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Relationship between Endogenous Abscisic Acid and Dry Matter Accumulation in Developing Seed and Pod of Pigeon Pea (*Cajanus Cajan*)

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Abstract: Two varieties of *Cajanus cajan* varying in sink size were selected for the study. Seeds were sown at botanical garden of Saurashtra University (Gujarat, India). Growth analysis in terms of dry weight and water content measurement was performed during the seed and pod development. From the growth data seed development was divided into four distinct phases, (i) cell division (ii) cell elongation (iii) dry matter accumulation and (iv) cell maturation. Antibodies against abscisic acid (ABA) were raised in rabbits and indirect ELISA was standardized to estimate endogenous levels of ABA. During seed and pod development, changes in endogenous free and conjugated ABA levels were measured. It was observed that levels of free ABA remained equal in seeds of both the varieties. The physiological age of ABA accumulation was different and this difference was remarkable in dry matter accumulation (DMA) phase. No significant difference of free ABA level was observed in developing pod of two varieties. The role of ABA in inhibition of cell elongation and promotion of DMA during seed development is discussed.

Key words: Abscisic acid . cell elongation . dry matter accumulation . seed development

INTRODUCTION

Every facet of plant growth from germination through differentiative growth and senescence is controlled by endogenous plant hormones. They have a fundamental role in embryo development, seed germination and the synthesis of storage substances [1]. A high level of assimilate availability during the reproductive growth stage is essential for high yield. It has been suggested that flower and pod set are regulated by the supply of assimilate to developing flowers and pods [2]. In many species, changes in hormonal content throughout the reproductive parts have been determined which suggest that at early reproductive stage when cell division is active in the embryo and endosperm, endogenous ABA concentration is normally low.

ABA regulates many agronomically important aspects of plant development including a major role in seed maturation and fruit development [3, 4]. However, evidences suggest a role of ABA in the partitioning of photoassimilates to developing fruit and seeds. It is well known that during seed development ABA acts differently and its content is developmentally regulated. From the number of reports it is clear that ABA is essential for normal growth and development of plant. During seed development, ABA plays several important roles including induction of storage proteins and lipid synthesis, desiccation tolerance and germination [1, 5-7].

Accumulation of ABA during seed development has been reported in many species [8]. ABA concentration in crop reproductive structures increases significantly when the plants are drought-stressed during flowering [9]. This increase of ABA concentration in the reproductive structures has been suggested to play a role in determining grain set in maize [10, 11] and wheat [12].

ABA levels increase in the initial stages of seed development and decrease during embryo maturation [4]. However, the highest concentration of ABA in the embryo occurs in many of the seeds at the time when their dry weight is increasing rapidly. The increase in ABA levels towards the end of grain filling and its rapid fall during maturation have raised questions about the role of ABA in controlling DMA [8, 13].

ABA concentrations in plant tissues are maintained dynamically by opposing forces of synthesis; transport and catabolism to inactive products. Inactivation of ABA in plant tissues can occur via two major pathways: oxidation and conjugation. In general active ABA can be rapidly metabolized to some inactive structures in higher plants through conjugation which involves formation of ABA-glucosyl ester (ABA-GE) or glucosyl ether (ABA-GS) [14]. The physiological

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significance of ABA conjugations in plants remains unclear. ABA-GE accumulates in plant tissues with age and during stress treatments and is known to be a physiologically inactive conjugated ABA and the end product of its metabolism rather than storage or transport form [15, 16]. Results from other plant systems show that a fall in free ABA is closely associated with a rise of conjugated ABA [17].

ABA has been shown to stimulate the movement of sugars and regulate sink strength in small grains, some horticultural crops and in legume seeds like soybeans, pea and bean [18, 19]. It has been proposed that ABA may stimulate sucrose transport into filling seeds of legumes, potentially regulating seed growth rate. However no data is available on endogenous ABA levels in *C. cajan* seed and pod. In this study, endogenous ABA was measured by raising antibodies against ABA-BSA conjugates in rabbits. The objective of this study was to determine the role of endogenous ABA in dry matter accumulation and sink size development in seeds and pods of pigeon pea.

MATERIALS AND METHODS

Certified seeds of *Cajanus cajan*, V_1 (Black seeded) and V_2 (B.D.N_2) were selected for the study and purchased from the commercial market, Rajkot, India. The growth experiment was studied during the month of July-February 2005-06. Seeds of both the varieties were soaked in water for three hours and sown 2 cm deep in black cotton soil at botanical garden in Saurashtra University, Rajkot (20°17′ N; 70°49′ E), India. Standard agricultural practices including irrigation, application of fertilizers and insecticides etc., were maintained throughout the crop growth to maximize the yield. NPK fertilizer was applied to the soil before planting while pesticide was given twice during the flowering period. Irrigation practices were followed at alternate days throughout the growth period. Flowers were tagged on the day of anthesis. Pods of equal size were harvested at the interval of three days for growth analysis and estimation of endogenous ABA.

