FREE AND CONJUGATED IAA AND PAA IN DEVELOPING SEEDS OF TWO VARIETIES OF PIGEON PEA (*CAJANUS CAJAN*)

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Summary. The endogenous levels of indole-3-acetic acid (IAA) and phenyl acetic acid (PAA) were estimated in developing seeds of two different varieties of *Cajanus cajan*: Black seeded variety (V_1, big seeded) and B. D. N_2 (V_2, small seeded). Antibodies against IAA and PAA were raised in rabbits. By competitive ELISA, free and conjugated IAA and free forms of PAA were estimated. Growth analysis of seeds was performed by measuring fresh weight, dry weight and water content. All growth parameters studied showed that V_1 had higher dry weight, rate of dry matter accumulation and water content when compared with V_2. In both varieties, free IAA increased gradually with seed age. The level of conjugated IAA increased approximately twice, but in the bigger seeds conjugated IAA was present at the time of seed maturation, while in smaller seeds, conjugated IAA synthesis started parallel to dry matter accumulation. The role of conjugated IAA in controlling seed size is discussed. PAA content was also higher in V_1 compared to V_2. The role of auxins in *C. cajan* seed development is discussed.

Key words: *Cajanus cajan*, ELISA, indole-3-acetic acid, phenyl acetic acid, seed development.

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*Abbreviation***s:** BSA - bovine serum albumin; 2,4-D - 2,4-dichlorophenoxy acetic acid; ELISA – enzyme-linked immunosorbent assay; IAA - indole-3-acetic acid; IBA - indole butyric acid; IPA - indole pyruvic acid; NAA - naphthalene acetic acid; PAA - phenyl acetic acid.

INTRODUCTION

Seeds are essential for plant propagation and crop production and must satisfy the increasing and diverse requirements of both modern agriculture and the agro-industry. Grain legumes are crop plants selected for high seed yield and characterized by high metabolic activity and fluxes in seeds (Weber et al., 2005). Seed development is genetically programmed and includes transcriptional and physiological reprogramming mediated by sugar and hormone responsive pathway (Wobus and Weber, 1999; Gibson, 2004). Accumulation of protein and starch in the seed is a key process determining seed yield and quality of crop plants. Under developing stages, endogenous plant hormone levels may change and have an impact on both the yield and quality. Understanding of hormonal control should offer opportunities to modify plant growth for better agricultural production.

Indole-3-acetic acid (IAA) is the most abundant naturally occurring auxin involved in the control of plant growth and development. It is well established that IAA occurs either as a hormonally active free form or in bound forms in which the carboxyl group is conjugated to sugars and *myo*-inositol via ester linkages and to the amino acids or peptides via amide linkages (Cohen and Bandurski, 1982; Bartel et al., 2001; Ljung et al., 2002). Evidence has been obtained indicating that amino acid conjugates play an important role in IAA metabolism, particularly as temporary storage reserves and by initiating the catabolism of IAA (Cohen and Bandurski, 1982). Only free IAA is established to be the direct biologically active compound, but its conjugates help to maintain IAA homeostasis both by inactivating IAA and by serving as a reservoir of IAA that can be released upon hydrolysis (Bandurski et al., 1995).

Besides IAA, several closely related plant compounds have also auxin-like activity and occur as free forms and in a variety of conjugated forms.

These include 4-chloroindole-3-acetic acid (4-Cl-IAA), indole butyric acid (IBA), (Epstein and Ludwig-Muller, 1993; Bandurski et al., 1995) and phenyl acetic acid (Ludwig-Muller and Cohen, 2002). Phenyl acetic acid (PAA) was identified as a natural auxin-like growth regulator in plants (Wightman and Lighty, 1982). PAA mimics IAA bioassays and it is active at much higher concentrations than IAA (Fitzsimons, 1989). To estimate endogenous levels from plant tissues, bioassays for hormones are sensitive enough at picogram (pg) levels, but they are nonspecific.

Earlier, Rao and Rao (1975) estimated endogenous auxin in developing and germinating seeds of *C. cajan* and observed that auxin was important in synthesis and accumulation of reserve substances in the embryo. However, no detailed study has been reported to distinguish IAA and PAA during the entire period of *C. cajan* seed development. Analysis of endogenous hormonal levels from developing seeds provides the opportunity to explain complex developmental processes. Therefore, it is interesting to evaluate the possible roles of IAA and PAA at all stages of seed development. Competitive ELISA was developed as a rapid and effective procedure to evaluate endogenous IAA and PAA levels during the seed development of *C. cajan*.

