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PEROXIDASE AND IAAOXIDASE ACTIVITIES DURING SINK DEVELOPMENT IN COTTON SEED

ABSTRACT

Seeds of the three cotton genotypes, H-6 and H-4 (*Gossypium hirsutum*),and V797 (*G. herbaceum*)] for their growth variations in IAA oxidase and peroxidase activities. The three genotypes varied in their final seed weight. The peroxidase analysis was done with four different hydrogen donors. Though the trends in peroxidase activity were almost similar in the three genotypes, there was variation with different hydrogen donors. These was an inverse correlation between Cytoplasmic peroxidase and Wall bound peroxidase activity and rate of water uptake of the seed. The peroxidase activity showed up increasing levels only after the rate of water uptake decreased. However, IAA oxidase activity did not show any discernible trend with water content. The role of cytoplasmic and wall bound peroxidase in seed development is discussed in detail.

Key words: cotton, IAA oxidase, peroxidase, seed development

Abbreviations: DMA – dry matter accumulation, DPA – days post anthesis, IAA – indole-3-acetic acid

INTRODUCTION

The final weight of the seed at physiological maturity, play an important role in determination of yield. Dry matter accumulation in the developing seed is regulated by the number and the size of the cells (Egli, 1990) which determines the yield. Endogenous hormones play a key role in the regulation of various metabolic processes which determine the final seed size. IAA is a principal auxin found in the seed of higher plants (Schneider and Wightman, 1974). Three status of the IAA are found in the plant cell (i) free (readily available for growth) (ii) bound form (released IAA in the cell as and when cell requires or participates in transport from cell to cell) and (iii) oxidised form (via IAA oxidase and peroxidase system). Role of auxin in regulation of seed size is well documented in the literature (Bangerth *et al.*, 1985). For an auxin to function as a regulator of cell expansion, its own concentration in the target tissue must be controlled (Pilet and Saugy, 1985). In the three cotton genotypes studied by us, the bigger seeds have higher auxin content as compared to middle and smaller sized seeds (unpublished). In this context, the existence of a specific enzymatic system which catalyses the oxidative degradation of IAA may

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be important. Peroxidases (E.C.1.11. 1.7) from numerous plant species have been shown to catalyze this oxidative decarboxylation (Sembdner *et al.*, 1980). There are numerous studies to show an inverse correlation between peroxidase activity and IAA content and/or elongation growth (Higuchi 1985, Gaspar, 1986, Reinecke and Bandurski, 1988).

In earlier studies from our laboratory on cotton fiber elongation (Rama Rao *et al.*, 1982a, b, Thaker *et al.*,1986), it was concluded that cytoplasmic and ionically wall bound peroxidases and IAA oxidases play an important role in the regulation of fiber elongation. Role of these enzymes in regulation of sink size in *H. esculentum* seed (Thaker, 1998) and wheat grain (Chanda and Singh, 1997) is also reported. However, no such studies have been done on cotton seed development. Therefore, in this study, changes in IAA oxidase and cytoplasmic and wall-bound peroxidases, during dry matter accumulation of seeds of three cotton genotypes varying in their final seed size were studied.

MATERIALS AND METHODS

Seeds of three cotton genotypes (*Gossypium hirsutum* L. cv. H-4 H-6, and *Gossypium herbaceum* L. cv. V_{797}) were grown under field conditions. Flowers were date-tagged on the day of anthesis and healthy bolls were harvested on desired dates post anthesis (DPA). To minimize the effect of environmental variations, data for each set of analysis were collected from the flowers that had bloomed during as narrow a period as possible. The experiments were repeated in two successive years.

Growth analysis

Freshly harvested bolls were opened with a sharp scalpel and fibers were separated from seeds. For fresh and dry weight measurements, seeds from four locules of four bolls were weighed before and after oven drying to a constant weight at 80°C. The difference between fresh and dry weights gave the seed water content in mg at each stage. The mean dry weight and water content per seed with ± SD was calculated. The data of mean water content per seed was fitted to an appropriate curve using a polynomial regression analysis programme.

