INFLUENCE OF LIGHT ON ENDOGENOUS HORMONAL CHANGES AND ASSOCIATED METABOLISM DURING GROWTH AND DEVELOPMENT OF LEGUMINOUS SEED

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(Rita S. Chudasama)

CONTENTS

Chapter 1

General Introduction

Legumes are broadly defined by their unusual flower structure and podded fruits. About 88% of the species examined to date have the ability to form nodules with rhizobia (de Faria et al., 1989) and are second to Graminae in their importance to humans (Grusak, 2002; Graham and Vance, 2003). They differ markedly from grasses, cereals and other non-legume crops because much of the nitrogen they require is produced through fixation of atmospheric nitrogen by nitrogen fixing bacteria residing in the root nodules. As a result, legumes are rich in protein. Legume seeds are put to a myriad of uses, both nutritional and industrial, and in some parts of the developing world they are the principal source of protein for humans (Wang et al., 2003). The majority of proteins in legume seeds consists of salt-soluble globulins, or storage proteins, that are synthesized during seed development, stored in protein bodies, and hydrolyzed during germination to provide nitrogen and carbon skeletons for the developing seedling.

In the present study, *Cajanus cajan* (Pigeon pea) was selected as a legume plant. It is a short-term perennial shrub which grows up to 4 meters high (usually 1-2 meters). The sowing of Pigeon pea is done during the Kharif season. This crop has a short-life and flowering begins 56 to 210 days after sowing (van der Maesen, 1989). Maturity ranges from 95 to 256 days. Ten maturity groups of Pigeon pea have been identified under Indian conditions. They are usually combined into four categories: extra early (120 days), early (145 days), medium (185 days) and late-maturing (200 days) cultivars (van der Maesen, 1989). It has slender, pointed trifoliate leaves and yellow or yellow and red flowers. Because of its deep roots, it grows well on semiarid land. It is a tropical grain legume with high tolerance of dehydration (Ford, 1984; Singal et

al., 1985; Flower and Ludlow, 1986; Lopez et al., 1987; Nandwal et al., 1991).

The cultivars of Pigeon pea are highly sensitive to photoperiods and thus this kind of study helps in prediction of date of flowering (Carberry et al., 2001). It is well known that light plays an important role in a number of plant developmental processes, including the initiation of cell differentiation in vegetative meristems, chloroplast development, hypocotyl elongation, leaf expansion and flowering (Dale, 1988; Halliday and Fankhauser, 2003). Quantity, quality and duration are the three principal characteristics of light which affect the plant growth. Light quantity refers to the intensity or concentration of sunlight, which varies with the seasons. Light quality refers to the color (wavelength) of light.

Light is functioning as an environmental signal that activates and modifies various growth and developmental processes in plants. All of these processes are regulated through plant hormones where effects of light and phytohormones can be synergistic, additive or antagonistic (Novakova et al., 2005). The relationship between the growth parameters and endogenous hormonal levels during fruit development can provide insights into yield-related processes. Understanding of hormonal control should offer opportunities to modify plant growth for better agricultural production. In legume crops both yield components (pod number and individual seed weight) are genetically determined (Tischner et al., 2003) and are subjected to environmental conditions that prevail during reproductive development (Fageria et al., 1997).

Plant hormones are signal molecules that regulate many developmental processes, including fruit development leading to mature fruit and viable mature seed. Growing fruits are metabolically very active and act as strong sinks for nutrients within the plant. Hormones may stimulate transport of nutrients through the phloem, modify the strength of the sink

by stimulating its growth and increase the ability for sucrose unloading from the phloem, or act on metabolism and compartmentalization of sucrose and its metabolites (Brenner and Cheikh, 1995).
From the literature, number of reports signifies that, growth regulators, i.e. auxins, gibberellins (GAs), and abscisic acid (ABA) are involved in regulating seed and fruit development (Ravishankar et al., 1995; Kende and Zeevaart, 1997; Sanvicente et al., 1999; Hansen and Grossmann, 2000; Naeem et al., 2004; Azhar et al., 2005; Knoche and Peschel, 2007).

Auxin is growth-promoting substance produces in plants and enhances cell division and cell elongation. It stimulates cell enlargement, which is regarded as necessary step in plant growth. Indole-3-acetic acid (IAA), the main auxin in plants, is known to be involved in various plant growth and developmental processes and plants have evolved a complicated network to precisely regulate auxin activities (Leyser, 2002; Ljung et al., 2002; Berleth et al., 2004; Dharmasiri and Estelle, 2004).

Auxin acts in two ways during cellular growth: (i) by stimulating the acidification of the cell wall resulting in increasing extensibility; and (ii) by inducing specific mRNAs transcription that codes for proteins associated with cellular growth (Stals and Inze, 2001; Richard et al., 2002). It has long been appreciated that the hormone auxin is also a regulator of developmental processes in seed plants and play an important role in embryo development (Geldner et al., 2000; Souter and Lindsey, 2000). It stimulates assimilate transport and partitioning to and within developing grains (Darussalam and Patrick, 1998; Agusti et al., 2002). Phenyl-acetic acid (PAA) is another auxin like substance and plays a direct role in plant growth regulation (Fries, 1977). As is the case for several phytohormones, PAA is produced by plants (Isogai et al., 1967; Wightman and Lighty, 1982). Similar to IAA, this compound is able to

stimulate cell enlargement (Milborrow et al., 1975; Gokani and Thaker, 2002).

In several systems, it is unequivocally demonstrated that increase in the auxin and gibberellic acid levels during the post fertilization period is due to their active synthesis by the embryo and not because of their translocation from the maternal tissue (Eeuwens and Schwabe, 1975; Pharis and King, 1985). Like auxin, gibberellins are important growth hormones that have been shown to participate in most, if not all, stages of plant development. They also mediate environmental signals such as light and photoperiod and induced physiological responses like stem elongation and flowering (Hedden and Phillips, 2000). Previous studies have suggested that these GA-mediated growth responses are regulated by the modulation of cellular GA concentrations and by altering the ability of cells to respond to this hormone (Richards et al., 2001). Several studies showed that GAs increase wall extensibility (Nakamura et al., 1975; Stuart and Jones, 1977; Cosgrove and Sovonick-Dunford, 1989) and induce cell elongation.

ABA is an important regulator of seed development and maturation and several transcription factors determining ABA sensitivity are involved in promoting reserve accumulation, developmental arrest, and the imposition of dormancy (Finkelstein et al., 2002; Koornneef et al., 2002). ABA has also been shown to inhibit cell division in endosperm tissue. In several systems, ABA inhibits the effects of growth promoting hormones. For example, ABA application reduced the gibberellic acid induced sucrose uptake in excised veins of *Pisum sativum* (Estruch et al., 1989) and fusicoccin induced glucose uptake in beet root protoplasts (Getz et al., 1987). It has also been demonstrated that ABA inhibits the mobilization of assimilates in several systems (Estruch et al., 1989;

Kasim, 1991) and is antagonistic to the effects of growth promoters (Alvin et al., 1976). Porter (1981) and Ober et al. (1991) clearly showed that ABA reduces the resource mobilizing ability of the sinks in lupins and in maize, respectively.

In recent years there has been interest in developing shorter duration cultivars of Pigeon pea with wider adaptation to a range of environments and intensive production systems because of rising demands for its grain, leaves for grazing, as well as a need for diversification of important production system (Chauhan et al., 2002). There is also an interest in cultivation of this crop in nontraditional areas because of its beneficial effects on soil fertility and organic matter (Whitbread et al., 1999). Except Pigeon pea most of the legume seeds have been used as a model system for cellular and molecular study. Analysis of endogenous levels of each hormone during seed and pod development are scanty. There are no reports regarding changes in endogenous level of hormones during seed and pod development of *C. cajan*. Therefore it is one of the two major objectives of this thesis to estimate endogenous level of phytohormones during entire period of seed and pod development. For this objective two released varieties of *Cajanus cajan* are selected which differ in their flower initiation time for approximately one month (Figure 1).

The mechanism of hormone action in seed development is discussed as increase in cell number/seed or sink size (Thaker, 1999). Sink strength or ability to import assimilates is controlled by both cell expansion and endogenous hormonal regulation. Considering this, to understand the influence of endogenous hormonal changes on cell wall elongation for sink development; changes in cell wall components during the entire period of *C. cajan* seed development is designed in the present study.

The growth of a plant cell is significantly influenced by the properties of the cell wall. During cell expansion, wall loosening and incorporation of

new materials are coordinated to maintain cell thickness. The plant wall is thought to be more like a polymer composite held together primarily by non covalent bonds. According to a cell wall model (McQueen-Mason, 1996; Cosgrove, 1997), the primary cell wall consists of three co-extensive polymer networks: the cellulose–xyloglucan framework, pectin, and structural protein. This model is based on the fact that these wall components can be extracted and separated from one another under conditions unlikely to break most covalent bonds (Talbott and Ray, 1992; Carpita and Gibeaut, 1993). In this study, esterified and non-esterified pectic substances along with low and high molecular weight xyloglucans are extracted and estimated from the seed.

Pectins are largely acidic polysaccharides that form gels in the extracellular matrix and are present in all cell walls as well as mucilage. The two most common pectins found in dicotyledonous plants are polygalacturonic acid (PGA) and rhamnogalacturonan I (RG I) (Brett and Waldron, 1990; Carpita and Gibeaut, 1993; Cosgrove, 1997). Many of the acidic residues in pectins are esterified with methyl, acetyl and other unidentified groups (Kim and Carpita, 1992; McCann et al., 1994). The pectic network is clearly involved in a range of functions relating to physiology, growth, development and defense. It was proposed that xyloglucan metabolism controls plant cell elongation. In the growing plant cell wall, xyloglucan oligosaccharides may provide positive or negative feedback control during cell elongation (Yaoi and Mitsuishi, 2002). Schopfer et al. (2001) supported the hypothesis that regulation of cell wall growth depends on xyloglucan metabolism.

The transition between seed development and germination is accompanied by large-scale changes in gene expression patterns and metabolic pathways (Bradford et al., 2003). Upon imbibition after seed maturation, different sets of genes are expressed for reserve mobilization, tissue weakening and embryo expansion associated with

seedling growth (Bradford et al., 2000; Gallardo et al., 2002). Among the several environmental factors known to affect plant gene expression, light is most important. During the transition from darkness to light, the rate of hypocotyl elongation is determined from the integration of light signals sensed through the phototropin, cryptochrome and phytochrome signaling pathways (Smith, 2000; Folta, 2004).

Response to these light signals in the form of altered plant growth and development is termed as photomorphogenesis, a process that is distinct from that of photosynthesis, where far greater quantities of light serve as a source of energy for the fixation of carbon (Parks, 2003). In plant stems photomorphogenesis has been described principally in terms of light induced changes in elongation rate and the biochemistry underlying these changes (Shinkle et al., 1998). It was suggested that hypocotyls provide a model system for studying the basic processes of cell elongation because they grow rapidly in length and this growth is under tight control of well-defined developmental and environmental signals (Gendreau et al., 1997). Light is one of the stimuli that reduce the growth rate of hypocotyls. The responses of seedlings to specific environmental parameters or growth conditions can be used to elucidate signal transduction pathways in plants (Smets et al., 2005).

Light has a dramatic effect on elongation growth. This elongation is expected to be associated with higher levels of cell wall-loosening enzymes (Caderas et al., 2000). The plant cell wall is a unique fabric that is strong but usually thin, flexible and capable of both plastic and elastic extension.

In recent years, the cell wall has been the subject of extensive research because of the role it plays in many developmental processes. Fujino and Itoh (1998) described a clear difference in cell wall architecture between elongating and non-elongating regions, suggesting a

modification in the molecular form of pectin polysaccharides during the elongation. It is considered that structural changes in cell wall networks are regulated by enzymatic modification and therefore wall-modifying enzymes would be expected to play an important role in regulating the elongation of the cell wall. Previous studies, mainly employing enzymes extracted from wall preparations (Nevins, 1970), reported that the amount of glycosidase activity correlates with the growth rate of the tissue from which the walls are prepared.

From the glycoside hydrolases (glycosidases or carbohydrases) are enzymes that catalyze hydrolytic cleavage of O-glycoside bond and are classed among enzymes of carbohydrate catabolism. In plants, the α-glucosidases are associated with a variety of functions, including: hydrolysis of α-linked oligosaccharides resulting in cell wall degradation in endosperm during seed germination (Leah et al., 1995). Murray and Bandurski (1975) reported a positive correlation between growth levels and glucosidase activity in pea cell walls. β-galactosidase is one of the enzymes that seem to play a role in the process of modification of pectin structure, removing the β-galactosyl linkages on the neutral sugar side chains (Dopico et al., 1990b; Konno and Tsumuski, 1993; Valero and Labrador, 1993). Gomez and coworkers (1995) showed close correlation between β-galactosidase activity and release of sugars from hemicellulose and suggested that hemicellulose is one of the endogenous substrate for β-galactosidase. A positive correlation between β-galactosidase and β-glucosidase and cell wall growth has been detected in epicotyl of *Cicer arietinum* (Dopico et al., 1990a).

Schopfer et al. (2001) supported the hypothesis that regulation cell wall growth depends on xyloglucan metabolism. The characterization of xyloglucan extracted from leaves of light-grown pea plants indicates that xyloglucan metabolism is tissue specific (Schopfer et al., 2001). Furuya et al. (1969) has shown that the decreased growth in red-irradiated rice

coleoptiles correlates with a change in the mechanical properties of the cell walls. Schopfer and coworkers (2001) observed inhibition of cell extension by light in the mesocotyl elongation zone of maize (*Zea mays* L.) seedlings.

Several photomorphogenic effects of blue light have been characterized in higher plants, including the suppression of epicotyl or hypocotyls elongation in dicots (Cosgrove, 1981; Baskin, 1986). Sale and Vince (1959) found that short term red light exposures were more effective than blue light in inhibiting stem elongation in etiolated pea seedlings, while blue light was more effective at longer exposure periods. In plant tissue culture several workers have reported the inhibition of callus growth under UV, blue and red light (Beauchesne and Poulain, 1966; Beauchesne, 1966; Polevaya, 1967).