Growth analysis

Fresh and dry weight measurements: For the measurement of fresh weight (FWt) and dry weight (DWt), pods were harvested at every three days intervals. Seeds were separated from the pods. Length of pod and number of seeds per pod were calculated. Pods and seeds were weighed before and after oven drying at 80°C for five days to a constant weight. Water Content (WC) was determined by differences in fresh and dry weights. Data were taken in triplicate and the mean fresh weight, dry weight and water content were calculated with ±standard deviations.

Raising of antibodies against ABA

Preparation of ABA-BSA and ABA-casein conjugate: To raise antibodies against ABA, ABA-BSA and ABA-casein conjugates were prepared as described by Gokani and Thaker [20]. Abscisic acid (132 mg) was dissolved in 3 ml of DMF (N-N,dimethyl formamide): distilled water (2 :1) and added drop wise in 250 mg BSA dissolved in distilled water and adjusted to the pH 8.5. After addition of ABA, the pH was readjusted to 8.0 with 1N NaOH. N-ethyl-n-(3-dimethylaminopropyl)-carbodiimide hydrochloride (210 mg) was added to the ABA-BSA mixture in 4 portions within 90 min. The complete preparation was then stirred constantly for 19 h in dark at 4°C. It was then dialyzed against tap water for 4 days and stored at 0°C.

Immunization and separation of IgG: The ABA-BSA conjugate was mixed with an equal volume of Freund's complete adjuvant and injected into two rabbits by intramuscular injection. Antigens are more immunogenic when presented in an insoluble form with an adjuvant. Booster doses of injections were given periodically to raise the antibody titer. Rabbits were bled periodically and every time about 10-15 ml of blood was collected. Blood was incubated at 37°C for 1 h and serum was separated. ?-immunoglobulin (IgG) was collected by passing the serum through DEAE cellulose pre-equilibrated with 0.01M phosphate buffer (pH 8.0). The purified IgG was concentrated to the original volume of serum taken, by 0.01M phosphate buffer (pH 8.0). Purified antibodies of each hormone were stored in glass vials at-20° C and used for estimation after appropriate dilution.

Extraction of ABA from seed and pod: Seeds and pods of different ages were crushed with liquid nitrogen. From the frozen samples 500 mg powder was mixed with 5 ml of 80% methanol containing 100 mg ascorbic acid as an antioxidant. The mixture was incubated for 48 h in dark. The mixture was centrifuged at 10,000 g for 10 min and supernatant was collected. Pellets were washed twice with 80% methanol, pooled supernatant was collected and kept for evaporation in dark. Final volume of the samples (10 ml) was prepared with phosphate buffer saline (pH 7.2) and directly used for the estimation of ABA.

Estimation of ABA content: Endogenous level of ABA was estimated by a comparatively more sensitive and specific technique i.e. indirect ELISA. To avoid

Table 1: Correlations among endogenous ABA Seed^{-1}, ABA Pod^{-1} and DWt, WC, rate of DMA, WAR in two varieties of *C. cajan*

	ABA Seed^{-1}		Conjugated ABA Seed^{-1}		ABA Pod^{-1}	
	V_1	V_2	V_1	V_2	V_1	V_2
DWt	0.52*	0.02	0.01	-0.54	-0.77	0.05
WC	0.72***	0.45	0.02	-0.49	-0.66	0.63*
Rate of DMA	0.72***	0.58*	-0.08	-0.37	-0.08	0.33
WAR	0.12	0.57*	0.13	0.04	0.47	-0.087

*Significant at $P \leq 0.05$, ** Significant at $P \leq 0.01$, *** Significant at $P \leq 0.001$

reaction with tagged protein, ABA-casein conjugate was diluted with coating buffer (10mM carbonate buffer, pH 9.7) and 300 µl coated on ELISA plate. The plate was incubated for overnight at 4°C, followed by washing with PBS-T (phosphate-buffered saline pH 7.2 containing 0.05% Tween 20). The next step involved was blocking of free protein binding sites of well with egg albumin and incubated for 1 h at 37°C then the plate was washed thrice with PBS-T. Antibodies against ABA mixed with samples were coated and incubated for 3 h at 37°C. Finally, the plate was coated with second antibody anti-rabbit goat IgG, tagged with peroxidase and the color was developed using O-phenylene diamine as a substrate. The reaction was terminated by addition of (50 µl) 6N sulfuric acid.