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 of *Cajanus cajan* was studied during July – February 2005/2006. Seeds of both varieties were soaked in water for 3 h and sown 2 cm deep in black cotton soil in the botanical garden of Saurashtra University, Rajkot, India (20° 17' N; 70° 49' E). 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 was done at alternate day throughout the growth period. Flowers were tagged on the day of anthesis and the number of flowers was recorded every day. Developing pods of equal size were harvested at an interval of 3 days for

growth analysis and estimation of hormones.

Growth analysis

For measurements of fresh and dry weights, seeds were separated from the freshly harvested pods of different ages and weighted before and after oven drying at 80 °C for five days to a constant weight. Water content 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 IAA and PAA

To raise antibodies against IAA and PAA, IAA-BSA and PAA-BSA conjugates were prepared as described by Weiler (1981) and Gokani and Thaker (2002).

Estimation of IAA and PAA content

Seeds of different ages were crushed with liquid nitrogen and endogenous level of IAA, and PAA were estimated by a comparatively more sensitive and specific technique i.e. indirect ELISA (Gokani and Thaker, 2002). Conjugated IAA level was determined according to the method of Bandurski and Schulze (1977). Data were taken in triplicate and the mean value of three replicates was calculated.

Statistical analysis

Correlation coefficients between growth parameters (i.e. DW, WC, rate of DMA and rate of water content) and endogenous IAA and PAA were worked out during the entire period of seed development. P values significant at 0.1 or less were considered for data interpretation.

RESULTS

Growth analysis

The size of seeds was greater in V_1 while V_2 had small-size seeds. The number of seeds per pod was 6-7 and 4-5 in V_2 and V_1, respectively.
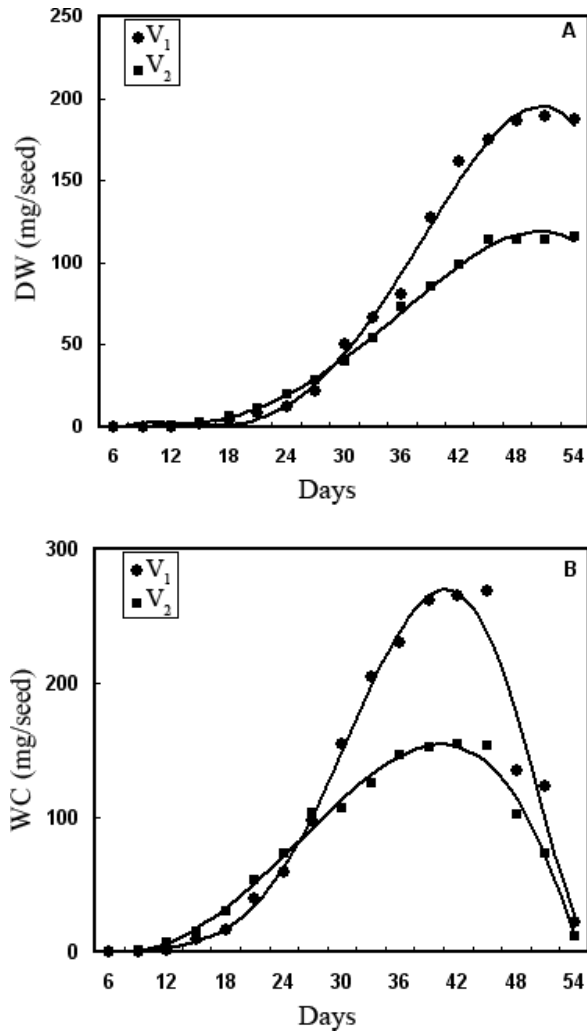

Fig. 1. Changes in dry weight (A) and water content (B) in developing seeds of V_1 (•) and V_2 (■).