Among the proteins encoded by the structural genes, phosphatase is a key component in these adaptive mechanisms and plays an important role in phosphate metabolism. Phosphatase is an enzyme that catalyzes the hydrolysis of a wide variety of phosphate esters to release Pi. Phosphatases have been traditionally classified as being acid or alkaline phosphatase according to whether their optimal pH for catalysis is below or above pH 7.0 (Vincent et al., 1992). Many roles have been ascribed to acid phosphatases in plants, including the releasing of inorganic phosphate in the environment (Duff et al., 1994). Acid phosphatases are constitutively expressed in seeds during germination and their activities increased with germination to release the reserve materials for growing embryo (Biswas and Cundiff, 1991). A genetic analysis of the light response in epicotyl to phosphatase may help to understand regulatory factors i.e. in *Escherichia coli* 25 genes have been identified that are induced by phosphate starvation, including an alkaline phosphatase (Shinagawa et al., 1987).

Considering aforesaid, the objectives of this thesis are divided into two major parts:

1 Evaluation of endogenous hormonal level during entire seed and pod development from two released varieties of *C. cajan* differ in their photoperiods

- **A.** Growth analysis of seed and pod during developmental period
- **B.** Preparation of hormone-protein conjugates for IAA, PAA, GA and ABA
- **C.** Immunization and raising of antibodies in rabbits against each plant hormone
- **D.** Collection of serum and purification of antibodies
- **E.** Standardization of indirect ELISA (Enzyme Linked Immuno Sorbent Assay)
- **F.** Estimation of endogenous hormones; IAA, PAA, GA & ABA from developing seeds and pods during the entire period of growth
- **G.** Studies on role of cell wall components for sink development in seeds of both the varieties

2 Influence of different intensities of light on seedling growth and development

- **A.** Growth analysis i.e. dry weight and water content of seedling growing under different lights
- **B.** Evaluation of role of cell wall components in elongation of epicotyl under different light intensities
- **C.** Standardization and estimation of wall loosening enzymes, i.e. α-galactosidase, β-galactosidase and β-glucosidase
- **D.** Studies on expression of reserve mobilization enzymes, i.e. acid and alkaline phosphatase under influence of different light intensities

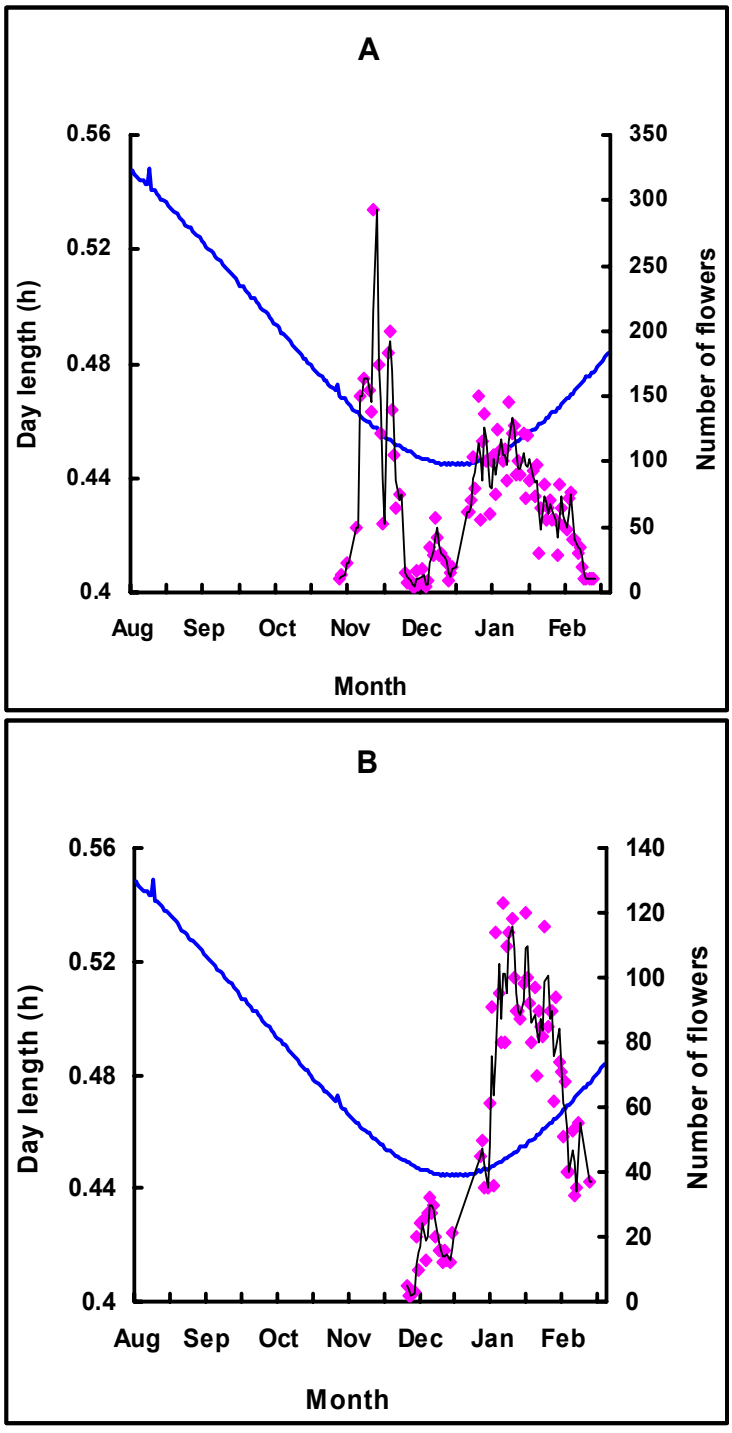


Figure 1:
Two varieties of Cajanus cajan; A (V_1, Black seeded) and B (V_2, B.D.N2) varying in their photoperiod (─)and flower numbers (-♦-)

Chapter 2

Changes in endogenous levels of IAA and PAA in developing seeds and pods

ABSTRACT

The endogenous content of Indole-3-acetic acid (IAA) and Phenyl acetic acid (PAA) were estimated in developing seeds and pods of *Cajanus cajan*. Antibodies against IAA and PAA were raised in rabbits. By competitive ELISA, free and conjugated IAA and free forms of PAA were estimated from the two different varieties; Black seeded variety (V_1, big seeded) and B.D.N_2 (V_2, small seeded). Growth analysis of seeds and pods was performed by measuring the fresh weight, dry weight and water content throughout the season. All the growth parameters studied showed that V_1 had more dry weight, rate of dry matter accumulation and water content than V_2. In both the varieties, the free IAA increased gradually with the seed age; value was near to double in bigger seed. Level of conjugated IAA was also approximately double but in the bigger seed, conjugated IAA was more at the time of seed maturation, while in the smaller seed, conjugated IAA synthesis started parallel to dry matter accumulation. PAA was also observed higher in V_1 than V_2. Significant difference was observed in PAA levels in pods of both the varieties. In V_1 pod PAA level showed significant correlation with WC while in V_2 IAA showed significant correlation with WC. Role of conjugated IAA and PAA in controlling seed size of *C. cajan* is discussed.

Abbreviations: BSA; Bovine serum albumin, 2,4-D; 2,4-Dichlorophenoxy acetic acid, DEAE; Diethylaminoethyl, DMF; N-N dimethyl formamide, DWt; Dry weight, 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, V_1; Variety –1, V_2; Variety –2, WC; water content

INTRODUCTION

Seeds are essential for plant propagation, crop production and satisfy the increasingly 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 seed is a key process determining seed yield and quality in crop plants. Under developing stages, endogenous plant hormone levels may change and have an impact on the yield and quality. Understanding of hormonal control should offer opportunities to modify plant growth for better agricultural production.

Auxin regulate numerous cellular and developmental responses in plants, including cell division, expansion, and differentiation; patterning of embryos, vasculature, and other tissues; and distribution of growth between primary and lateral root and shoot meristems (Casimiro et al., 2001; Marchant et al., 2002; Aloni et al., 2003). It is the major hormone controlling all stages of fruit development. The stages of the development are characterized by changes in fruit length, dry matter and fresh weight of seed. The growth of pollinated fruit is promoted by the hormones synthesized mainly in seeds (Ozga et al., 2003). It is probable that developing seeds produce signal molecules that regulate cell division and cell expansion of the surrounding fruit tissue.

It has been suggested that, like growing vegetative tissues, auxin promotes cell expansion in fruit by causing an increase in cell wall extensibility (Gillaspy et al., 1993). The process of cell enlargement in response to auxin requires water uptake (Ray et al., 1972). Indole-3-acetic acid (IAA) is one of the naturally occurring auxin involved in the control of plant growth and development and it has been successively used to improve many cash crops. Previously IAA content of seeds has been correlated with fruit growth (Varga and Bruinsma, 1976) and water uptake (Boyer and Wu, 1978). It also stimulates photoassimilates transport and increases sucrose concentration in developing wheat grains (Darussalam and Patrick, 1998)

It is well established that IAA can occur either as the hormonally active free forms 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). Evidences indicate 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 also have 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 (PAA), (Ludwig-Muller and Cohen, 2002). 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 concentration than IAA (Fitzsimons, 1989). To estimate endogenous levels from the 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 is important in synthesis and accumulation of reserve substances in the embryo. However, no detailed study has been reported to distinguished IAA and PAA during the entire period of *C. cajan* seed and pod development. Analysis of endogenous hormonal levels from the developing seeds provides the opportunity to explain complex developmental processes. Therefore, it is interesting to evaluate the probable roles of IAA and PAA in all the stages of seed development. To evaluate endogenous IAA and PAA level throughout the seed and pod development of *C. cajan*, a rapid and effective procedure, competitive indirect ELISA was developed.

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 commercially from Rajkot, India. The growth experiment of *C. cajan* was studied during the month of July – February 2005-06. Seeds of both varieties were soaked in water for three hours and sown 2 cm deep in black cotton soil at botanical garden of Saurashtra University, Rajkot, India (20^o 17' N; 70^o 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 applications were done at alternate day throughout the growth period. Flowers were tagged on the day of anthesis and numbers of flowers were recorded every day. Developing pods of equal size were harvested at the interval of three days for growth analysis and estimation of hormones.

Growth analysis

Fresh and dry weight measurements

For the measurement of fresh and dry weights, pods were harvested at every three days intervals (From the day of anthesis- 0 d to maturation - 54 d). Seeds were separated from the pods of different ages. Length of pod and number of seeds per pod were calculated. Pods and seeds were weighed before and after oven drying at 80 ^oC for five days to a constant weight. Water content was determined by differences in fresh and dry weights. Data were taken in triplicates and the mean fresh weight, dry weight and water content were calculated with $\pm$ standard deviations.

Raising of antibodies against IAA and PAA

Preparation of IAA–BSA and IAA–casein conjugate

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), respectively. IAA (52.3mg) or PAA (100mg) was dissolved in 2 mL of DMF and reacted with 75-µl tri-n-butyl amine and the solution was cooled to (0 ^{o}C). After this 40 µl isobutyl chlorocarbonate was added and incubated for 8 min at room temperature. This reaction mixture was added with constant stirring to 421 mg BSA dissolved in 22 ml of DMF: water (1:1, v/v) and 420 µl 1M NaOH. After 1 h incubation at 0 ^{o}C another 0.2 ml of 1M NaOH was added and stirring was continued for 5 h. The mixture finally dialyzed against 10% DMF for 24 h and against distilled water for 4 day.

Immunization and separation of IgG

The IAA-BSA and PAA-BSA conjugates were mixed with an equal volume of Freund's complete adjuvant and injected into two rabbits, respectively by intramuscular injection. Booster injections were given periodically to raise the titer. Rabbits were bled periodically and every time about 10-15 mL of blood was collected. Blood was incubated at 37 ^{o}C for 1 h and serum was separated. IgG (γ-immunoglobulin) was collected by passing the serum through DEAE cellulose pre-equilibrated with 0.01 M phosphate buffer (pH 8.0). The purified IgG was concentrated to the original volume of serum taken, by 0.01 M phosphate buffer (pH 8.0). Purified antibodies were stored in glass vials at 0 ^{o}C and used for estimation after appropriate dilution.

Antibody sensitivity test

To check accuracy and specificity of the polyclonal antibodies developed, the antibodies were reacted with the compounds having similar structure with that of IAA and/or PAA. To check the cross reactivity of antibodies developed against IAA-BSA conjugate, plate was coated with IAA-casein conjugate; while for the test of PAA-BSA conjugate, the plate was coated with PAA-casein conjugate. The antibodies against IAA and PAA were reacted with different naturally occurring and synthetic compounds having similar structure like IAA, PAA, IBA, IPA, NAA and 2, 4-D before coating on the plate. The percentage of cross reactivity was calculated by comparing absorbance value of different compounds with that of IAA and PAA, respectively. Antibodies against IAA and PAA did not react significantly with other naturally occurring auxins (Table 2) and hence they were used for estimation of endogenous IAA and PAA content from seed and pod sample.

Extraction of IAA and PAA from the seeds and pods

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 and 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 IAA and PAA.

Estimation of IAA and PAA contents

Endogenous level of hormones viz. IAA and PAA were estimated by a comparatively more sensitive and specific technique i.e. indirect ELISA (Gokani and Thaker, 2002). IAA-casein or PAA-casein conjugate (300 µl) was coated on ELISA plate and incubated for overnight at 4 ^{o}C, followed by washing with PBS-T. The next step involved was blocking of free protein binding sites of well with egg albumin and incubated for 1 h at 37 ^{o}C then the plate was washed thrice with PBS-T. Antibodies against IAA or PAA mixed with samples were coated and incubated for 3 h at 37 ^{o}C. Finally, the plate was coated with anti rabbit 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. After each coating, the ELISA plate was washed thoroughly with PBS containing 0.05% tweeen-20.