Preparation of enzyme extract

Seeds were removed and fibers were removed with a scalpel on an ice bath. After dissection, the ovules were counted, weighed and frozen. The frozen material was crushed in a cooled mortar with sterilized sand in pre-chilled extraction buffer 0.1 M K-phosphate buffer (pH 6.4) containing diethyldithiocarbamate (0.15%), cysteine-HCl (0.1%), EDTA (2.5 mM) and PEG-6000 (8%) as suggested by Hawker (1969). The homogenate was centrifuged at 15,000 g_n for 20 minutes. The resulting supernatant was passed through 15 mL column of Sephadex G-25 pre-equilibrated with 0.02 M K-phosphate buffer (pH 6.4). The desalted preparations were then used for the assay of all cytoplasmic enzymes. For IAA oxidase assay, the desalted enzyme extracts were incubated with insoluble PVP for 20 min in

an ice bath. The filtered extracts were then used for estimation of IAA oxidase activity.

Preparation of ionically wall-bound enzyme

The wall fraction separated after PEG extraction was washed several times with K-phosphate buffer (0.02 M, pH 6.4) by resuspending the wall material and was centrifuged. The residue was then extracted in 1 M NaCl for 1 h at room temperature with constant shaking to release ionically wall-bound enzyme and was centrifuged at 15000 g for 20 min. The supernatant was decanted and the resulting pellet extracted as before. The combined supernatants were used as the source of salt-extracted enzyme. The preliminary studies showed that 1 M NaCl was appropriate and that higher concentrations did not improve the extraction of ionically wall-bound enzyme.

Peroxidase assay

A spectrophotometric assay employed for peroxidase activity was similar to that described by Thaker *et al.* (1986). The reaction mixture consisted of 14 mM K-phosphate buffer (pH 6.4), 3 mM hydrogen donors viz., ferulic acid, caffeic acid, chlorogenic acid or 11 mM guaiacol, 1 mM H_2O_2 and the enzyme in a final volume of 3.5 cm^3. The reaction was triggered by adding H_2O_2 and increase in the absorbance was monitored at ?400 nm for ferulic acid, caffeic acid and chlorogenic acid, and at 470 nm for guaiacol. The linear phase of each reaction was considered for the calculation of mean values. The activity is expressed as ΔA_{400} or ΔA_{470} nm × min^{-1} × $seed^{-1}$.

IAA oxidase assay

IAA oxidase activity was determined by a modified method of Gordon and Weber (1951). The reaction mixture of 6 cm^3 consisted of 0.2 mM 2-4 dichlorophenol, 0.2 mM $MnCl_2$, 10 mM K-phosphate buffer (pH 6.4), 400 µg IAA and the enzyme. The reaction was initiated by the addition of IAA and immediately (after gentle shake) thereafter, 1 cm^3 of reaction mixture was added to 4 cm^3 of Salkowaski reagent (50 cm^3 35% perchloric acid + 1 cm^3 500 mM $FeCl_3$) to serve as a control. Both the reaction and control sets were incubated in dark at room temperature. After each 10 min, 1 cm^3 of the mixture was added to 4 $cm^3$of Salkowaski reagent and color was allowed to develop for 20 min. The absorbance of the pink solution was measured at 530 nm. The values of the activity was obtained from the linear part of the reaction and expressed as µg IAA oxidised.h^{-1}.$seed^{-1}$.

Statistical analysis

Seed dry weight data for the three genotypes were fitted to polynomial curves of different degrees and the best-fit equations were determined statistically. All estimations were done in triplicate and mean value with ± standard deviations were presented.