Relative binding values were calculated as B/Bo, where B and Bo are the values of absorbance in the presence (B) and absence (Bo) of internal standard hormone or sample, respectively. A standard curve of ABA was prepared in a range of 100-800 ng for each plate and values falling on the curve were taken. To test the sensitivity of the assay, each sample was mixed with known amount of ABA (400 ng) as an internal standard before reacting with the antibodies.

Conjugated ABA contents were determined according to the method of Bandurski and Schulze [21]. In brief, the hormone extract was allowed to hydrolyze with an equal amount of 2M KOH at 25°C for 60 min. The hydrolyzed samples were then used for the determination of total ABA content by immunoassay. The amount of conjugated ABA was calculated from the difference between total (hydrolyzed) and free ABA (unhydrolyzed) content at each stage of development. Data were taken in triplicates and mean value of three replicates was calculated. Endogenous level of ABA in developing seed and pod were expressed as µg ABA/mg seed or pod dry weight.

Statistical analysis: The statistical significance between means of endogenous ABA and growth parameters in seed and pod of two varieties was analyzed using analysis of variance (ANOVA). Correlation coefficient was worked out between growth parameters (i.e. DWt, WC, rate of DMA and WAR) and endogenous ABA (Table 1) during the entire period of seed development. P values significant at 0.05 or less than that were considered for the data interpretation. Values were presented as mean±SD.

RESULTS

Growth analysis: On the basis of seed weight, size of seed was more in V_1 than V_2. In V_1 DWt per seed

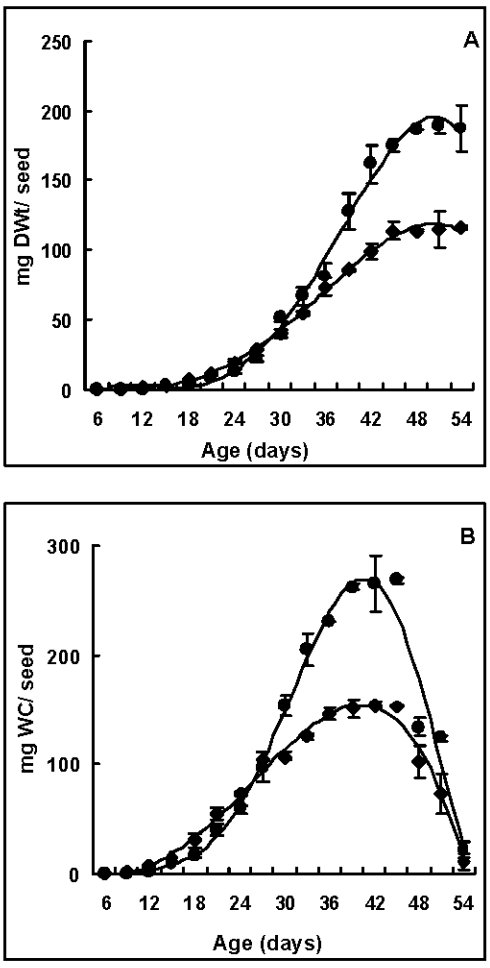

Fig. 1: Changes in dry weight (A) and water content (B) in developing seeds of V_1 (•) and V_2 (♦)

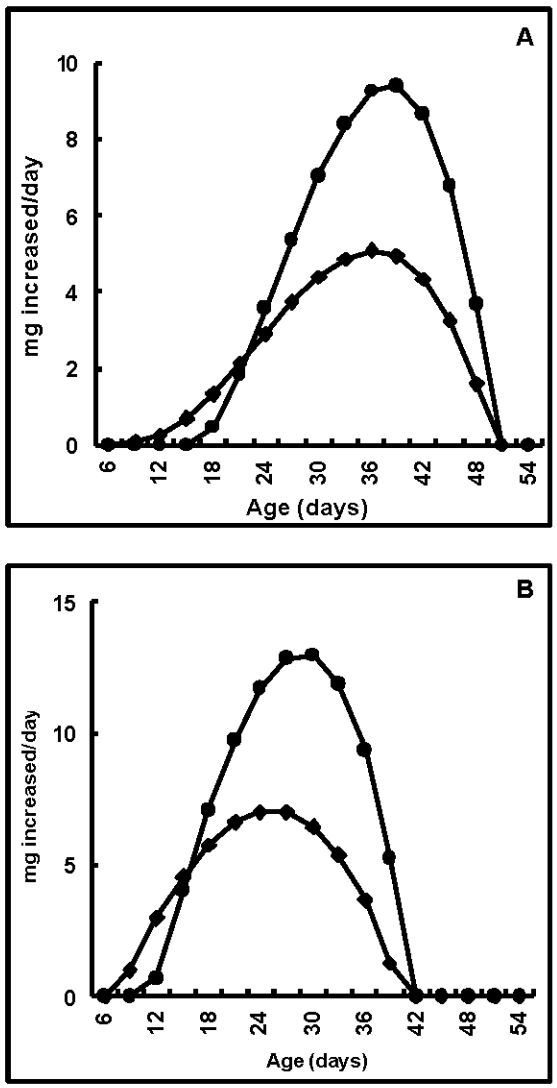

Fig. 2: Changes in rate of Dry Matter Accumulation (DMA) (A) and Water Accumulation Rate (WAR) (B) in developing seeds of V_1 (•) and V_2 (♦)