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 dry weight per seed increased up to 45th day and stabilized in later stages (Fig 1A). The maximum dry weight was 187 mg/seed. Water content per seed increased up to 39th day, stabilized up to 45th day and then declined at later ages (Fig. 1B). The maximum water content was measured
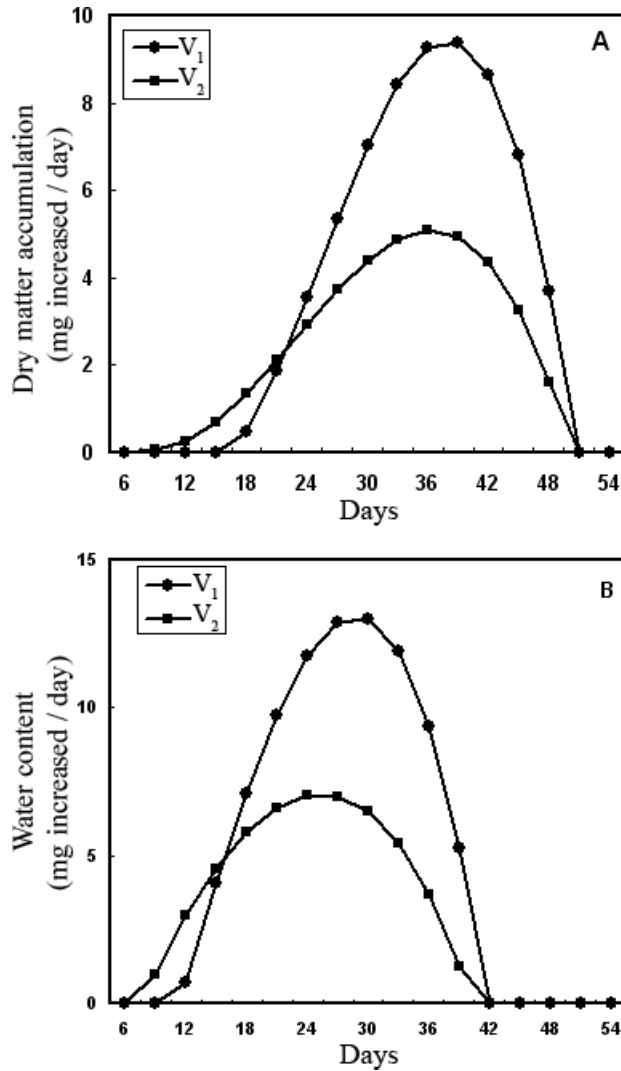

Fig. 2. Rate of dry matter accumulation (A) and water content (B) in V_1 (•) and V_2 (■).

to be 268.4 mg/seed at 45th day. In V_2 the dry weight of seeds increased up to 45th day and stabilized in later stages (Fig. 1A). The maximum dry weight was 115.8 mg/seed measured on 54th day. Water content per seed increased gradually up to 36th day, stabilized up to 45th day and declined in later ages (Fig. 1B). The maximum value of water content was 153.25 mg/
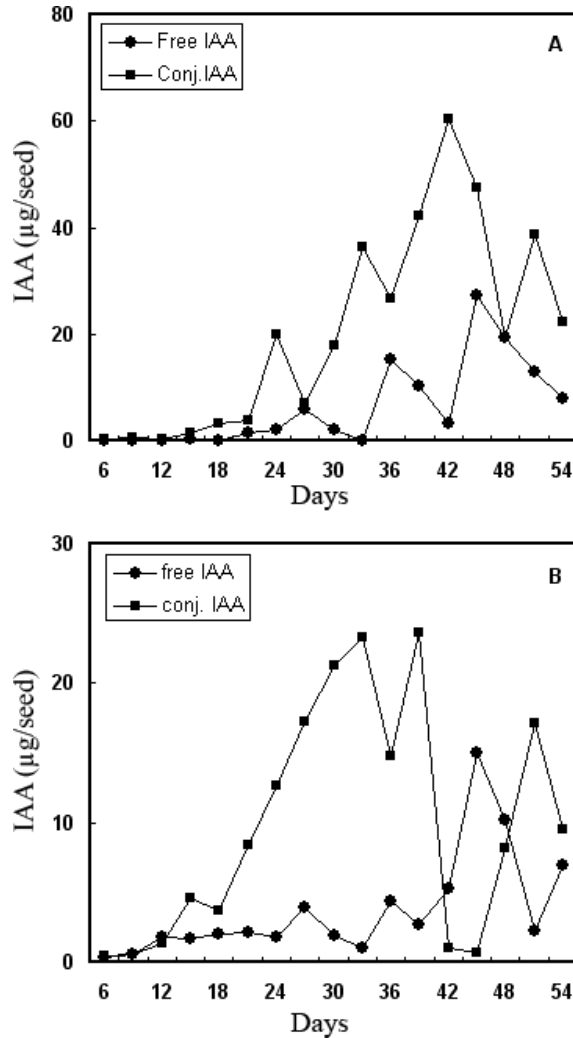

Fig. 3. Changes in free (•) and conjugated IAA (■) in developing seeds of V_1 (A) and V_2 (B).

seed measured on 45th day. The maximum rate of dry matter accumulation (DMA) was 9.3 on 39th day in V_1 and 5.07 on 36th day in V_2 (Fig. 2A). In V_1 the rate of water content increased up to 27th day and the maximum value (12.99) was measured on 30th day. In V_2 the rate of water content increased up to 21st day and stabilized on 27th day. The maximum rate of water content in V_2 was 7.011 measured on 27th day (Fig. 2B).