RESULTS AND DISCUSSION

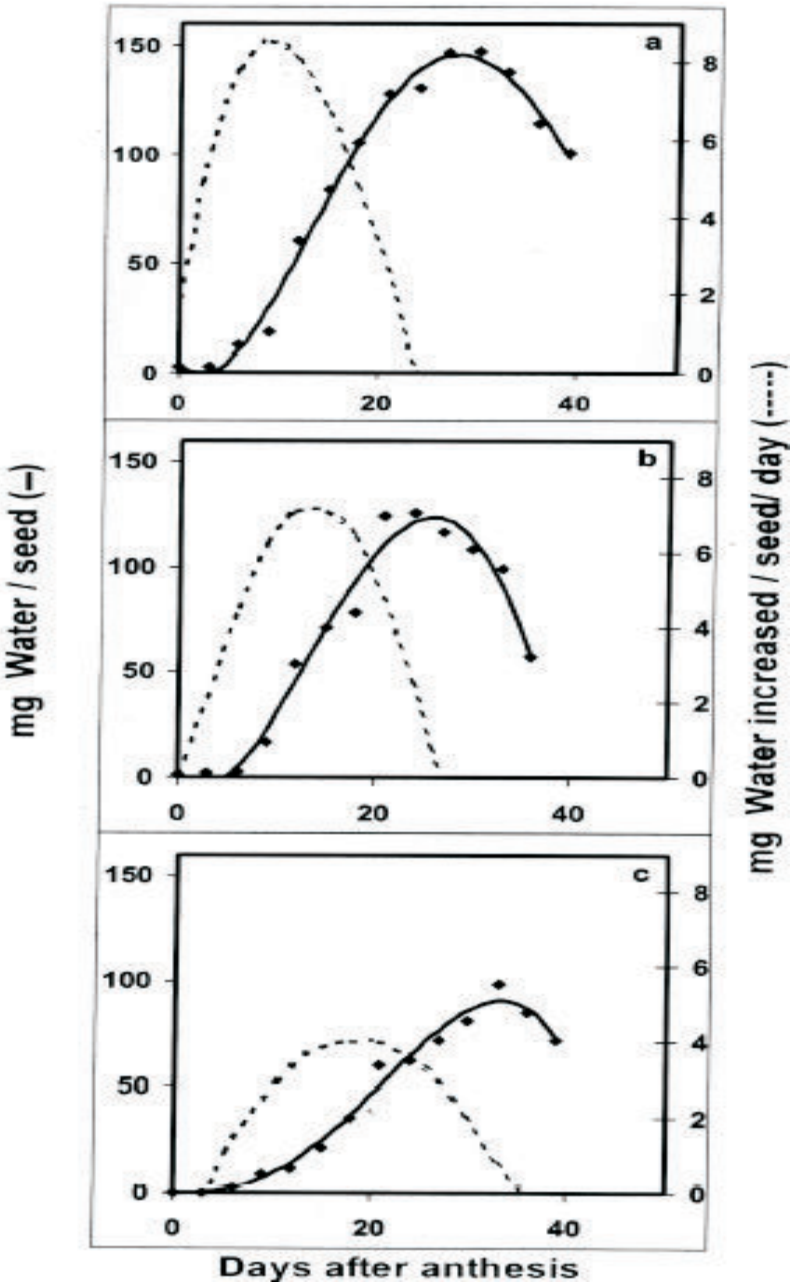

Fig.1. Changes in dry matter accumulation in seeds against boll age (continuous line) and its rate (broken line) in H-4, (a) H-6 (b) and $_{V797}$ (c)

In all the three genotypes, a cubic polynomial explained the data appropriately (Fig.1). In H-4, dry matter accumulation showed lag up to 10 DPA initially and then it

entered a linear phase of dry matter accumulation, and maximum seed dry weight was achieved around 39 DPA. Subsequently, a slight decrease in seed dry weight was observed. A similar trend was noted in seeds of H-6 and V_{797}. However, in the latter genotype, the initial lag phase was extended up to 12 DPA. Amongst the three genotypes, at physiological maturity, H-4 had maximum seed weight (102 mg/seed) followed by H-6 (74 mg/seed) while V_{797} had minimum seed weight (43 mg/seed). Further, H-4 had more rate of dry matter accumulation (4 mg/day) than H-6 (3 mg/day) and V_{797} (2 mg/day).