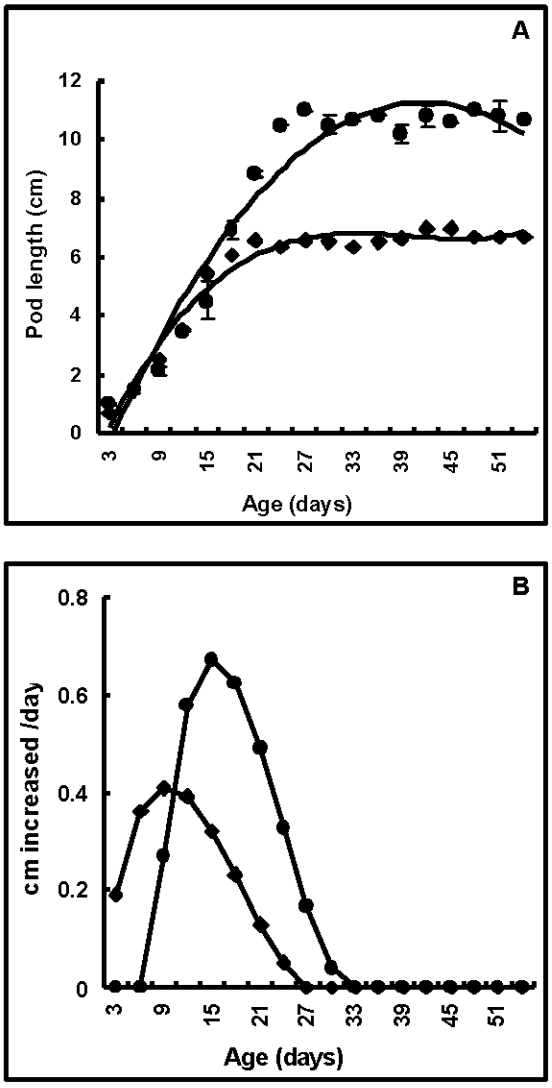

Fig. 3: Changes in length of pod (A) and rate of pod length (B) in developing pods of V_1 (•) and V_2 (♦)

increased up to 45 d and then it was stabilized in later stages. Maximum DWt was 187 mg/seed at 54 d (Fig. 1A). WC per seed increased up to 39 d, stabilized up to 45 d and then declined at later ages. Maximum WC was 268.4 mg/seed at 45 d (Fig. 1B). In V_2 DWt of seed increased up to 45 d, stabilized in later stages and peaked at 54 d (115.8 mg). WC per seed increased gradually up to 36 d, stabilized up to 45 d and declined in later ages. Maximum value of WC was 153.25 mg/seed at 45 d (Fig. 1B). The maximum rate of DMA was observed at 39 d (9.3mg) in V_1 and at 36 d (5.07 mg) in V_2 whereas the maximum WAR was observed at 30 d (12.99 mg) in V_1 and at 27 d (7.011 mg) in V_2 (Fig. 2A and 2B).

The number of seeds per pod was 6-7 in V_1 and 4-5 in V_2. Therefore, the length of pod was also double (11-12 cm) in V_1 than in V_2 (5-6 cm). In V_1 the length of pod increased up to 27 d and at later stages it was stabilized (Fig. 3A). In V_1 the maximum length of pod was 11 cm at 27 d and the maximum rate of length was 0.672 cm at 15 d (Fig. 3B). In V_2 the length of pod was increased up to 21 d and at later stages it stabilized. In V_2 the maximum length of pod was 6.95 cm at 42 d (Fig. 3A) whereas the maximum rate of length was 0.471 cm at 9 d (Fig. 3B).

