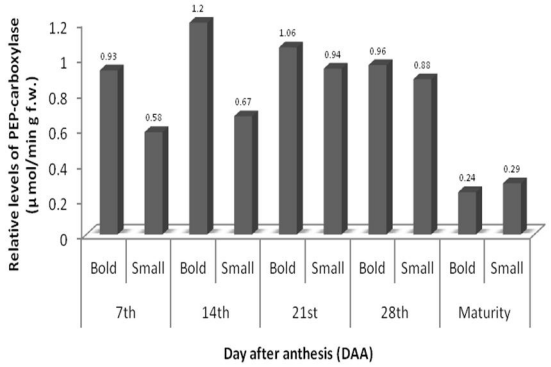

Figure 4. Relative levels of PEP-carboxylase (µ mol/min g fresh weight) at different location within developing grains of wheat (*Triticum aestivum* L. var. *PBW-343*) as influenced by salicylhydroxamic acid

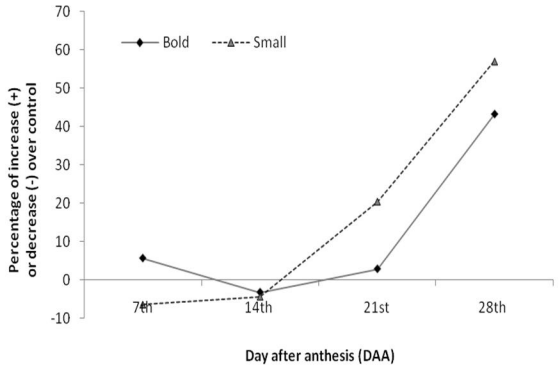

Figure 5. Percentage of increase (+) or decrease (-) in the level of PEP-carboxylase over control at different location within developing grains of wheat (*Triticum aestivum* L. var. *PBW-343*) as influenced by salicylhydroxamic acid

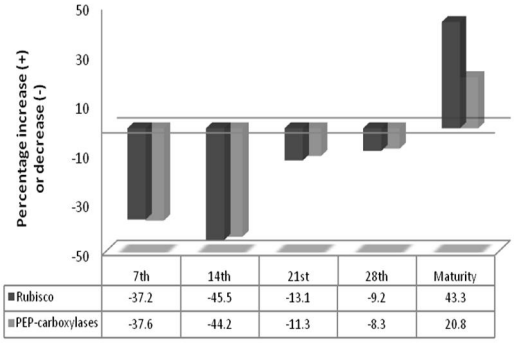

Figure 6. Percentage of increase (+) or decrease (-) in relative levels of Rubisco and PEP-carboxylase in small grains over their counterparts bold grains

4. Discussions

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. This axiom was advocated by Abolina (1959) and is in line with the observations of innumerable workers (Cook and Evans 1978; Larsson and Hensen 1992; Wang *et al.* 1998; Yang *et al.* 2003). Nevertheless, the sequence of events, piloting the yielding ability, is the metabolic profile and if augmented through the use of plant growth regulators (Yang *et al.* 2000; Houshmandfar and Eradatmand-Asli 2011) or by imposing a shift in metabolic events (Dua *et al.* 1990) promotery effects are achievable (Hayashi 1961; Michael and Beringer 1980). In present context, the central point which came to light in the present endeavor is that an unusual path of aerobic respiratory chain (CN-resistant respiration) plausibly switches-on during the grain filling stage and if checked, through the immaculate use of salicylhydroxamic acid, can increase the relative levels of Rubisco and PEP-carboxylase in the grains. Of course, SHAM or regulator of alternate oxidase pathway was not successful in eliminating the disparities between the two types of grains.

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