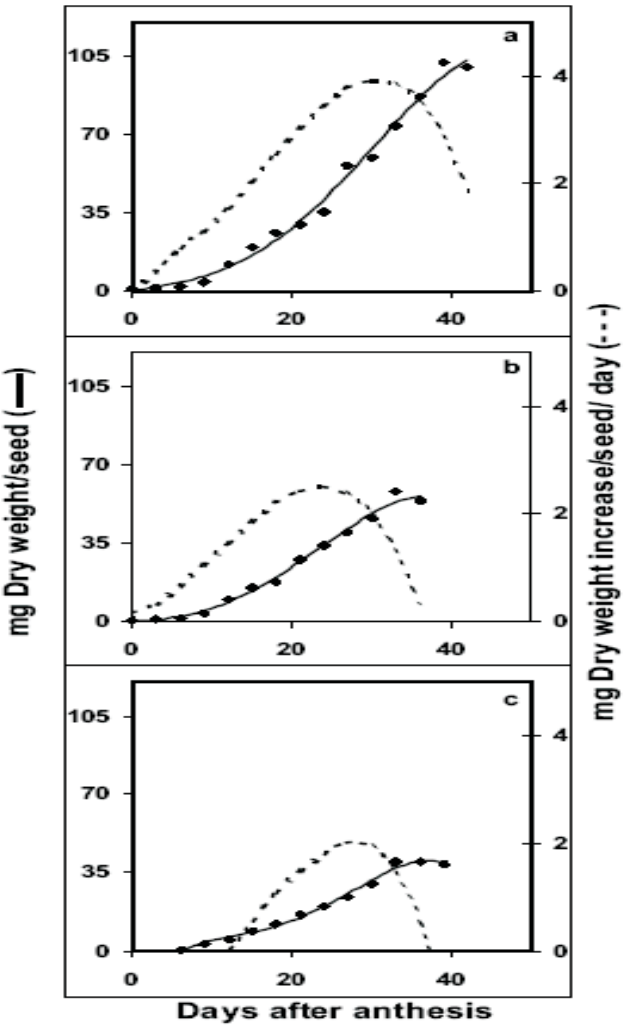

Fig.2. Changes in water amount in seeds against boll age (continuous line) and its rate (broken line) in H-4, (a) H-6 (b) and V_{797} (c)

Data of water amount was also fitted to a polynomial equation and presented in Fig. 2. In H-6 and H-4, water amount was low up to 6 DPA and increase up to 30 DPA and

decreased thereafter. In V_{797}, on the other hand, the initial lag phase of water uptake was around 10 DPA after which it increased sharply and attained a peak around 33 DPA. Amongst the three genotypes, maximum water amount recorded in H-4 (173 mg/seed) followed by H-6 (138 mg/seed) and V_{797} (110 mg/seed), respectively (Fig.2).

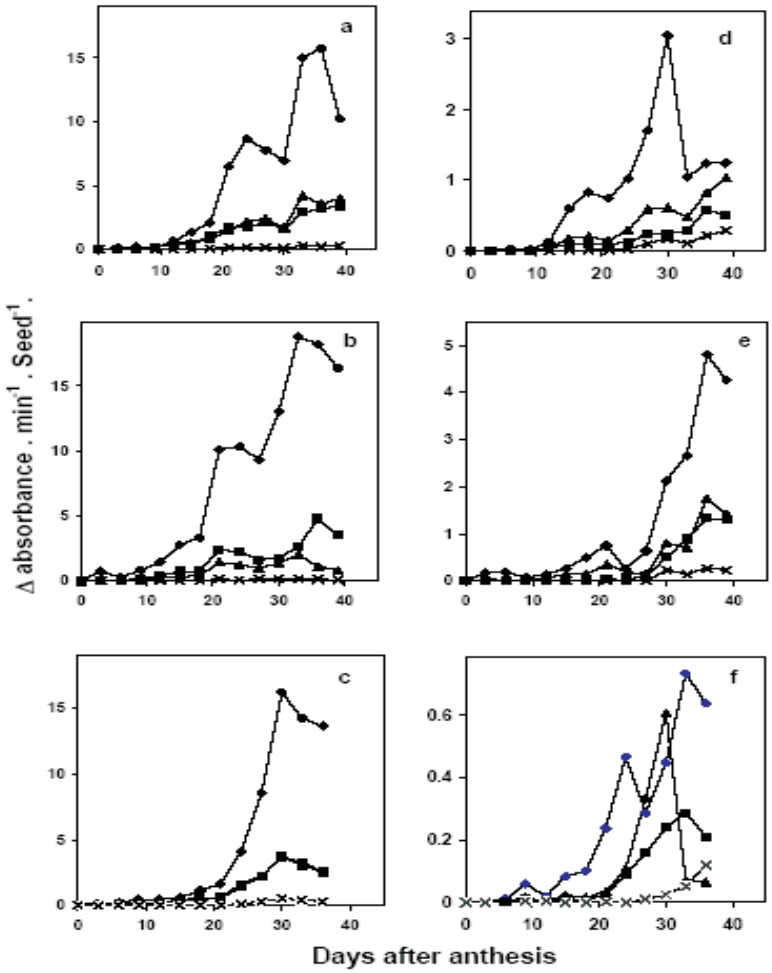

Fig.3. Changes in cytoplasmic (a,b,c) and wall bound (d,e,f) peroxidase activity against boll age using guaiacol (◆), chlorogenic (■) caffeic acid (▲) and ferulic acid (✖) as substrates