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 hormone standard or sample, respectively. To test the sensitivity of the assay, each sample was mixed with known amount of IAA or PAA (200 ng) as an internal standard before reacting with the antibodies.

Conjugated IAA content was determined according to the method of Bandurski and Schulze (1977). In brief, the hormone extract was allowed to hydrolyze with an equal amount of 2M KOH at 25 ^{o}C for 60 min. The hydrolyzed samples were then used for the determination of total IAA content by immunoassay. The amount of conjugated IAA was calculated from the difference between total (hydrolyzed) and free IAA (unhydrolyzed) content at each stage of development. Data were taken in triplicates and mean values were calculated. Each endogenous level of IAA and PAA in developing seed and pods were expressed as µg IAA/PAA seed^{-1} and µg IAA/PAA pod^{-1}.

Statistical analysis

Correlation coefficient was worked out between growth parameters (i.e. DWt, WC, rate of DMA and rate of water accumulation) and endogenous IAA and PAA during the entire period of seed and pod development (Table 3A, 3B). P values significant at 0.1 or less than that were considered for the data interpretation.

RESULTS

Growth analysis

Size of seed was more in V_1, while in V_2 it was small. In V_1 dry weight per seed increased up to 45 d and stabilized in later stages (Figure 2A). Maximum dry weight was 187 mg seed^{-1}. Water content per seed increased up to 39 d, stabilized up to 45 d and then declined at later ages (Figure 2B). Maximum water content was 268.4 mg seed^{-1} at 45 d. In V_2 dry weight of seed increased up to 45 d and stabilized in later stages (Figure 2A). Maximum dry weight was 115.8 mg seed^{-1} on 54 d. Water content per seed increased gradually up to 36 d, stabilized up to 45 d and declined in later ages (Figure 2B). Maximum value of water content was 153.25 mg seed^{-1} at 45 d. The maximum rate of dry matter accumulation (DMA) was 9.3 at 39 d in V_1 and 5.07 at 36 d in V_2 (Figure 3A). In V_1 the rate of water accumulation increased up to 27 d and the maximum value was 12.99 at 30 d. In V_2 the rate of water accumulation increased up to 21 d and stabilized at 27 d. The maximum rate of water accumulation in V_2 was 7.011 at 27 d (Figure 3B).

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. 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 (Figure 4A and Figure 4B). 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 whereas the maximum rate of length was 0.471 cm at 9 d (Figure 4A and Figure 4B).

In V_1 dry weight per pod increased gradually up to 36 d, stabilized at later stages and maximum value was observed on 45 d (591 mg pod^{-1}) (Figure 5A). Water content per pod increased up to 27 d, stabilized up to 42 d then declined at later ages. Maximum water content per pod was 1254 mg at 30 d (Figure 5B). In V_2 dry weight of pod increased gradually up to 42 d, stabilized at later stages and achieved maximum value at 42 d (201.66 mg pod^{-1}) (Figure 5A). Water content per pod increased gradually up to 27 d, stabilized up to 42 d and declined in later ages. Maximum value of water content was 339 mg pod^{-1} at 39 d (Figure 5B).

The maximum rate of dry matter accumulation of pod was 28.84 mg at 21 d in V_1 and 6.9 mg at 18 d in V_2 (Figure 6A). The maximum rate of water accumulation was 77.15 mg at 18 d in V_1 and 21.3 mg at 15 d in V_2 (Figure 6B).

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

Free and conjugated forms of IAA were estimated from the seeds of both the varieties. In V_1, free IAA content remained low up to 24 d and increased up to 45 d (27.19 µg $seed^{-1}$). It declined gradually at later period of seed growth (up to 54 d). Conjugated forms of IAA were also low initially up to 21 d and then increased gradually and peaked on 42 d (60.28 µg $seed^{-1}$, Figure 7A). During later period conjugated IAA content declined gradually. In V_2 free IAA content remained low up to 24 d and increased slowly up to 42 d. Peak was observed at 45 d (14.99) and declined in later period of seed. Conjugated IAA increased gradually up to 39 d. Maximum value of conjugated IAA was 23.67 µg $seed^{-1}$ at 39 d and then decreased gradually in later stages (Figure 7B).

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

In V_1 during initial period of seed development (up to 21 d) PAA levels remained negligible and there after days increased gradually (Figure 9A). A peak value was observed at 45 d (32.35 µg seed^{-1}). At later stages the value declined and reached to 14.18 µg seed^{-1}at 54 d. In V_2, PAA level increased gradually till 42 d then decreased at later ages (Figure 9B). A peak value was 17.30 µg seed^{-1} at 42 d and at 54 d it was 3.64 µg seed^{-1}. PAA level was higher during later stages of seed development in V_1 than V_2.

Changes in endogenous IAA and PAA levels in developing pod

Indole acetic acid (IAA)

In V_1 IAA level was not showed clear trend with pod development (Figure 10A). Maximum amount of IAA was present only at early days, peaked at 18 d (43.11 µg pod^{-1}) and declined later on. In contrast to V_2 the IAA level remained lower during initial days and maximum accumulation was observed at pod elongation period (18d to 45 d) and declined at maturation ages (Figure 10B).

Phenyl acetic acid (PAA)

In both pods PAA level showed same trend but remained five times higher in V_1 (Figure 11A, 11B). In both pods it remained lower initially, increased gradually till maximum DMA and declined at later. In V_1 peaks were observed at 27d (85.70 µg pod^{-1}) and 42 d (90.62 µg pod^{-1}) while in V_2 peaks were observed at 24 d (15.93 µg pod^{-1}) and 39 d (17.36 µg pod^{-1}).

DISCUSSION

Changes in growth pattern i.e. dry weight and water content (mg seed^{-1}); and rate of dry matter accumulation and water accumulation (mg increased day^{-1}) are presented in Figure 2 and 3, respectively. Based on growth pattern seed development was divided into four distinct phases i.e. (i) Cell division (0-15 d), (ii) Cell elongation (12-36 d in V_1 and 9-36 d in V_2), (iii) Dry matter accumulation (21-42 d in V_1 and 18-39 d in V_2), and (iv) Cell maturation (42-54 d in V_1 and 39-54 d in V_2). During embryogenesis, zygote divides repeatedly to form embryo (phase I), these divided cells enters to cell enlargement (phase II) and is characterized by deposition of storage product (phase III) and the acquisition of desiccation tolerance along with water removal from the maturing seed (phase IV) (Consonni et al., 2005). Since these phases continued for stipulated time period, marked overlap in these phases was observed. Similar overlapping phases were also observed with cotton (Rabadia et al., 1999), Jojoba (Bagatharia, 2001) and *Hibiscus* (Thaker, 1999) seed growth.

A close correlation was observed between water content and the rate of dry matter accumulation of seeds in both the varieties (Table 3A), suggesting the important role of water content in dry matter accumulation. This is further supported by double rate of dry matter accumulation in V_1 than V_2 (Figure 3A). Similarly, the rate of water accumulation was also double in V_1 as compared to V_2 (Figure 3B). These results suggest that high rate of water accumulation has increased 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). 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).

Role of auxin in cell division and cell elongation is well documented (Reed, 2001). Similar pattern of free IAA changes during seed development was observed in both the varieties (Figure 7A, 7B), although the value of free IAA was double in V_1 than V_2. During the initial days (up to 24 d) free IAA level remained low, increased gradually (up to 45 d) and declined thereafter as the seed matured (Figure 7A, 7B). Studies on auxin during embryogenesis have been established in various seeds, (Hocher et al.,1992; Gregorio et al., 1995 and Fisher-Iglesias et al., 2001). In this study, the IAA level was not estimated from the separated embryonic tissues, but the presence of IAA levels during developmental phase suggests that IAA may have decisive 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 also increased during the cell elongation and dry matter accumulation phase (Figure 7A, 7B). Peak level of IAA content was observed at 45 d where amount of water content was maximum. The action of IAA is to cause loosening of the cell wall and 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 the 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 compare to V_2 but the time of accumulation of conjugated IAA was different in both the varieties. The conjugated IAA remained lower during the initial age of seed (up to 21 d) in V_1 and afterward showed high accumulation with the seed development (Figure 7A). In V_2 there was a gradual accumulation of conjugated IAA from 15 d to 33 d (up to cell

elongation phase) and at later stages the content was declined (Figure 7B). These results suggest that less free IAA during cell elongation phase of V_2 might have restricted the size of seed. Similarly higher accumulation of conjugated IAA at later stages in big seed may be because of higher amount of storage protein accumulation in the seed. Earlier, Yadav (1983) has also reported maximum amount of free amino acids between 21-28 days and the subsequent decrease in free amino acids was accompanied by a rapid accumulation of protein up to 42 d in the developing seed of *C. cajan*.

The ratio of conjugated to free IAA decreased with seed development in V_1 (Figure 8A). This result suggests that during division and elongation phases, cells hydrolyze conjugated IAA to free forms, while at later stages the IAA is stored in conjugated forms. Similarly conjugated IAA showed highly significant correlation with water content of seed (Table 3A). The ratio of conjugated to free IAA increased gradually with seed development in V_2 and declined at later stages (Figure 8B). Similarly conjugated IAA showed less significant correlation with water content (Table 3A). 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 parts of plants 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 found 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) have demonstrated in developing Bean seeds that free IAA almost disappears during seed maturation and amide-linked IAA increases and becomes the major form of IAA.

In the present study, PAA level increased gradually in V_2 and than decreased during seed maturation (Figure 9B). In V_1 the level of PAA was almost absent during initial days (up to 21 d) but accumulation of PAA was observed at later days of seed growth (Figure 9A). Similarly, PAA showed significant correlation with dry weight of seed (Table 3A). To date free and conjugated IAA have been reported from 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 there are no reports on quantitative interrelations between IAA and PAA in developing seeds. Occurrence of PAA was obtained from the vegetative tissue of plants including peas, wheat, maize, sunflower, tobacco and barley from HPLC and GLC analysis (Wightman and Lighty, 1982). PAA also showed auxin like activity by stimulating elongation of wheat coleoptiles and hypocotyls of sugar beet seedlings (Wheeler, 1977). Earlier, Gokani and Thaker (2002) have also found a close relation between PAA and cotton 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.

Further, IAA levels measured from the developing pod of two varieties showed statistically insignificant difference while PAA levels showed significant difference. In pea fruit pod elongation normally depends upon the presence of seeds (Ozga et al., 2002). Here negligible levels of IAA and PAA at initial days of seed development suggest that during these days pod may acquire source of auxins from the seeds. Previously Bialek and Cohen (1989) observed in soybean that IAA content, which was relatively high in rapidly growing pods, declined to low levels as the seeds matured.

Recently Ashraf and co-workers (2006) showed that IAA treatment alleviated the adverse effect of water stress and enhanced the growth and yield of barley crops. Water content of V_2 pod showed significant correlation ($P<0.001$) with IAA while rate of DMA and rate of water accumulation showed negative correlation (Table 3B). In V_1 pod, PAA showed significant correlation with WC while rate of DMA and rate of water accumulation showed negative correlation. Uptake of water is regarded as the primary process influenced by auxin, close correlation of IAA and PAA with water content suggest its role in water uptake and pod development.

Table 1:

Results of Analysis of Variance between means of growth parameters in two varieties of *Cajanus cajan*

	DF	Mean (V_1)	Mean (V_2)	F-value
DWt Seed^{-1}	1 , 32	74.88	51.85	1.09
DWt Pod^{-1}	1 , 32	361.57	125.88	16.68***
WC Seed^{-1}	1 , 32	111.07	77.03	1.41
WC Pod^{-1}	1 , 34	667.9	201.25	12.9***
Rate of DMA Seed^{-1}	1 , 24	5.21	2.99	4.63*
Rate of DMA Pod^{-1}	1 , 34	10.97	3.69	6.62**
Rate of WCA Seed^{-1}	1 , 18	8.57	5.03	5.78*
Rate of WCA Pod^{-1}	1 , 34	24.02	6.9	5.27*
Length Pod^{-1}	1, 32	8.09	5.51	6.62**

***** Significant at $P<0.05$

****** Significant at $P<0.01$

******* Significant at $P<0.001$

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

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 3A:

Correlation coefficient between endogenous free and conjugated IAA seed^{-1}, PAA seed^{-1} and growth parameters in two varieties of *Cajanus cajan*

	Free IAA seed^{-1}		Conjugated IAA seed^{-1}		PAA seed^{-1}	
	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***
Rate of DMA	0.34	0.14	0.68**	0.58*	0.30	0.67**
Rate of WCA	-0.29	-0.43	-0.13	0.37	-0.45	0.002

***** Significant at $P<0.05$

****** Significant at $P<0.01$

******* Significant at $P<0.001$

Table 3B:
Correlation coefficient between endogenous free and conjugated IAA pod^{-1}, PAA pod^{-1} and growth parameters in two varieties of *Cajanus cajan*

	Free IAA pod^{-1}		PAA pod^{-1}	
	V_1	V_2	V_1	V_2
Dry weight	0.12	0.36	0.32	0.37
Water content	0.01	0.78***	0.60**	0.36
Rate of DMA	0.22	-0.18	0.35	0.09
Rate of WCA	0.15	-0.16	0.03	-0.29