In V_1 DWt per pod increased gradually up to 36 d, stabilized at later stages and maximum value was observed on 45 d (591 mg) (Fig. 4A). WC per pod increased up to 27 d, stabilized up to 42 d and then

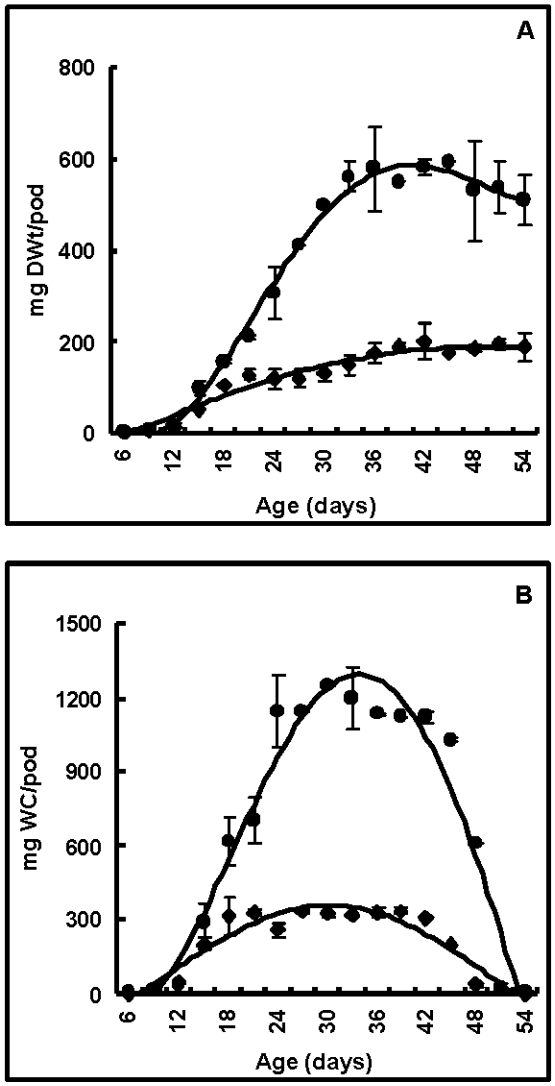

Fig. 4: Changes in dry weight (A) and water content (B) in developing pods of V_1 (•) and V_2 (♦)

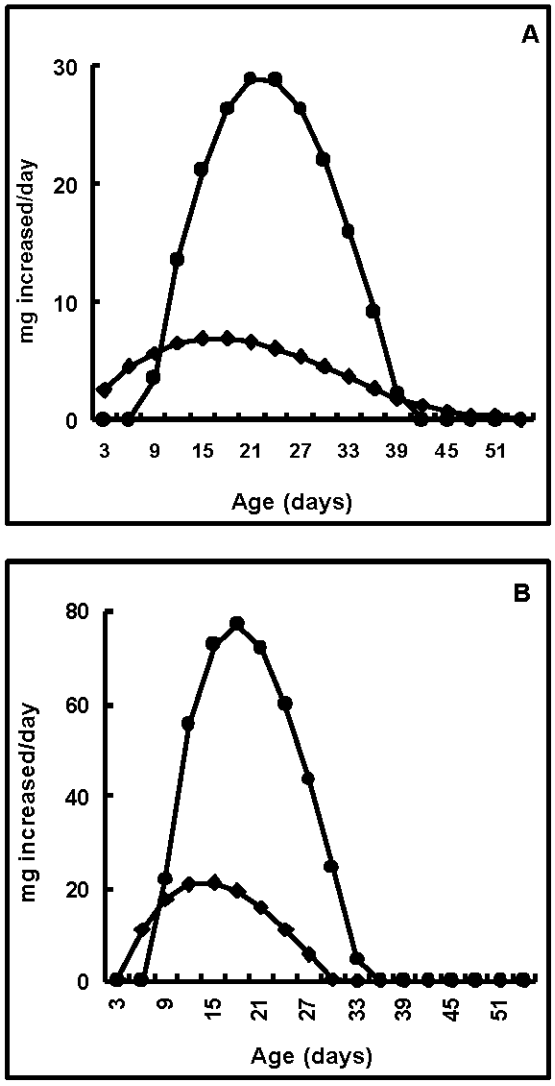

Fig. 5: Changes in rate of Dry Matter Accumulation (DMA) (A) and rate of water accumulation (B) in developing pods of V_1 (•) and V_2 (♦)

declined at later ages. Maximum WC per pod was 1254 mg at 30 d (Fig. 4B). In V_2 DWt of pod increased gradually up to 42 d, stabilized at later stages and achieved maximum value at 42 d (201.66 mg) (Fig. 4A). WC per pod increased gradually up to 27 d, stabilized up to 42 d and declined in later ages. Maximum value of WC was 339 mg at 39 d (Fig. 4B).

The maximum rate of DMA of pod was 28.84 mg at 21 d in V_1 and 6.9 mg at 18 d in V_2 (Fig. 5A). The maximum WAR was 77.15 mg at 18 d in V_1 and 21.3 mg at 15 d in V_2 (Fig. 5B).