Changes in IAA levels in developing seeds of *C. cajan* during the entire period of seed development

In V_1 free IAA content remained low up to 24th day and increased up to 45th day (27.19 µg/seed). It declined gradually at later stages of seed growth (up to 54th day). Conjugated forms of IAA were also low up to 21st day and then increased gradually up to 42nd day. A peak was observed on 42nd day (60.28 µg/seed). During later stages conjugated IAA content gradually declined. In V_2 free IAA content remained low up to 24th day and increased slowly up to 42nd day. A maximum was observed on 45th day (14.99 µg/seed) followed by a decline thereafter. Conjugated IAA increased gradually up to 39th day. The maximum value of conjugated IAA was 23.67 µg/seed fresh weight on 39th day and then decreased gradually thereafter.

Changes in PAA contents in developing seeds of *C. cajan* during the entire period of seed development

In V_1 during the initial period of seed development (up to 21st day) PAA levels remained negligible followed by a gradual increase thereafter (Fig. 5A). A maximum value was observed on 45th day (32.35 µg/seed). At later stages PAA content declined and on 54th day it was 14.18 µg/seed. In V_2 PAA level increased gradually till 42nd day and then decreased at later ages (Fig. 5B). A maximum value was measured on 42nd day (17.30 µg/seed) and on 54th day it was 3.64 µg/seed. PAA level was higher in V_1 than in V_2 during the later stages of seed development.

DISCUSSION

Based on the growth pattern (Fig. 1 and Fig. 2) seed development can be divided in four distinct phases: (i) cell division (0-15 day), (ii) cell elongation (12-36 day in V_1 and 9-36 day in V_2), (iii) dry matter accumulation (21-42
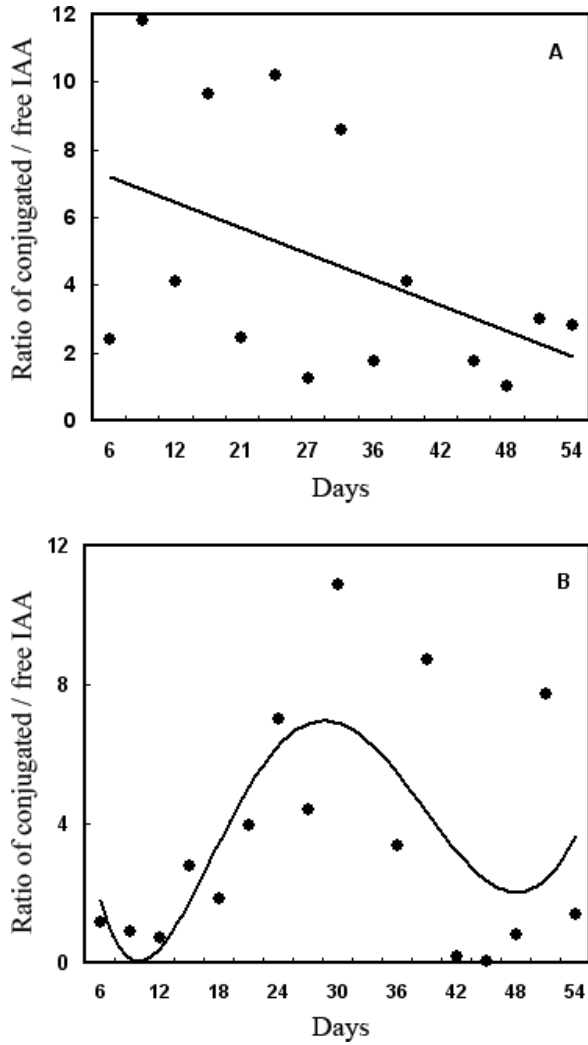

Fig. 4. Ratio of conjugated to free IAA in developing seeds of V_1 (A) and V_2 (B).