***** Significant at $P<0.05$

****** Significant at $P<0.01$

******* Significant at $P<0.001$

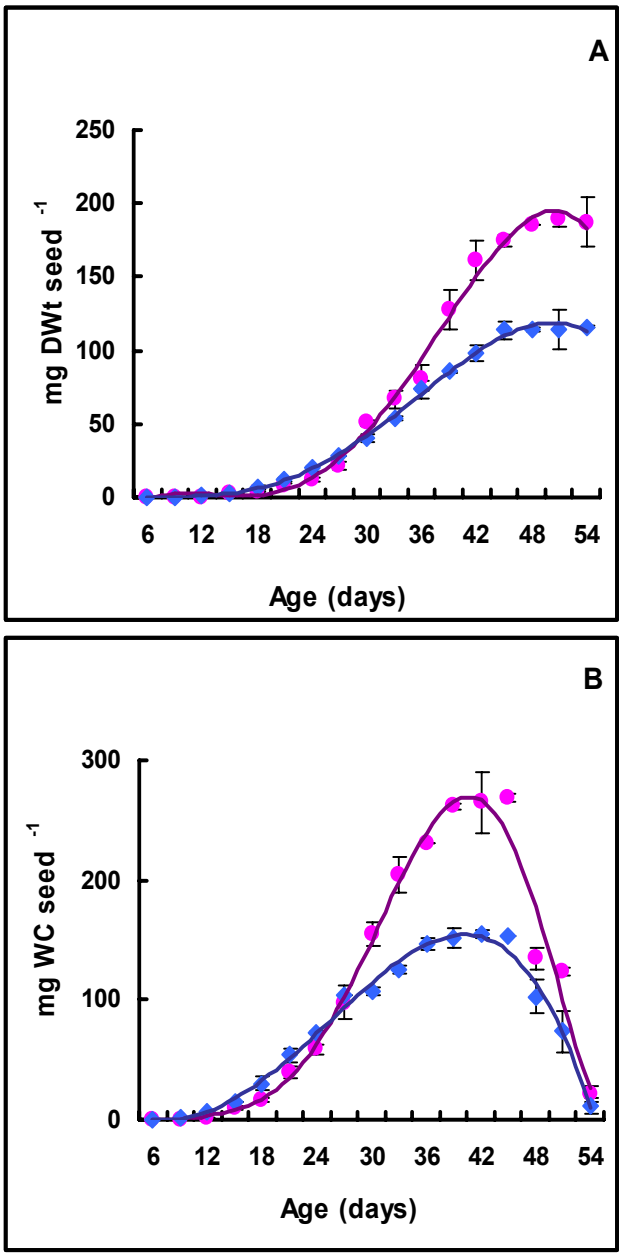


Figure 2:
Changes in Dry weight (A) and Water content (B) in developing seeds of V_1(●) and V_2 (♦)

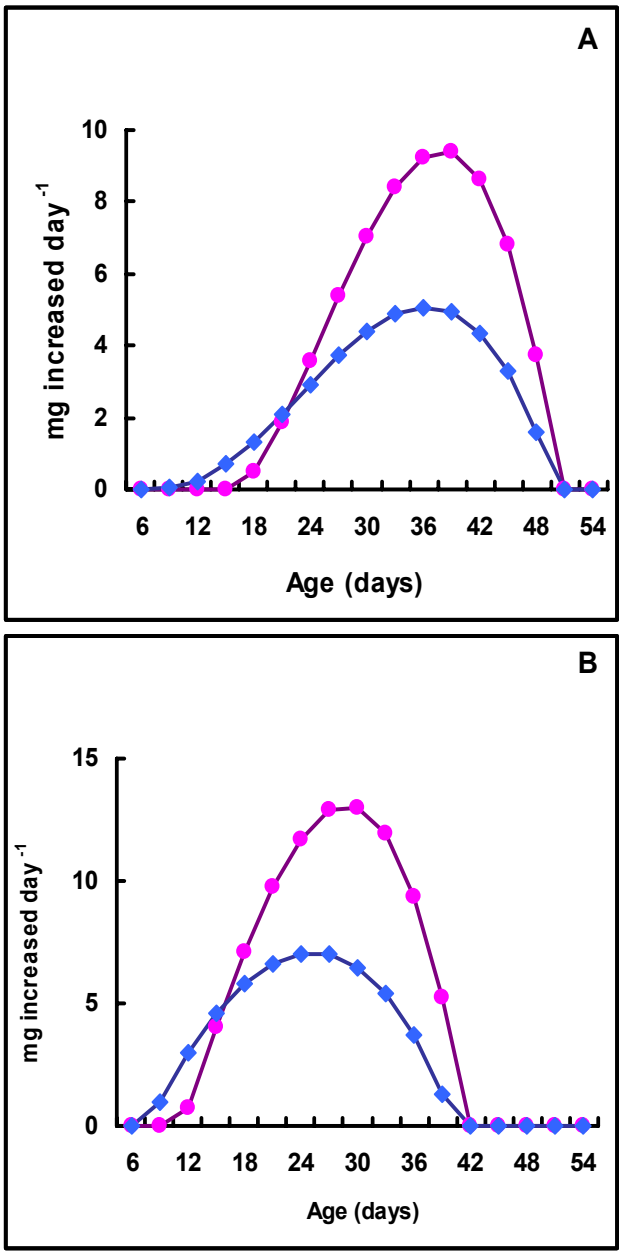


Figure 3:
Changes in rate of dry matter accumulation (DMA) (A) and rate of water accumulation (B) in developing seeds of V_1 (●) and V_2 (♦)

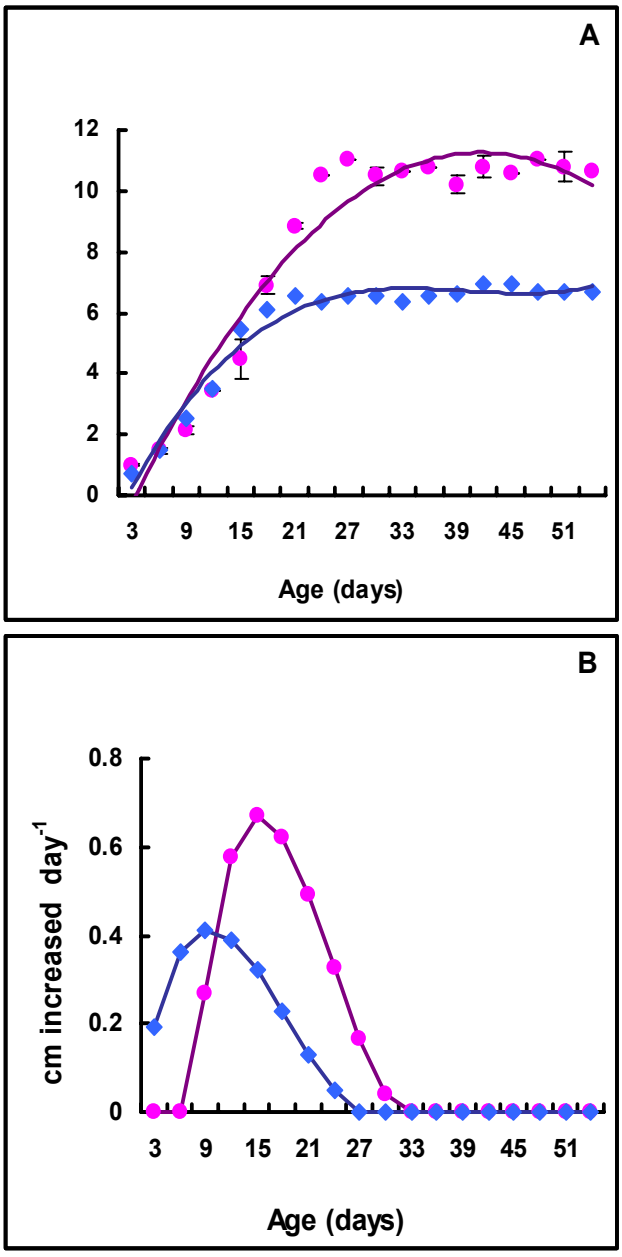


Figure 4:
Changes in length of pod (A) and rate of pod length (B) in developing pods of V_1 (●) and V_2 (♦)

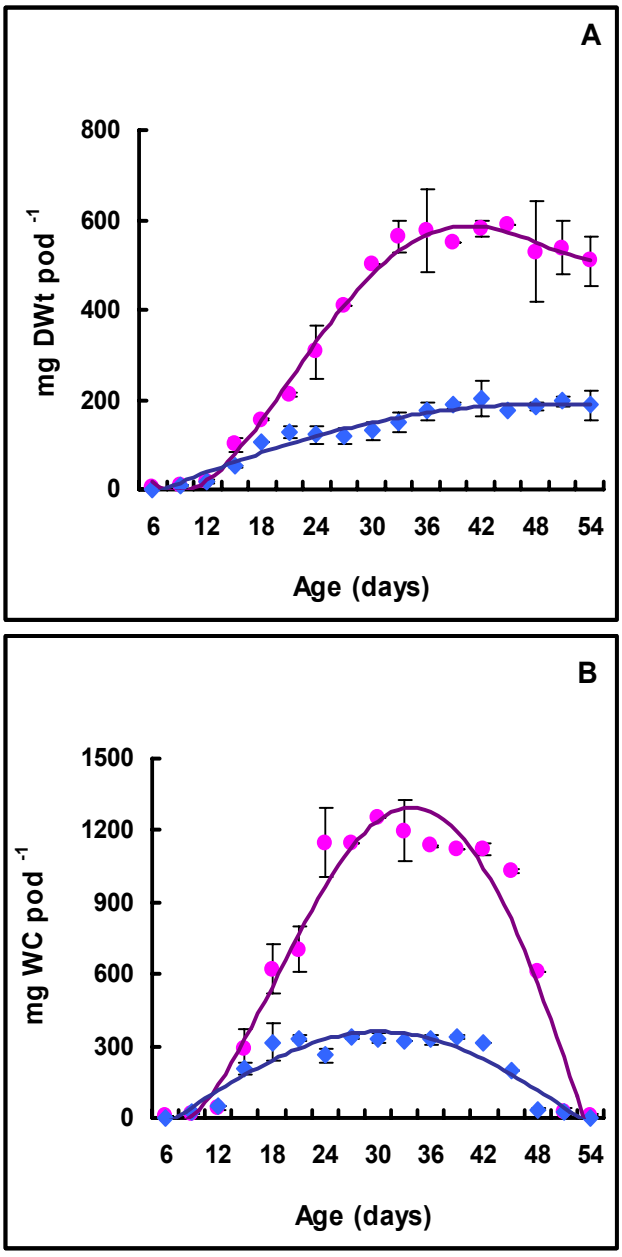


Figure 5:
Changes in dry weight (A) and water content (B) in developing pods of V_1 (●) and V_2 (♦)

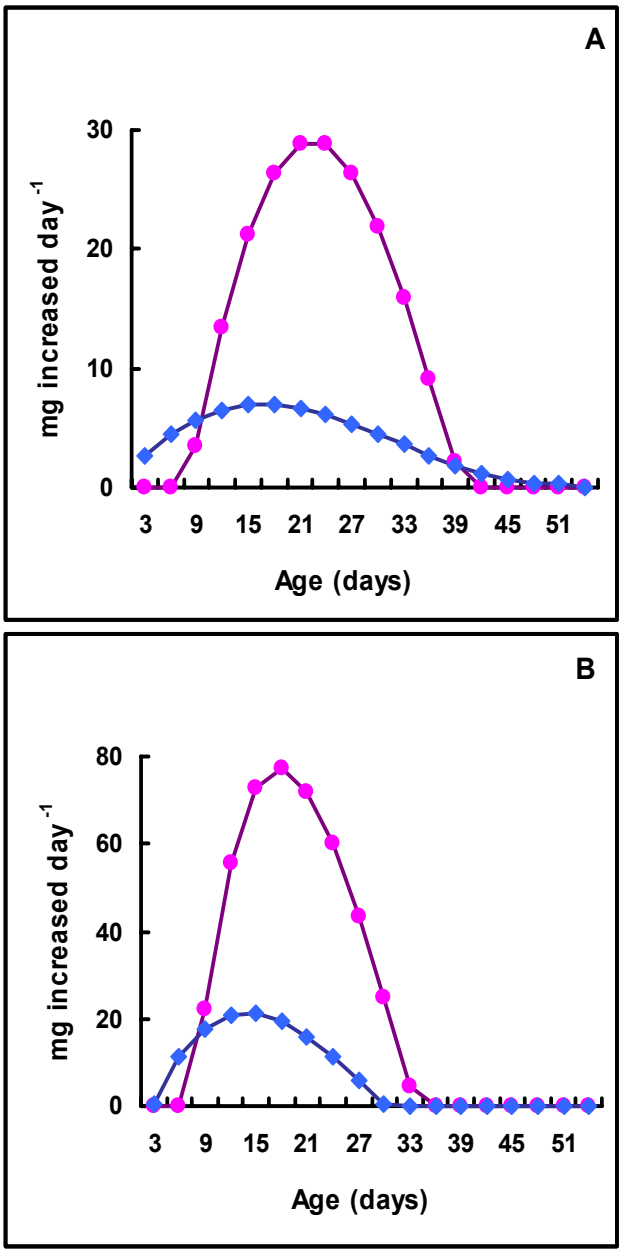


Figure 6:
Changes in rate of dry matter accumulation (DMA) (A) and rate of water accumulation (B) in developing pods of V_1 (●) and V_2 (♦)

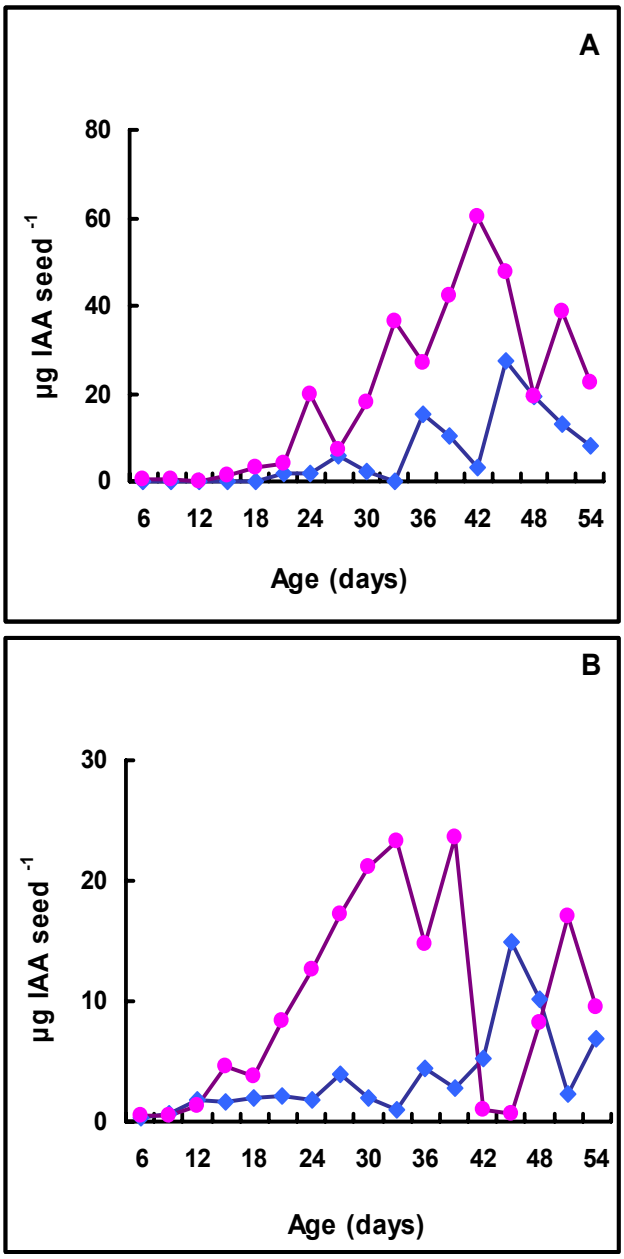


Figure 7:
Changes in free IAA (♦), µg seed^{-1} and conjugated IAA (●), µg seed^{-1} in developing seeds of V_1 (A) and V_2 (B)