The data on rate of dry matter accumulation showed close parallelism with the water amount (Figs.1, 2). However, the rate of water uptake was more (9.5 mg/day) in H-4 followed by H-6 (7.4 mg/day). A peak was observed at d 20 after which, declined gradually. In V_{797} the rate was low (4 mg/day) compare to H-4 and H-6 and showed peak at d 30 (Fig. 2). From this growth analysis, seed development divided into four distinct phases of (i) cell division (0-9 DPA), (ii) cell elongation (3-21 DPA), (iii) rapid dry matter accumulation (12-39 DPA) and (iv) maturation (33-39 DPA onwards) phases.

Further, significant correlation was observed with the data on rate of DMA and water amount (H-4 N=14, r =0.98; H-6 N=13, r = 0.874: V_{797} N=13, r =0.82). Water status has multifunctional regulation in seed development. For example, it has been observed that in cereals, a water deficit imposed during grain filling caused a large decrease in final endosperm and embryo mass. The duration of dry matter accumulation was shorter (in both endosperm and embryo mass) in water deficit plant (Egli 1990). The rapid synthesis of end products such as starch, protein, oil during seed development requires optimum coordination between substrate availability, enzyme synthesis and activation (Bewley and Black 1993). A decrease in these factors during desiccation could lead to cessation of dry matter accumulation (Wesgate 1994). The elongating cell requires water for cell expansion (Taiz 1984) and thus water content may regulate sink size (Thaker 1999). The close parallelism between water amount and rate of dry matter accumulation of developing seeds reported in the present study, therefore, suggest that rapid uptake of water may be required for increased cell volume and to support rapid rate of dry matter accumulation.

Changes in cytoplasmic and wall bound peroxidase activities, in the three cotton genotypes, using four hydrogen donors viz., guaiacol, chlorogenic, caffeic and ferulic acid are presented in Fig. 3. The cytoplasmic peroxidase activity was low until the rate of water uptake and attained maximum value with a decline in the rate of water uptake in all three genotypes (Fig. 2 & 3) thus showed inverse relationship with sink size development phase. In H-4, wall bound peroxidase recorded low levels up to 27 DPA i.e. during massive DMA phase with all the four hydrogen donors used. The activity showed a significant rise in the subsequent periods and maximum levels were attained around 36 DPA. Similar trends were observed in H-6 and V_{797}.

Although the general trends in peroxidase activity, estimated with different hydrogen donors, during cotton seed ontogeny considerable variation in the levels of peroxidase activity was discernible with different hydrogen donors. It is well-known that most peroxidase activity can be fractioned into large number of isozymes, the precise role of which often remains uncertain. However, there have been numerous reports in literature with respect to peroxidase involvement in lignin biosynthesis (Mader *et al.*, 1986; Bruce and West, 1989; Pang *et al.*, 1989) and oxidation of endogenous IAA (Reinecke and Bandurski, 1988; Beffa *et al.*, 1990; Pressey, 1990). An inverse correlation between cytoplasmic peroxidase activity and rate of water uptake, in the present study, suggests an important role of peroxidases in the process of sink development and dry matter accumulation (Figs.1-4). Similar inverse correlations have also been reported (Gaspar *et al.*, 1985, Chanda and Singh, 1997, Thaker, 1998).

Changes in ionically wall-bound peroxidase activity in the three cotton genotypes, using four hydrogen donors are presented in Figs. 3 d,e,f. In H-4, wall-bound peroxidases, recorded low levels up to 27 DPA i.e. during massive dry matter accumulation phase with all the four hydrogen donors used. The activity showed a significant rise in the subsequent periods and maximum levels were attained around 36 DPA. Similar trends were observed in H-6 and V_{797} however, values remained very low in V_{797}.

The inverse correlation of ionically wall bound peroxidases with the rate of water uptake in the present study, suggests an important role of these peroxidases in the overall mechanism of sink size development. There are suggestions that wall peroxidases can catalyze the formation of cross-links between extensin (Lamport, 1986; Fry, 1982

a,b, 1987), lignin (Higuchi, 1985; Bolwell, 1988; Bruce and West, 1989) and feruloylated polysaccharides (Fry, 1982a, Smith *et al.*, 1984) at the expense of H_2O_2. Indeed, earlier studies from this laboratory on ionically wall-bound peroxidases in cotton fibre (Rama Rao *et al.*, 1982 a,b; Thaker *et al.*, 1986) and other tissues (Saroop *et al.*, 1987; Thaker, 1998), have shown inverse correlation between wall-located activity and cell elongation growth/ sink development.