day in V_1 and 18-39 day in V_2), and (iv) cell maturation (42-54 day in V_1 and 39-54 day in V_2). During embryogenesis zygote divides repeatedly to form an embryo (phase I), these divided cells further enlarge (phase II) together with deposition of storage products (phase III) and the acquisition of desiccation tolerance along with water removal from the maturing seed
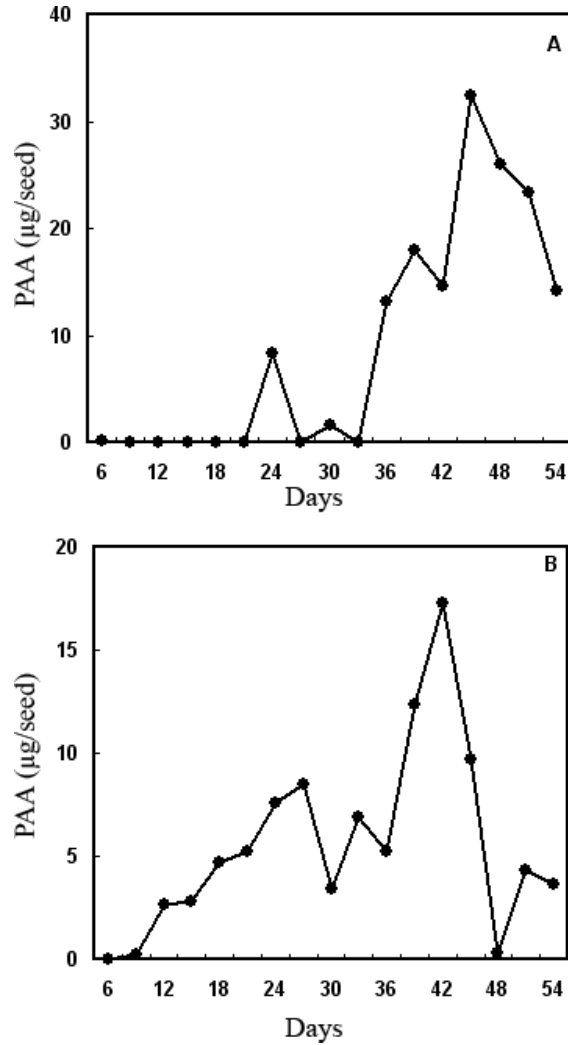

Fig. 5. Changes in PAA in developing seeds of V_1 (A) and V_2 (B).

(phase IV). Since these phases continued for a stipulated time period, a marked overlapping of these phases was observed (Fig. 1). Similar overlapping phases were also observed during seed growth of cotton (Rabadia et al., 1999), jojoba (Bagatharia, 2001) and *Hibiscus* (Thaker, 1999).

A close correlation between water content and the rate of dry matter accumulation was observed in both the varieties (Table 1), suggesting the important role of water content in dry matter accumulation. This is further supported by a double rate of dry matter accumulation in V_1 as compared with V_2 (Fig. 2A). Similarly, the rate of water content was also twicw as high in V_1 as compared to V_2 (Fig. 2B). These results suggest that high rate of water content can increase the rate of dry matter accumulation. The status of water content has been reported to play an important role in cotton and jojoba seed development (Rabadia et al., 1999; Bagatharia, 2001). The control of seed size involves complex interactions among the zygotic embryo and endosperm, the maternally derived seed coat and the parent plant (Schruff et al., 2006). As the water relations of the developing seed play a fundamental role in seed filling, and seed filling is sensitive to water shortage (Davies et al., 1999).

The role of auxin in cell division and cell elongation is well documented (Reed, 2001). A similar pattern of free IAA changes during seed development was observed in both varieties (Fig. 3), although the value

	Free IAA		Conjugated IAA		PAA		DMA	
	V_1	V_2	V_1	V_2	V_1	V_2	V_1	V_2
Dry weight	0.74***	0.70***	0.77***	0.24	0.89***	0.37		
Water content	0.58*	0.45*	0.88***	0.48*	0.60**	0.71***	0.88***	0.88***

Degrees of freedom (d, f) is 32   * Significant at $P<0.1$.
      ** Significant at $P<0.01$.
      ***Significant at $P<0.001$.