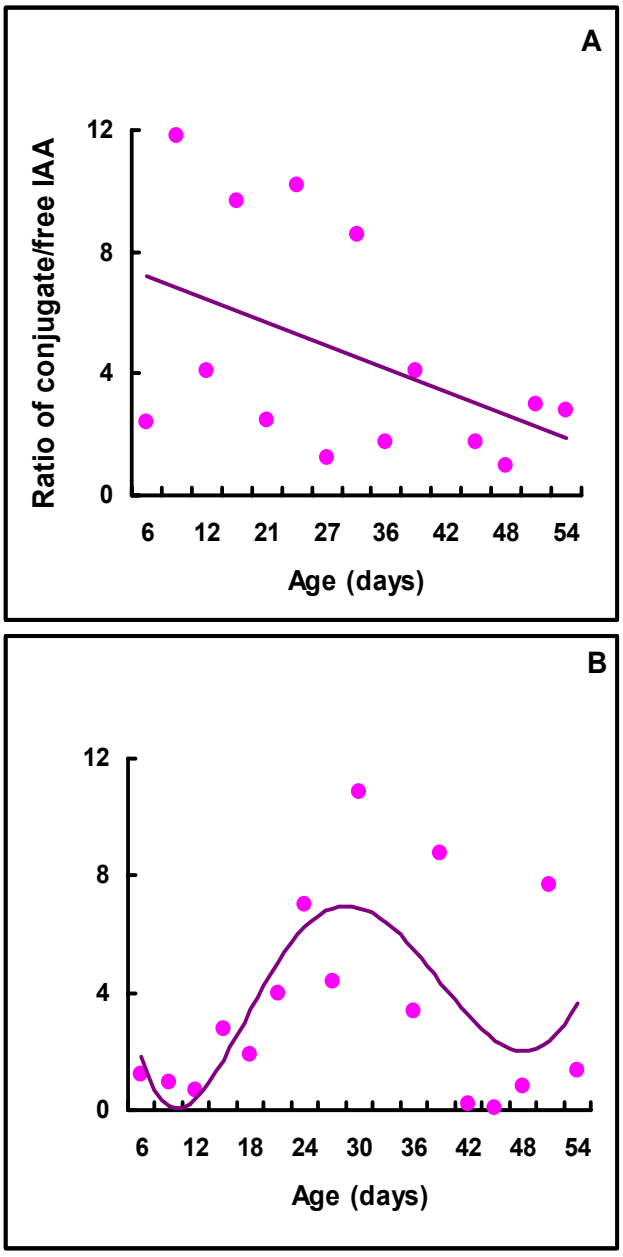


Figure 8:
Ratio of conjugated to free IAA (●) in developing seeds of V_1 (A) and V_2 (B)

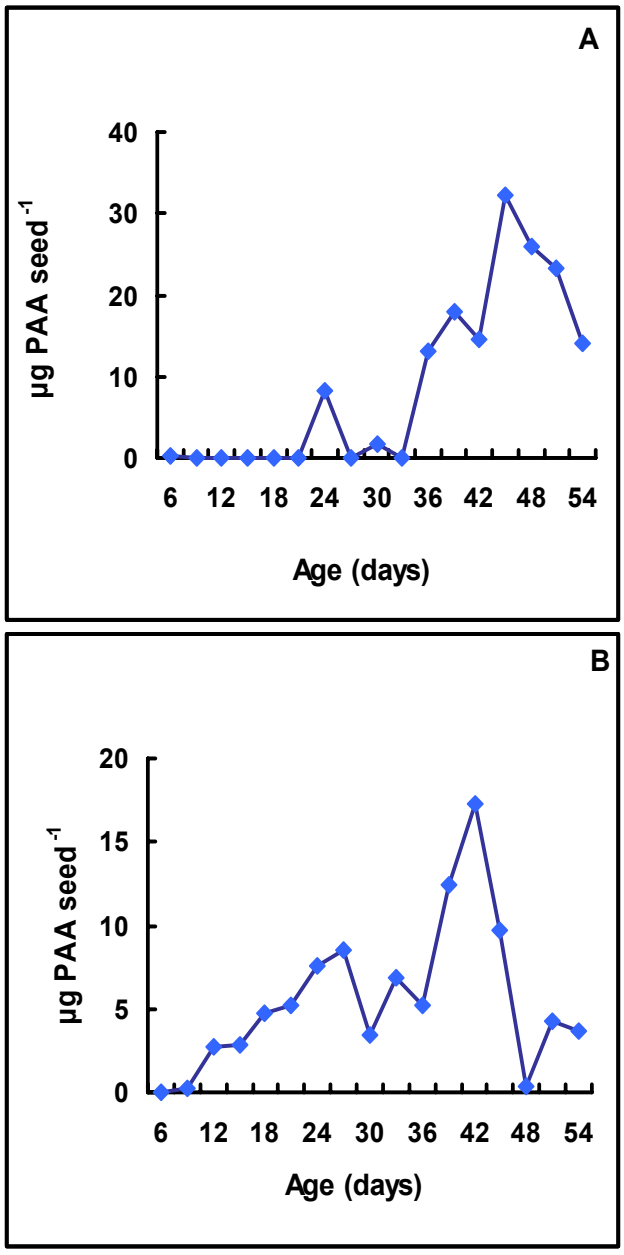


Figure 9:
Changes in PAA (♦), µg seed^{-1} in developing seeds of V_1 (A) and V_2 (B)

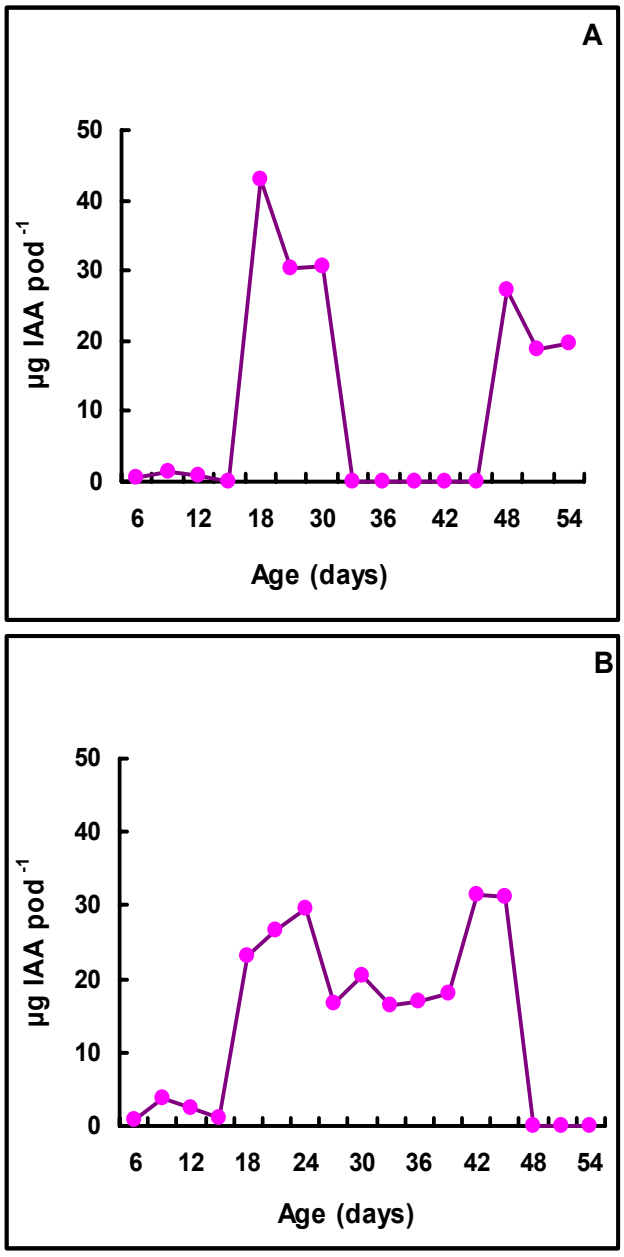


Figure 10:
Changes in IAA (●), µg pod^{-1} in developing pods of V_1 (A) and V_2 (B)

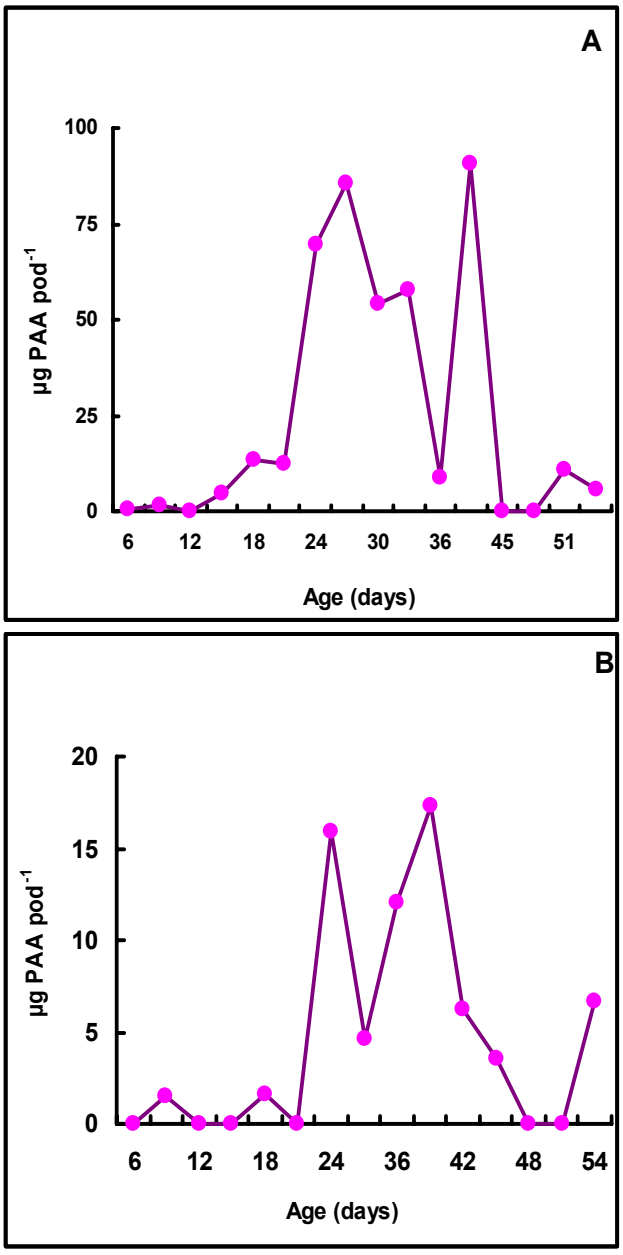


Figure 11:
Changes in PAA (●), µg pod^{-1} in developing pods of V_1 (A) and V_2 (B)

Chapter 3

Changes in endogenous levels of GA in developing seeds and pods

ABSTRACT

Changes in endogenous gibberellic acid (GA) levels were determined in developing seeds and pods of *Cajanus cajan.* Antibodies against GA_3 were raised in rabbits and indirect ELISA developed to estimate endogenous level. In seeds of both the varieties, higher GA content was observed during cell elongation and DMA phases, respective to their varietals differences in growth properties. Relationship between WC and rate of DMA, WC and GA was statistically significant in seeds and pods. The results suggest role of GA and WC in sink size development in both the varieties studied.

INTRODUCTION

Gibberellins (GAs) are a large family of tetracyclic diterpenoid plant growth substances. The function of GA as a hormone in regulating plant growth was known as early as the 1950s (Brian and Hemming, 1955; Vlitos and Meudt, 1957) which are associated with various plant growth and development processes such as seed germination, stem and hypocotyl elongation, leaf expansion, floral initiation, floral organ development, fruit development and induction of some hydrolytic enzymes in the aleurone of cereal grains (Matsuoka, 2003). Hooley (1994) has proposed that the types of responses of plant cells and tissues to GAs are classified into three categories: cell growth in vegetative tissues, seed reserve mobilization by aleurone cells and flower and fruit development.

Fruit size is considered to be one of the important characteristics in new cultivars selection. Gibberellins control fruit development in various ways and at different developmental stages. Fruit development is a complex and tightly regulated process. Growing fruits are very active metabolically and acts as strong sink for nutrients with hormones possibly modulating the process (Brenner and Cheikh, 1995). Plant hormones play a significant role in the process that leads to mature fruit and viable mature seed (Nitsch, 1970). Exogenous applications of various hormones to different stages of developing fruit and endogenous quantifications have highlighted their importance during fruit development. The development of a fruit can be separated into phases that include pre-pollination, pollination, fertilization and fruit set, post fruit set, ripening and senescence stages. The successful fertilization of the ovule is followed by cell division and cell expansion resulting in the growth of fruit. Gibberellins are known to influence both cell division and cell enlargement (Adams et al., 1975; Kamijima, 1981).