Changes in endogenous ABA in seed: Free and conjugated forms of ABA were measured from seeds of both the varieties. The ABA content was not detected till 24 d in V_1 seed, later on it raised and peaked at 36 d (0.457) (Fig. 6A). During the maturation phase ABA decreased and at 51 d it became zero. Similarly in V_2 seed, ABA level was not detected till 18 d, increased rapidly thereafter and peaked at 21 d (0.526). From the 24 d gradual declined trend was observed (Fig. 6B). It was observed that in V_1 and V_2 seed, the value of free ABA remained almost equal but their accumulation ages were different.

Conjugated ABA remained four times higher in V_2 seed as compared to V_1 but their age of accumulation was different (Fig. 7). In V_1, conjugated ABA peaked at 18 d (0.727), declined gradually and second peak was observed at maturation phase (0.427) (Fig. 7A). In V_2, conjugated

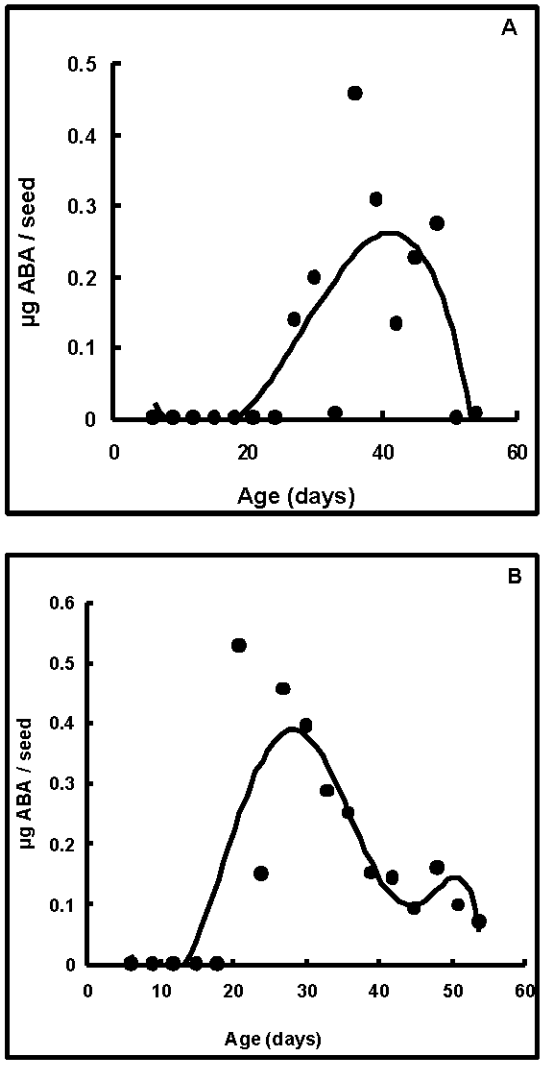

Fig. 6: Changes in free ABA (•), µg/seed in developing seeds of V_1 (A) and V_2 (B)

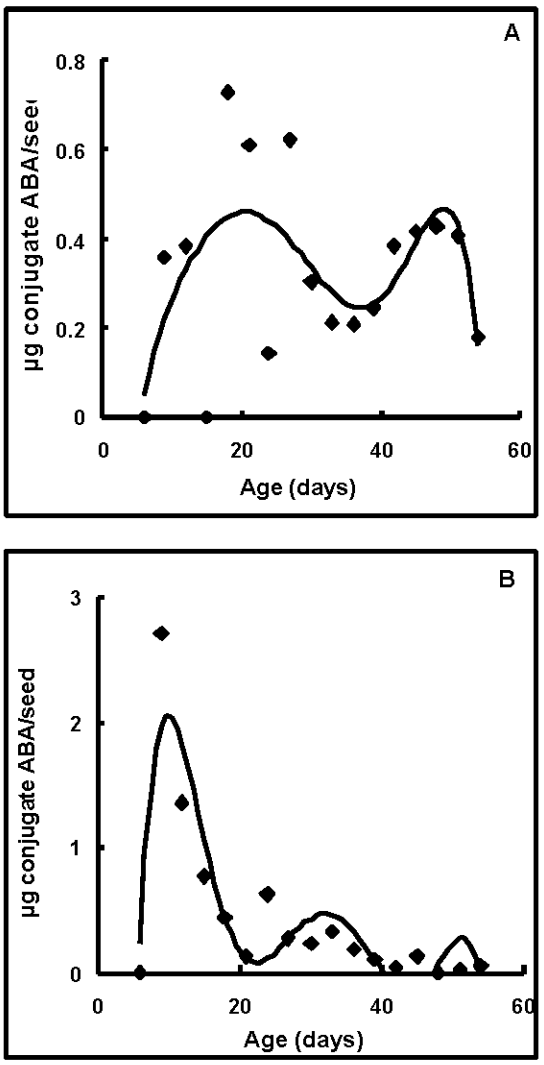

Fig. 7: Changes in conjugated ABA (♦), µg/seed in developing seeds of V_1 (A) and V_2 (B)

ABA increased gradually, peaked at 9 d (2.70) and decreased gradually up to 18 d (0.44). From 27 d the value remained lower and constant (Fig. 7B).