Table 1. Correlation coefficients between endogenous free and conjugated IAA , free PAA, dry weight and water content, and rate of dry matter accumulation and water content in two pigeon pea varieties.

of free IAA was double in V_1 than in V_2. During the initial stage (up to 24th day) free IAA level remained low, then it increased gradually (up to 45th day) and declined thereafter during seed maturation (Fig. 3). Studies on auxin during embryogenesis have been performed in various seeds (Hocher et al., 1992; Gregorio et al., 1995). In this study, IAA level was not estimated from the separated embryonic tissues, but the presence of IAA during this developmental phase suggests that IAA may have a crucial role in seed growth. The suspension culture of *C. cajan* cotyledons showed high frequency of somatic embryogenesis when supplemented with 2, 4-D in MS liquid medium (Anbazhagan and Ganapathi, 1999). However, the level of IAA increased also during the cell elongation and dry matter accumulation phase (Fig. 3). A maximum level of IAA content was observed on 45th day when there was a maximum amount of water content. The action of IAA is to cause loosening of the cell wall, thus resulting in increased water uptake and cell enlargement. Previously, the presence of 4-Cl-IAA has been reported from the developing bean seeds, which induced cell enlargement and water uptake to permit dry matter accumulation (Pless et al., 1984).

The tight control of IAA concentration is necessary for proper plant development. IAA is stored in conjugated forms that are mostly considered to be inactive. The level of conjugated IAA was more in V_1 as compared to

Compound	Antibodies against	
	IAA-BSA	PAA-BSA
IAA	100	0.6
PAA	1.0	100
IBA	0.5	-
IPA	-	-
Tryptophan	-	-
NAA	11.0	-
2,4-D	39.0	2.74

Table 2. Cross reactivity (%) of natural and synthetic auxins with antibodies against IAA and PAA conjugates.

V_2, but the time of accumulation of conjugated IAA was different in both varieties. In V_1 the conjugated IAA remained lower during the initial stage of seed development (up to 21st day) showing high accumulation with further seed development (Fig. 3A). In V_2 there was a gradual accumulation of conjugated IAA from 15th till 33rd day (up to cell elongation phase) followed by a decline at later stages (Fig. 3B). These results suggest that less free IAA during cell elongation phase of V_2 might have restricted seed size. Similarly, higher accumulation of conjugated IAA in the big seeds at later stages may be due to higher storage protein accumulation in the seed. Earlier, Yadav (1983) reported also maximum amount of free amino acids between 21-28 day and a subsequent decrease in free amino acids accompanied by a rapid accumulation of proteins up to 42nd day in developing *C. cajan* seeds.

The conjugated/free IAA ratio decreased with seed development in V_1 (Fig. 4A). This result suggests that during cell division and cell elongation phase cells hydrolyzed conjugated IAA to free forms, while at later stages the IAA was stored in conjugated forms. Similarly, conjugated IAA showed highly significant correlation with seed water content (Table 1). The conjugated/free IAA ratio increased gradually with seed development in V_2 and declined thereafter (Fig. 4B). Similarly, conjugated IAA showed less significant correlation with water content (Table 1). These results suggest that cells may not have sufficient IAA during cell division and cell elongation phases of seed growth and thus the size of seed remained smaller in V_2. IAA conjugates have been found in different plant parts, however, they are more abundant in mature seeds. It is believed that the major source of free IAA for young seedlings are IAA conjugates stored in the seeds during their maturation (Bialek et al., 1992). In legumes, the major portion of IAA present in seeds is present as amide-linked conjugates. Several IAA-peptide conjugates have been identified in bean seeds (Bialek and Cohen, 1986; Walz et al., 2002) and *Arabidopsis* (Walz et al., 2002). Bialek and Cohen (1989) demonstrated that free IAA almost disappeared during seed maturation of bean seeds whereas amide-linked IAA increased and became the major form of IAA.

In the present study, PAA levels gradually increased in V_2 followed by a decrease during seed maturation (Fig. 5B). In V_1 PAA was almost absent during the initial days (up to 21st day), however, accumulation of PAA was

observed during seed growth thereafter (Fig. 5A). Similarly, PAA showed significant correlation with seed dry weight (Table 1). To date free and conjugated IAA have been found in the developing seeds of *Vicia faba*, (Pless et al., 1984), *Phaseolus vulgaris* (Bialek and Cohen, 1989), *Lycopersicon esculentum* (Hocher et al., 1992) and *Sechium edule* (Gregorio et al., 1995), but no data are available on the quantitative correlations between IAA and PAA in developing seeds. PAA was found in the vegetative tissues of peas, wheat, maize, sunflower, tobacco and barley based on HPLC and GLC analysis (Wightman and Lighty, 1982). PAA showed also auxin-like activity by stimulating elongation of wheat coleoptiles and hypocotyls of sugar beet seedlings (Wheeler, 1977). Earlier Gokani and Thaker (2002) found a close corelation between PAA and fiber elongation. In the present study, it may be possible that at later stages of seed development, when IAA is conjugated, cells may utilize or synthesize PAA as an alternative source of auxin.

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