Previous studies have suggested that these GA-mediated growth responses are regulated in part by the modulation of cellular GA concentrations and by altering the ability of cells to respond to this hormone (Richards et al., 2001). Seeds, a rich source of many hormones (Crane, 1969), are known to be essential for normal development of fruits; the size and shape of many fruits being determined by seed number and distribution. For many species, GA_3 produced by developing seeds stimulates the growth and maturation of that fruit. Studies on endogenous GAs and GA metabolism in developing and mature seeds and grain revealed that the extremely large amount of GAs accumulate during dry matter accumulation (Pharis and King, 1985). Viable seeds are an important sink signal for driving fruit development and seed fill duration often correlates with yield (Egli, 1994). Thus for high yield it is important to maintain a steady sink activity throughout the seed-filling period (Hanson, 1991; Jenner et al., 1991).

The leguminosae are second to cereal crops in agricultural importance based on area harvested and total production. Studies on physiological roles of GAs in seeds of dicotyledonous plants showed that developing seeds of Leguminosae contain large amount of GAs (Nakayama et al., 2002). Pigeon pea (*Cajanus cajan*) is an important pulse crop of India. It is a rich source of proteins, carbohydrates and certain minerals (Salunkhe et al., 1986) and used for food, feed and fuel. Since long times hormonal regulation of many legume fruit development was studied, till to date there is no report on endogenous regulation of gibberellic acid in Pigeon pea fruit development. This study aimed to evaluate the role of GA in seed development in *Cajanus cajan*. Endogenous levels of GA from the seed and pod were estimated by indirect ELISA, during entire period of growth and development. To understand the probable role of GA, two varieties, distinct in their growth parameters were selected.

MATERIALS AND METHODS

Growth analysis

Pods of equal size were harvested at the interval of three days for growth analysis as described in Chapter 2. Seeds and pods were collected and samples were prepared for estimation of endogenous GA.

Raising of antibodies against GA_3

Preparation of GA–BSA and GA–casein conjugate

To raise antibodies against GA_3, GA_3-BSA conjugate was prepared as described by Weiler (1981). GA_3 (106 mg) was dissolved in 2.5 mL of DMF and reacted with 75-µl tri-n-butyl amine and the solution was cooled (0 ^{o}C), 40 µl isobutyl chlorocarbonate was added and incubated for 20 min at room temperature. This reaction mixture was added with constant stirring to 420 mg BSA dissolved in 22 ml of DMF: water (1:1, v/v) and 420 µl 1M NaOH. After 1 h incubation at 0 ^{o}C another 0.2 mL of 1M NaOH was added and stirring was continued for 5 h. The mixture finally dialyzed against 10 % DMF for 24 h and against distilled water for 4 day.

Immunization and separation of IgG

The GA_3-BSA conjugate was mixed with an equal volume of Freund's complete adjuvant and injected into two rabbits. Antibodies against GA were purified as described in Chapter 2.

Extraction of GA from seeds and pods

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 and centrifuged at 10,000 g for 10 min. Supernatant was collected and pellet was washed twice with 80% methanol. Pooled supernatant was collected and kept for evaporation. Final volume of the samples (10 mL) was prepared with phosphate buffer saline (pH 7.2) and used for the estimation of GA.

Estimation of GA content

Endogenous level of GA was estimated by indirect ELISA. GA_3-casein conjugate (300 µl) was coated on ELISA plate and remaining steps were followed as described in Chapter 2.

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 hormone standard or sample, respectively. A standard curve of GA_3 was prepared in a range of 50-500 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 GA_3 (200 ng) as an internal standard before reacting with the antibodies.

Data were taken in triplicates and mean values were calculated. Endogenous levels of GA in developing seeds and pods were expressed as µg GA_3 equivalent $seed^{-1}$ and µg GA_3 equivalent pod^{-1} fresh weight, respectively.

Statistical analysis

Mean values are presented with standard deviation of the mean (SD). The statistical significance between means of endogenous GA and growth parameters in seed and pod of two varieties was analyzed using analysis of variance (ANOVA). Correlation coefficient was determined between growth parameters (DWt, WC, rate of DMA and WCA) and endogenous GA during the entire period of seed and pod development (Table 4). P values significant at 0.05 or less than that were considered for the data interpretation.

RESULTS

Changes in GA content in seed

In V_1, negligible value of GA was recorded during early stages of seed development (up to 18 d). From the 24 d the GA content increased gradually, peaked (40.29 µg seed^{-1}) at 45 d and at later period of seed development it decreased. In mature seed (54 d), the value of GA was 11.09 µg seed^{-1} (Figure 13A). In V_2 the level of GA increased gradually with the seed development with a peak (15.8 µg seed^{-1}) at 45 d and decreased gradually at later stages (Figure 13A).

At later stages of seed development the same pattern of GA level was observed in both the varieties. However, it remained three times higher in V_1. A difference in GA content was also observed during the initial stages; up to 18 d, where in V_2 the value of GA was 2.27 µg seed^{-1} and in V_1 it was negligible.

Changes in GA content in Pod

In V_1 pod the GA content increased gradually with age and it showed peak at 30 d (153.09 µg pod^{-1}). At later days it decreased gradually and at 54 d the GA level was 35.29 µg (Figure 13B). In V_2 GA level increased gradually up to 39 d and at later age it showed declined trend. The maximum value was 43.55 µg pod^{-1}at 39 d (Figure 13B).

DISCUSSION

Changes in endogenous levels of GA showed similar pattern in developing seeds of both varieties and also showed statistically significant difference ($P<0.05$). On the basis of seed growth phases, in V_1 seed, the endogenous GA level increased rapidly during cell elongation phase till the maximum value for DMA was achieved. Maximum level of GA was observed at 45 d, the day where WC peaked. Similarly in V_2, the level of GA increased gradually till 45 d where water content was maximum (Figure 13A).

It is well documented that gibberellic acid plays a significant role in seed development in various plant species. Gibberellins have been suggested to be involved in early stages of seed development in pea (Swain et al., 1997) and *Phaseolus* (Yeung and Meinke, 1993). In a number of plant species, early seed growth was correlated with increase in bioactive GAs (Pharis and King, 1985; Rock and Quatrano, 1995). It has been proposed that gibberellin (GA) is closely related to cell division and cell enlargement during fruit development in Japanese peer (Zhang et al., 2005). Exogenous application of GA increased the seed weight and delayed the seed dehydration; suggest a role of GA in later stages of fruit and seed development (Groot et al., 1987).

In V_1 seed dry weight and water content showed significant correlation with endogenous gibberellic acid (Table 4). In V_2 seed, water content showed significant correlation with endogenous GA, while dry weight was less significant. These results suggest that endogenous GA level has increased the level of WC and thus enhanced the cell elongation. Similarly it was observed in V_1 seed, that the GA level was double during dry matter accumulation and cell maturation phase (Figure 13A). In both seeds, water content showed close correlation with GA suggests that more GA increased water uptake. Nanda and Dhindsa (1968) suggested

that enhanced elongation of internode is brought by enhanced mobilization of reserve food by GA_3. Similarly, increasing the concentration of GA_3 in alfa alfa (*Medicago sativa*) and brome grass increased the growth and total yield (Bidlack and Buxton, 1995).

In the present study, at initial stage (cell division phase and elongation phase), 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) the difference was significant ($P<0.05$). Additionally the rate of DMA and water accumulation in seeds also showed significant differences (Figure 3). A close correlation was observed between water content and the rate of dry matter accumulation (DMA) in both the varieties suggesting the role of water content in DMA. The rate of DMA and rate of water accumulation was double in V_1 (Figure 3). These results suggest that high rate of water uptake has increased the rate of DMA in seeds and hence both the seeds were different in weight.

An important role of water content is reported in various plant species. The effect of water stress decreased the growth and elongation of internodes in *Tactona grandis* (Rajendrudu and Naidu, 1997). Villela (1998) suggested that during seed development, the water content after fertilization is typically high and decreases as the physiological maturity occurs. In legume seed development, due to higher water uptake, transgenic cotyledons take up more amino acids, leading to higher protein content (Borisjuk et al., 2003).

Further, length of pod was different in both the varieties and showed significant difference ($P<0.01$). Statistical analysis of these two varieties showed highly significant difference in pod DWt and WC ($P<0.001$). In V_1 pod, the endogenous level of GA increased gradually with pod age. Maximum GA was present at 30 d, where pod achieved maximum WC (1257 mg Pod^{-1}) (Figure 13B).

Rate of length showed close correlation with rate of water accumulation (Figure 12). This suggests that increase in length was due to higher water uptake; enhancing the accumulation of dry matter. Gibberellins enhance longitudinal growth in shoot and increase water uptake (Banyal and Rai, 1983). This is mainly due to GA mediated increase in cell expansion. Here, water content of pod showed significant correlation with GA, thus suggesting the important role of GA in water uptake and pod development. In V_2 pod, maximum GA value was observed at 39 d, where pod achieved maximum water content (339 mg Pod^{-1}) (Figure 13B). Similar correlation between endogenous level of GA and WC per pod was observed in V_2 but the level of GA remained lower suggesting that low GA may decrease the water uptake during the pod development. Similarly difference in rate of fiber length and water content was observed in three cotton cultivars (Rabadia et al., 1999). In legume plants, pod development is characterized by active cell division in the young ovule and is marked by rapid pod expansion; both processes are very sensitive to water uptake. Inglese et al. (1998) suggested that high levels of gibberellins produced by the fruits at the time of anthesis/fertilization induce physiological changes in the maternal tissue that ensure the supply of water and dry matter. Mahouachi et al. (2005) found that endogenous level of GA is closely associated with water availability during citrus fruit development. Water stress usually reduces fruit yield (van Iersel et al., 1994; Nerd and Mizrahi, 1995; Pessarakli, 1995), leading in some cases to the abscission of immature fruit (Pessarakli, 1995).

Fruit development requires a major investment of carbon and water (Galen et al., 1999). Photosynthate supply plays an important role in controlling crop reproductive development under well-watered conditions. Pods are part of source-sink pathway that can produce photosynthates and deliver nutrients to the seeds. In this study, pods are bigger in V_1 and therefore number of seeds per pod is also more. Higher amount of GA in bigger pod and seed may increase uptake of water in total fruit. It is proposed that endogenous GA and water content of seed and pod play an important role in increasing fruit size of *C. cajan*.

Table 4:

Correlations among endogenous GA Seed^{-1}, GA Pod^{-1} and dry weight, water content and rate of dry matter accumulation in two varieties of *Cajanus cajan*

	GA Seed^{-1}		**GA Pod^{-1}**	
	V_1	**V_2**	**V_1**	**V_2**
Dry weight	0.81***	0.55*	0.42*	0.42*
Water content	0.91***	0.93***	0.60**	0.86***
Rate of DMA	0.69***	0.83***	0.34	-0.03

* Significant at $P<0.05$

** Significant at $P<0.01$

*** Significant at $P<0.001$

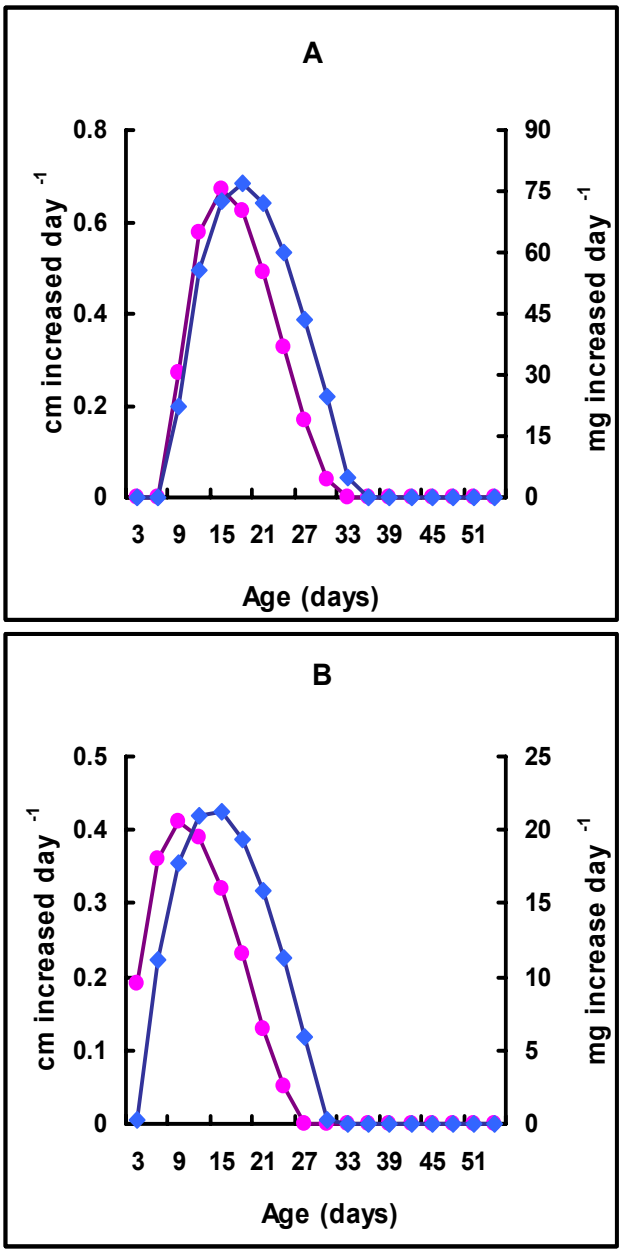


Figure 12:
Changes in rate of pod length (●) and water content accumulation (♦) in developing pod of V_1 (A) and V_2 (B)