Changes in endogenous ABA in pod: In V_1, up to 15 d of pod development, maximum ABA level (0.237) was observed and declined gradually up to 36 d (0.005). Again second peak was observed at 42 d (0.161) and afterwards it declined (Fig. 8A). In V_2, ABA level was undetectable initially (up to 12 d), accumulated from 15 d onwards, remained higher up to 42 d (0.244) and later on declined to zero (Fig. 8B).

DISCUSSION

Data on dry weight and water content of seed and pod were fitted to appropriate polynomial equation. The rate of DMA and WAR were obtained from the fitted curve. On the basis of growth analysis seed development in two varieties is divided into four distinct stages. Initial lag phase of DMA (0-15 d) is considered as cell division phase (i), rapid water uptake (12-36 d in V_1 and 9-36 d in V_2) as cell elongation phase (ii), rapid rate of DMA (21-42 d in V_1 and 18-39 d in V_2) as DMA phase (iii) and stabilization of DMA (42-54 d in V_1 and 39-54 d in V_2) as maturation phase (iv). Overlap between these phases was observed in both the varieties studied (Fig. 1 and 2). Since these phases continued for stipulated time period, marked overlap in these phases was observed in both the varieties studied (Fig. 1). Similar growth phases are described for seed development in cotton [22] and Hibiscus [23].

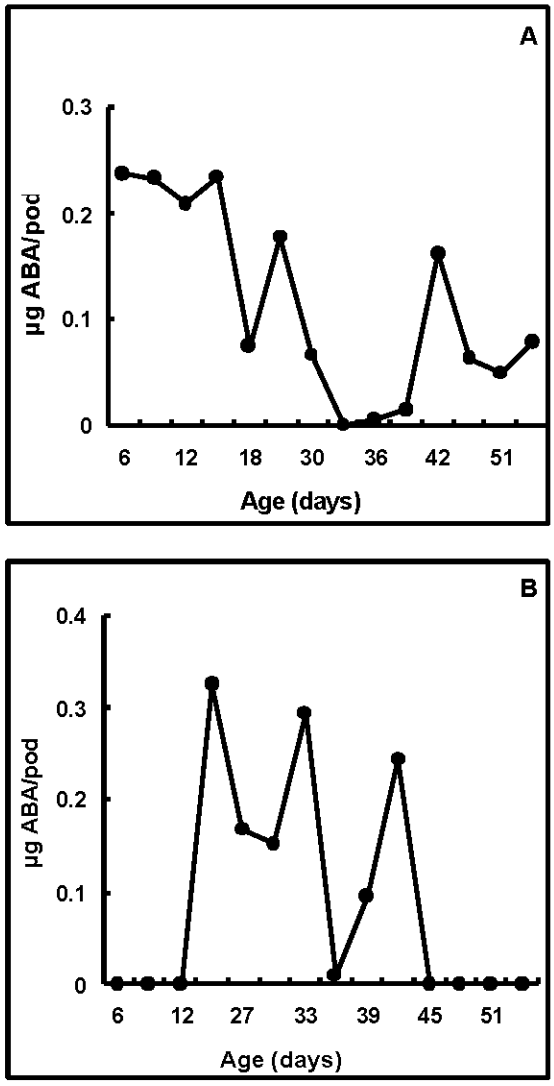

Fig. 8: Changes in free ABA (•), µg/pod in developing pods of V_1 (A) and V_2 (B)

Free ABA level in seed: Changes in free ABA levels in seeds of V_1 and V_2 are presented in Fig. 6A and 6B. Analysis of variance of these two varieties showed insignificant difference in endogenous ABA level during seed development. During cell division phase, ABA levels remained low in both the varieties. It increased in early elongation phase (21 d) in smaller seed (V_2) and at 27 d in bigger seed (V_1). Thus cell elongation phase of V_2 seed was remarkably affected by ABA (Fig. 6A and 6B). Though the values of free ABA remained almost equal in both the varieties, the difference was observed at the growth phases suggesting the role of ABA in cell size development in seeds.

It was proposed in soybean seeds that genotypes with high seed growth rates would be characterized by high concentrations of ABA and sucrose in their tissues [19]. In this study, the rate of DMA and rate of water accumulation in seeds showed significant difference (Fig. 2A and 2B) and remained double in V_1. Though, the growth rate was higher in V_1, ABA level remained similar in both the varieties. Remarkable difference in ABA level during DMA phase suggests the role of ABA in dry matter accumulation (Fig. 6) in seed development.