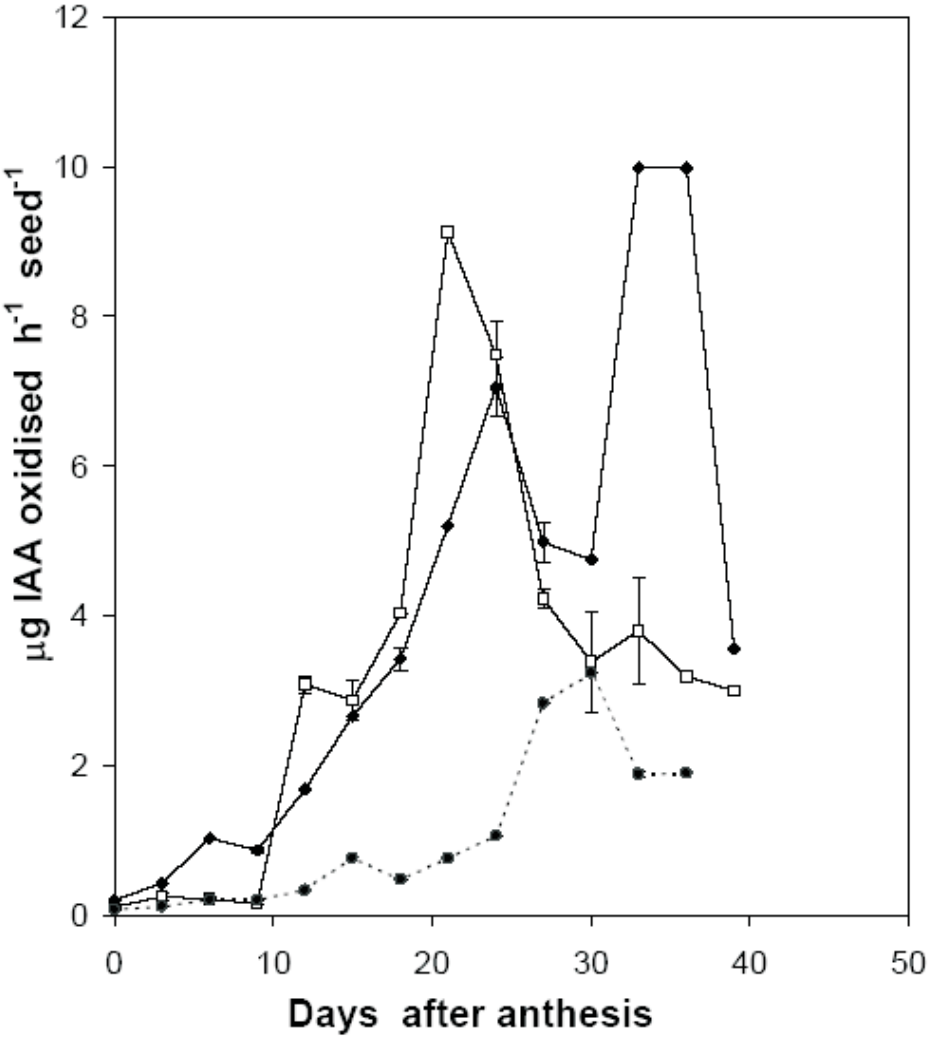

Fig.4. Changes in IAA oxidase activity in seeds against boll age; in (◆) H-4, (□) H-6 and (●) V_{797}

Changes in cytoplasmic IAA oxidase activity are presented in Fig. 4. The activity did not reveal any correlation with the water uptake phase in genotypes H-6 and H-4. The activity in both these genotypes was low up to 10 DPA and showed a close parallelism with the water amount during subsequent periods. In genotype V_{797}, however, IAA oxidase activity was low until the rate of water uptake was higher i.e. up to 21 DPA,

while in subsequent periods it increased significantly (Fig. 4). A correlation between the low levels of IAA and high levels of peroxidase activity led many workers to suggest that peroxidase mediated IAA oxidation may regulate the auxin concentration in tissues (see Gaspar, 1986).

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