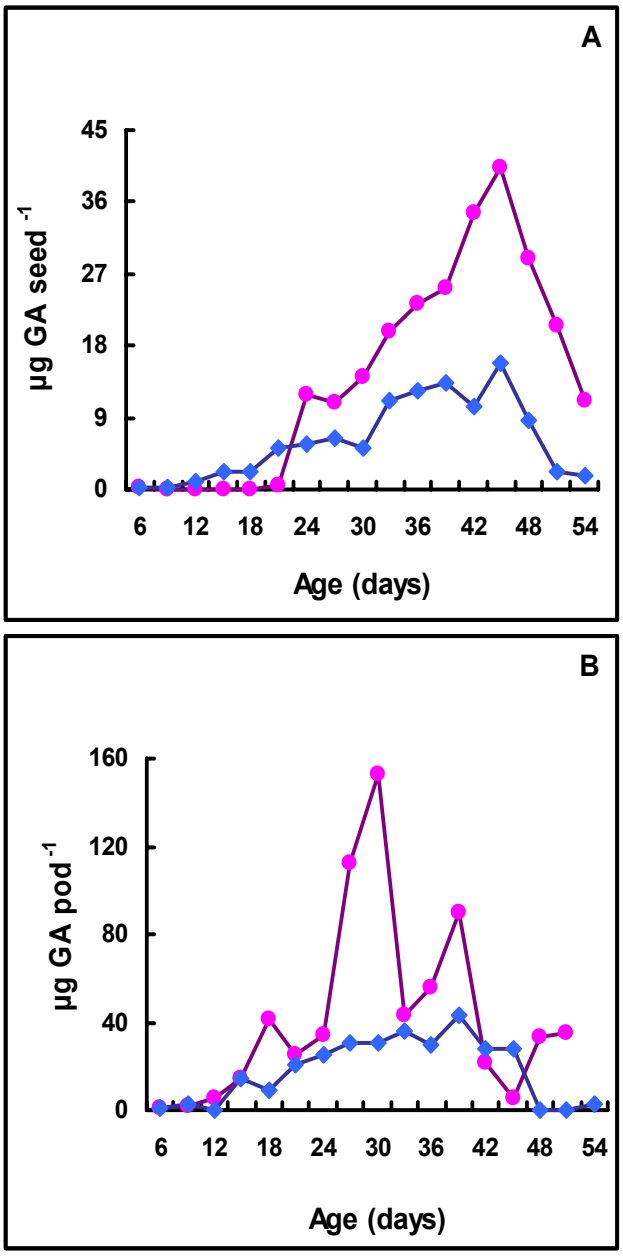


Figure 13:
Changes in endogenous levels of GA, µg seed^{-1} (A) and µg pod-1 (B) in V_1 (●) and V_2 (♦)

Chapter 4

Changes in endogenous levels of ABA in developing seeds and pods

ABSTRACT

During seed and pod development, changes in endogenous free and conjugated abscisic acid (ABA) levels were measured. Antibodies against ABA were raised in rabbits and indirect ELISA was standardized to estimate ABA. It was observed that levels of free ABA remained equal in seeds of both the varieties. The physiological age of ABA accumulation was different in seeds of both the varieties 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 and pod development is discussed.

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 (Silveira et al., 2004). 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 (Schou et al., 1978). 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 (Davies and Zhang, 1991; Rock and Quantrano, 1995). 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 (Schmitz et al., 2000; Suzuki et al., 2000; Tian and Brown, 2000; Silveira et al., 2004).

Accumulation of ABA during seed development has been reported in many species (King, 1982). ABA concentration in crop reproductive structures increases significantly when the plants are drought-stressed during flowering (Setter et al., 2001). This increase of ABA concentration in the reproductive structures has been suggested to play a role in determining grain set in maize (Ober et al., 1991; Artlip et al., 1995) and wheat (Westgate et al., 1996).

ABA levels increase in the initial stages of seed development and decrease during embryo maturation (Rock and Quatrano, 1995). 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 (King, 1982; Bewley and Black, 1994).

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) (Zeevaart and Creelmann, 1988). The physiological 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 (Neill et al., 1983; Lehman and Vlasov, 1988). Results from other plant systems show that a fall in free ABA is closely associated with a rise of conjugated ABA (Philips and Hofmann, 1979).

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 (Jones and Brenner, 1987; Schussler et al., 1991). 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

Growth analysis

Pods of equal size were harvested at the interval of three days for growth analysis as described in Chapter 2. Seeds and pods were collected and samples were prepared for estimation of endogenous ABA.

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 (2001). 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 ^{o}C. It was then dialyzed against tap water for 4 days and stored at 0 ^{o}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. Booster injections were given periodically to raise the titer. Antibodies against ABA were purified as described in Chapter 2.

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 indirect ELISA. ABA-casein conjugate (300 µl) was coated on ELISA plate and remaining steps were followed by as described in Chapter 2.

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 (1977). In brief, the hormone extract was allowed to hydrolyze with an equal amount of 2M KOH at 25 ^{o}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 seed^{-1} or pod^{-1} 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 (DWt, WC, rate of DMA and rate of water accumulation) and endogenous ABA (Table 5) 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

Changes in endogenous ABA in seed

Free and conjugated forms of ABA were measured from seeds of both the varieties. The changes in endogenous ABA levels in seeds and pods were expressed as µg ABA per seed or pod dry weight. The ABA content was not detected till 24 d in V_1 seed, later on it raised and peaked at 36 d (0.457) (Figure 14A). 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 (Figure 14B). 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 (Figure 15). In V_1, conjugated ABA peaked at 18 d (0.727), declined gradually and second peak was observed at maturation phase (0.427) (Figure 15A). In V_2, conjugated 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 (Figure 15B).

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 (Figure 16A). 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 (Figure 16B).

DISCUSSION

Free ABA level in seed

Changes in free ABA levels in seeds of V_1 and V_2 are presented in Figure 14A and 14B. 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 (Figure 14A, 14B). 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 (Schussler et al., 1991). In this study, the rate of DMA and rate of water accumulation in seeds showed significant difference (Figure 3A, 3B) 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 (Figure 14) in seed development.

Earlier Gokani et al. (1998) 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 dry matter accumulation in the seeds of several grain crops indicates a role of the hormone in promoting assimilates unloading (Yarrow et al., 1988).

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 (King, 1982; Bewley and Black, 1994). Presence of ABA at later stage of seed development suggests a role of ABA in the physiology of seed maturation and germination (Crouch et al., 1985).

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 (Figure 15A, 15B). 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 (Figure 14B, 15B). 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 5).

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 (Bano et al., 1993, 1994; Hartung and Jeschke, 1999). Conjugation of ABA is thought to be irreversible and may represent a mechanism for protecting tissue from the physiologically active free form of ABA (Zeevaart and Creelman, 1988).

It was observed that ABA has stimulated the movement of sugars in various economically important crop plants (Schussler et al., 1991) and thus improved fruit yield and quality (Xia et al., 2000). 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 (Karssen et al., 1990) but large concentrations of ABA probably inhibit cell division by depression of cell-cycle gene expression (Setter et al., 2001). 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 (Myers et al., 1990; Mambelli and Setter, 1998).

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 (Figure 16A). DWt and WC showed negative correlation with ABA suggest that with increase in DWt and WC the level of ABA decreased (Table 5). 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 (Figure 16B). 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 (1987) 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 (Addicott, 1983). However, evidence suggests that ABA inhibits pod abscission and play a role in partitioning of photoassimilates to developing soybean fruit (Yarrow et al., 1988). A positive relationship between exogenously applied ABA and sink activity has been shown in wheat (Dewdney and Mcwha, 1979) and barley (Tnrz et al., 1981).

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 (Thomas, 1986). ABA has been implicated in the regulation of sink strength in small grains, soybeans and some horticultural crops through mediation of phloem unloading (Jones and Brenner, 1987).

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.

Table 5:

Correlations among endogenous ABA seed^{-1}, ABA pod^{-1} and dry weight, water content, rate of DMA, rate of water content in two varieties of *Cajanus cajan*

	ABA Seed^{-1}		**Conjugated ABA Seed^{-1}**		**ABA Pod^{-1}**	
	V_1	**V_2**	**V_1**	**V_2**	**V_1**	**V_2**
Dry weight	0.52*	0.02	0.01	-0.54	-0.77	0.05
Water content	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
Rate of WCA	0.12	0.57*	0.13	0.04	0.47	-0.087

* Significant at $P< 0.05$

** Significant at $P< 0.01$

*** Significant at $P<0.001$

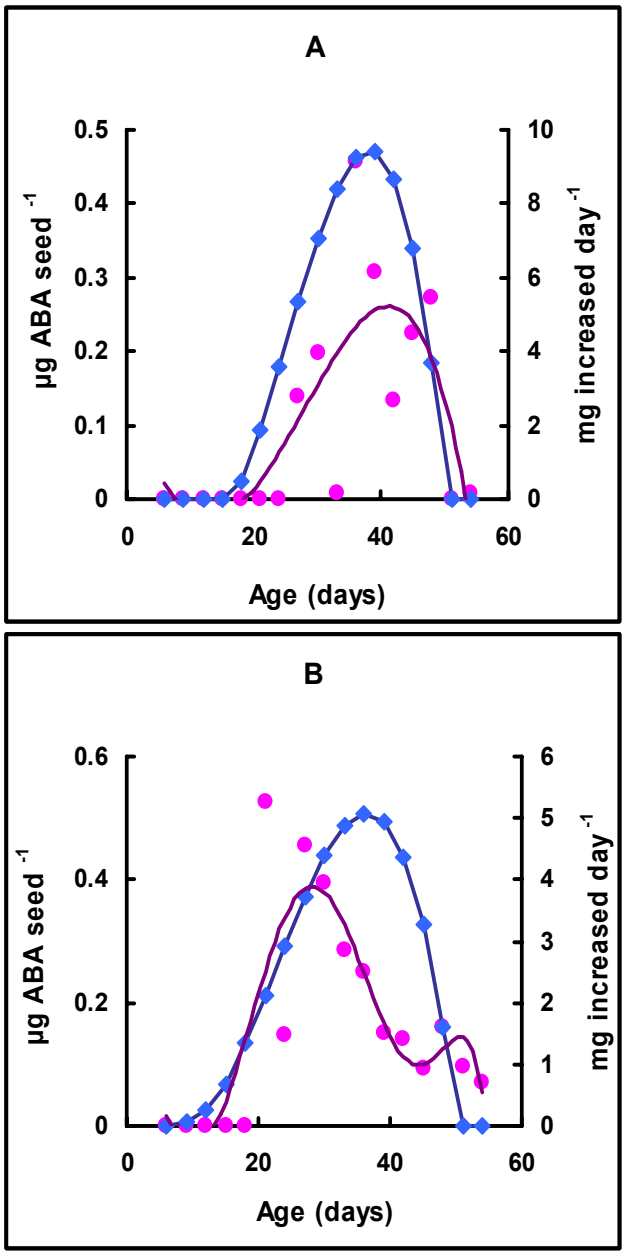


Figure 14:
Changes in free ABA (●), µg seed^{-1} and DMA (♦) in developing seeds of V_1(A) and V_2 (B)

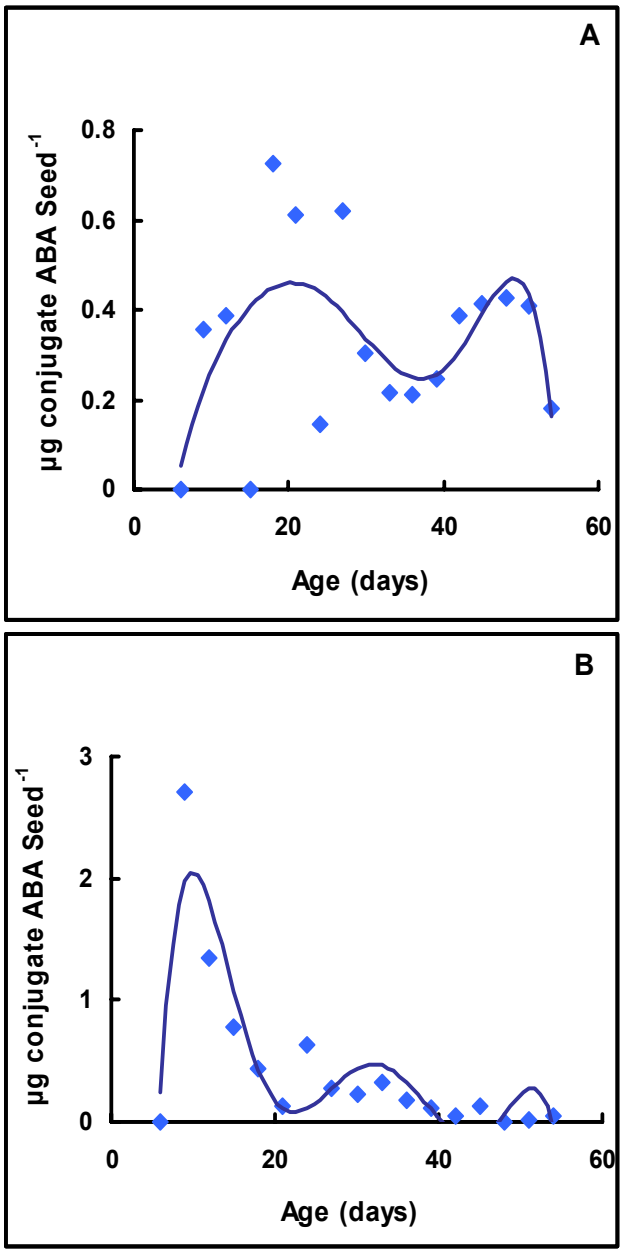


Figure 15:
Changes in conjugated ABA (♦), µg seed^{-1} in developing seeds of V_1 (A) and V_2 (B)