Earlier Gokani *et al.* [24] has also observed in developing cotton seed that ABA content was negligible during the early phases of seed development but increased at later phases. In this study, at initial stage (cell division and elongation phases), the difference in DWt and WC was not statistically significant in V_1 and V_2; whereas at later stages (from 30 d to 54 d) seed DWt and WC showed difference in both the varieties ($P<0.05$). The parallelism between ABA concentration and the rate of DMA in the seeds of several grain crops indicates a role of the hormone in promoting assimilates unloading [25]. Earlier various workers have suggested that increase in ABA content with increase in grain weight is an indication of the involvement of ABA in grain development [8, 13]. Presence of ABA at later stage of seed development suggests a role of ABA in the physiology of seed maturation and germination [26].

Conjugated ABA level in seed: In this study, ABA remained in bound form up to 27 d (during cell division and cell elongation phases) in V_1 and up to 15 d (cell division) in V_2, suggesting its regulatory role in cell division and cell elongation phases (Fig. 7A and 7B). In V_1, second peak was observed at maturation phase while in V_2 it peaked at cell division phase only and decreased gradually from the cell elongation phase. From 27 d, the value remained lower and almost equal. In V_2 seed as the conjugated ABA declined rapid increase in free ABA started (Fig. 6B and 7B). The bound form of ABA was very high in V_2 than V_1. In general no statistical significant correlation was observed with growth parameters and conjugated ABA level (Table 1).

The hormonal status in cells found as free (readily available form) or conjugated (which is stored or bound) forms, regulate the growth and developmental phases. Generally in plants, ABA conjugates accumulate with age and during stress treatment [27-29]. Conjugation of ABA is thought to be irreversible and may represent a mechanism for protecting tissue from the physiologically active free form of ABA [14].

It was observed that ABA has stimulated the movement of sugars in various economically important crop plants [19] and thus improved fruit yield and

quality [30]. Considerable events revealed that ABA levels increase sharply, rise during maturation and then fall to low levels in the dry seed in number of varieties. However it has been proved that embryonic ABA plays an important role in seed development [31] but large concentrations of ABA probably inhibit cell division by depression of cell-cycle gene expression [9]. Many workers have reported that inhibition of cell division by ABA in the developing embryo/endosperm results in a weak sink for assimilates, causing abortion of the young ovaries [32, 33].

ABA level in pod: No significant difference of free ABA level was observed in developing pod of two varieties. In V_1 pod, high accumulation of ABA was recorded during early days of development whereas, in later stages it deceased gradually and again accumulated at maturity (Fig. 7A). DWt and WC showed negative correlation with ABA suggest that with increase in DWt and WC the level of ABA decreased (Table 1). In contrast to this in V_2, ABA level was not detected during the early and later pod development. The accumulation of ABA was observed only in between 15 d to 42 d (Fig. 7B). A positive and significant correlation of WC and DMA and presence of ABA during the pod development in V_2 suggests the role of ABA in growth and development. Brenner [34] proposed a model in which soybean development may be correlated through the production of ABA in leaves and transport to other sinks including the fruit. ABA has been reported to be related to abscission of various plant structures [35]. However, evidence suggests that ABA inhibits pod abscission and play a role in partitioning of photoassimilates to developing soybean fruit [25]. A positive relationship between exogenously applied ABA and sink activity has been shown in wheat [36] and barley [37]. ABA stimulates the accumulation of sucrose in a range of tissues and a correlation has been observed between dry matter accumulation and endogenous ABA levels in the sink regions of some plant species [38]. ABA has been implicated in the regulation of sink strength in small grains, soybeans and some horticultural crops through mediation of phloem unloading [18].

CONCLUSION

Thus the data collected in this experiment and earlier studies on seeds of other higher plants done elsewhere lead to the conclusion that ABA has an important role in inhibition of cell elongation and promotion in DMA in seeds. A negative correlation of ABA in pod and positive and significant correlation of ABA in seed of V_1 suggest the role of ABA in seed development. It is further suggested that though the amount of ABA was equal but the difference in physiological age (for ABA accumulation) is responsible for the variation in seed size of both the varieties.

Abbreviations

ANOVA	Analysis of variance
BSA	Bovine serum albumin
DEAE	Diethyl amino ethyl cellulose
DMA	Dry matter accumulation
DWt	Dry weight
ELISA	Enzyme linked immunosorbent assay
PBS	Phosphate buffer saline
V_1	Variety-1
V_2	Variety-2
WAR	Water accumulation rate
WC	Water content.

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