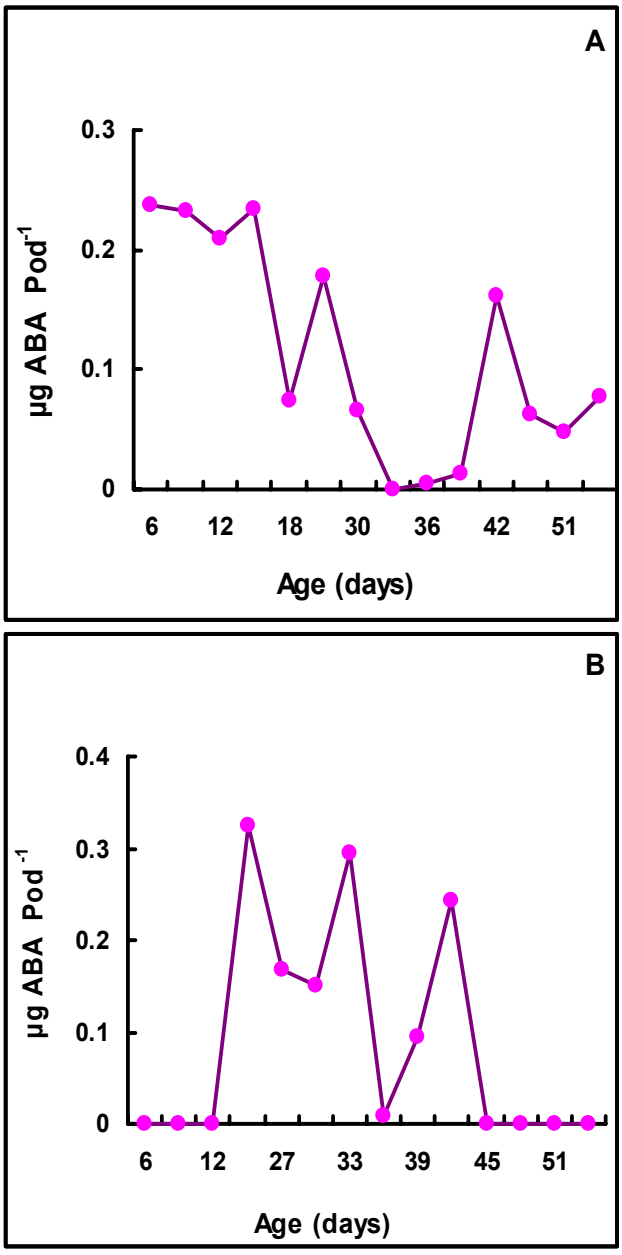


Figure 16:
Changes in free ABA (●), µg pod^{-1} in developing pods of V_1 (A) and V_2 (B)

Chapter 5

Role of wall components in sink size development

ABSTRACT

Esterified and non esterified pectins along with low and high molecular weight xyloglucans were estimated from the developing seeds of both the varieties. As both seeds showed distinct difference in their growth phases, a remarkable difference was also observed in cell wall components during their developmental period. Esterified and non esterified pectins remained higher in V_1 seed throughout the development. At cell division phase both pectic substances were detected while low and high molecular weight xyloglucans remained in negligible amount. During cell elongation phase a remarkable difference in pectins and xyloglucans was observed. Dry weight and water content showed a significant correlation with both pectic substances and xyloglucans. The probable role of wall components in sink size development is discussed.

Abbreviations: DMSO; Dimethyl sulphoxide, EDTA; Ethylene diamine tetra acetic acid, SLS; Sodium lauryl sulfate

INTRODUCTION

The grain legumes represent an important group of crop plants with exceptional nutritional value as food and feed. Legume crops are selected for high seed yield and characterized by high metabolic activity and fluxes in seeds (Weber et al., 2005). Pigeon pea is the second largest pulse crop of India, accounting for 20% of total pulse production. Desirable improvements of seed quality and yield may be achieved with understanding of the seed composition. During the earlier part of seed development increase in weight may occur because of increase in size. Later, when the seed has attained its full size, the increase in dry weight is due to accumulation of storage material. This accumulation can be measured by changes in dry weight of seed. The higher dry matter accumulation capacity of the harvestable organs has been accomplished mainly by increasing either the number of grains (Tanaka and Yamaguchi, 1972) or the size of individual grain (Evans and Dunstone, 1970).

The use of larger seed of a seed stock results in increased germination, speedier emergence and improved seedling growth (Wood et al., 1977). Physiological and biochemical aspects of fruit and seed development in large seeded legumes have been intensively studied (McCarty, 1995). However for high yield it is important to maintain a steady sink activity throughout the seed filling period (Hanson, 1991; Jenner et al., 1991). In legume seeds the cell wall polysaccharides represent the significant portion of dry matter (Stombaugh et al., 2000). In soybean cotyledons cell wall polysaccharides accounted 12% of dry matter (Daveby and Aman, 1993) and represent a substantial amount of dry matter deposited during seed development. Plant cell walls play an important role in regulating physiological events in sink development (Thaker, 1998).

Plant development involves a coordinated series of biochemical processes that results in the biosynthesis and degradation of cell wall components (Stolle-Smits et al., 1999). Different cell types within a plant can be distinguished from each other by the chemistry and organization of their walls, and walls differ in a way that is related to developmental stage or to their exposure to different environmental conditions (Fincher, 1993; Pennel, 1998; Cosgrove, 1999; Carpita et al., 2001). Biochemical modifications of the cell wall, such as changes in the molecular size and quantities of cell wall polysaccharides have been considered to be possibly involved in the regulation of cell wall extensibility (Sakurai, 1991, Kaku et al., 2002). When plant cells grow, the wall is biochemically “loosened” to permit turgor-driven cell expansion (Cosgrove, 2000). Cell expansion requires the constant rearrangement of the bonds within this network, to allow the movement of wall polymers and the incorporation of new wall material into the existing cell wall architecture.

Cell wall of the plant is composed of many complex carbohydrates and proteins, which undergoes rapid turnover during the process of cell elongation. Pectic polysaccharides comprise between 30 and 50% of the cell walls of dicotyledonous plants (Carpita and Gibeaut, 1993). The pectic matrix of plant cell walls is a complex mixture of homogalacturonan (HGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) polymers (Voragen et al., 1995). The major hemicellulose in dicotyledons is xyloglucan, which is thought to coat and tether the cellulose microfibrils together, forming an extensive cellulose-xyloglucan network (Hayashi and Maclachlan, 1984; McCann et al., 1990). This network is believed to represent a major constraint to turgor-driven cell expansion (Cosgrove, 2000). The increased deposition of one of these matrices generates accumulation of carbohydrates which functions as a storage compound (Silvatine et al., 2000). In this study, changes in wall components, i.e. pectic polysaccharides and xyloglucans were studied from two different varieties of *Cajanus cajan* varying in seed size during the entire period of seed development.

MATERIALS AND METHODS

Seeds of V_1 (Black seeded) and V_2 ($B.D.N_2$) were grown in botanical garden of Saurashtra University, Rajkot during the period of 2003-2004. Cultural practices including irrigation, application of fertilizers and insecticides etc., were maintained throughout the 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 wall components.

Growth analysis

Fresh and dry weight measurements

Growth analysis of seeds used in this experiment was done as described in Chapter 2.

Extraction and estimation of wall components

The method described by Selvendran et al. (1985), was followed for preparation of cell wall material as well as further extraction of the native wall components which can be summarized as follows:

Preparation and purification of cell wall material

Freshly harvested seeds were powdered with liquid nitrogen in pre-chilled mortar-pestle and stored at –20 ^{o}C prior to use. From each developmental stage 500 mg of crushed seeds were suspended in 1.5% aqueous SLS (Sodium Lauryl Sulfate) containing 5mM $Na_2S_2O_5$, mixed thoroughly and centrifuged at 10,000 g for 10 min followed by washing with 0.5% SLS containing 5mM $Na_2S_2O_5$. The supernatant, containing SLS soluble polymers of intracellular origin was discarded and residue was washed thrice with distilled water. The residue was then suspended in 0.5% SLS containing 3mM $Na_2S_2O_5$ and incubated for 16 h at 2 ^{o}C.

The supernatant after centrifugation contained cold, water soluble pectic substances. The residue was again washed two times with distilled water and re-suspended in PAW (Phenol, Acetic acid, Water, 1:1:1 w/v/v) to remove residual proteins, lipids, adsorbed SLS and some starch. This was followed by two washes of distilled water and two washes with DMSO (Dimethyl Sulphoxide) to remove starch from residues. For removal of adsorbed DMSO the residue was then washed six times with distilled water. The starch free residue was finally taken as purified Cell Wall Material (CWM).

Extraction and estimation of pectic polysaccharides

Different fractions of pectic polysaccharides were extracted by using EDTA (Ethylene Diamine Tetra Acetic acid) as a chelating agent and Na_2CO_3. The CWM was stirred with 50mM EDTA (pH 6.5) for 6 h at 20-22 ^oC, centrifuged and washed with the distilled water. The pooled supernatants served as source of non esterified pectic polysaccharides (fraction 1). The residue was again subjected to the same EDTA treatment for 2 h at 20-22 ^oC followed by washing with distilled water (fraction 2). The pooled supernatant of both fractions was used as residual non esterified pectic polysaccharides soluble in EDTA.

The residue was then suspended with 50 mM Na_2CO_3 containing 20mM $NaBH_4$, incubated for 16 h at 1 ^oC. The supernatant was collected and the residue was washed once with distilled water. The pooled supernatant was used for estimation of cold Na_2CO_3 soluble esterified pectic substances (fraction 3). The residue was finally re-extracted with 50mM Na_2CO_3 as above for 3 h at 20-22 ^oC and washed with distilled water (fraction 4). The pooled supernatant of both the fractions served as a source of esterified pectic substances soluble in Na_2CO_3.

Color development procedure was adopted as Dubois et al. (1956). One mL of the extract was mixed with 1 mL of 5% phenol and 5 mL sulfuric acid. The mixture was incubated for 10 min at room temperature with stirring and 20 min at 30 ^{o}C in water bath. Absorbance values were measured at 490 nm.

The controls were prepared by addition of respective extractant and remaining additions were kept similar. The pectic polysaccharides were expressed as ΔA_{490} seed^{-1}.

Extraction of Hemicelluloses

The depectinated residue was then used for extraction of low and high molecular weight xyloglucan fractions. The residue was stirred with 1M KOH containing 10mM $NaBH_4$; incubate for 2 h at 1 ^{o}C. The supernatant was collected that mainly contained cold 1M KOH soluble low molecular weight xyloglucans (fraction 1). The second fraction was collected in similar way with the same solution but at room temperature (fraction 2). The pooled supernatant of both the fractions served as a source of KOH soluble low molecular weight xyloglucans.

Thereafter, the residue was treated with 4M KOH containing 10mM $NaBH_4$ for 2 h at 20-22 ^{o}C. The supernatant served as a source of bulk of 'free' cold alkali/KOH soluble high molecular weight xyloglucans (fraction 3). The residue was finally treated with 4M KOH containing 3-4 % Boric acid for 2 h at 20-22 ^{o}C. The supernatant was collected and estimated for 4M KOH + Borate soluble high molecular weight xyloglucans (fraction 4). The pooled supernatant of fraction 3 and 4 was used as a source of high molecular weight xyloglucans. The acidification of each xyloglucan fractions was done prior to estimation and pH adjusted to 5.0 with glacial acetic acid.

Colorimetric estimation was done by Iodine staining method of Kooiman (1960) with a slight modification (Nishitani and Masuda, 1981). One mL of the extract was mixed thoroughly with 250 µl of I_2KI and 2 mL 15% Na_2SO_4. The mixture was incubated for 1 h at 4 ^oC and optical density of the resultant color solution was measured at 640 nm.

The controls were prepared by addition of the extractant instead of the xyloglucan fractions. The non-specific absorption measured from these controls was used for correction of the reaction. The low and high molecular weight xyloglucan contents were expressed as ΔA_{640} $seed^{-1}$.

RESULTS

Changes in growth pattern i.e. dry weight and water content mg seed^{-1} and rate of dry matter accumulation and water content are presented in figure 17, 18. Based on growth pattern seed development is divided into four distinct stages i.e. (i) Cell division (0-15 d), (ii) Cell elongation (9-36 d), (iii) Dry matter accumulation (DMA) (18-39 d), (iv) Cell maturation (39-54 d). In V_1, dry weight (DWt) per seed increased up to 39 d and stabilized later. Maximum DWt was 177.5 mg seed^{-1} at 45 d. In V_2, DWt of seed increased up to 45 d and stabilized in later stages. Maximum DWt was 115.8 mg seed^{-1} on 54 d (Figure 17A). In V_1 water content (WC) increased up to 30 d, stabilized up to 36 d and later it declined. The maximum WC per seed was 269.33 mg at 39 d. In V_2, WC per seed increased gradually up to 36 d, stabilized up to 45 d and declined at later ages. Maximum value of water content was 153.25 mg seed^{-1} at 45 d (Figure 17B).

In V_1, rate of DMA increased up to 24 d (10.56), declined later on and in V_2, the rate of DMA increased up to 36 d (5.07), (Figure 18A). In V_1, the rate of water accumulation increased up to 21 d, while in V_2 the rate of water accumulation increased up to 27 d. The maximum rate of water content was 17.27 mg seed^{-1} (at 21 d in V_1) and 7.04 mg seed^{-1} (at 24 d in V_2), (Figure 18B).

Changes in wall components in V_1 seed

The amount of EDTA soluble non esterified pectic polysaccharides (extracted in the fraction 1 and 2) is presented in figure 19A. The total non-esterified pectic substances remained lower up to 18 d (1.20), increased rapidly up to 36 d (20.19) and declined slightly at later ages of seed development (17.07).

Figure 19B demonstrates the Na_2CO_3 soluble esterified pectic substances extracted in fraction 3 and 4. The total content of esterified pectic polysaccharides showed same pattern but its content was slightly lower than the non esterified pectins. The esterified pectic substances remained in low amount up to 12 d (0.55) then increased gradually. A peak value was observed at 36 days (17.29) while in later ages the amount declined (13.92, 54 d).

Low molecular weight xyloglucans extracted in fraction 5 and 6 is presented in figure 20A. The total low molecular weight xyloglucans remained very low up to 21 d (2.41), increased sharply till maturation and peaked at 30 d (68.81). High molecular weight xyloglucans showed a small increase up to 30 d (3.87), peaked at 36 d (63.73) and declined thereafter (5.60, Figure 20B).

Changes in wall component in V_2 seed

The total non-esterified pectic substances remained low up to 15 d (0.86) and showed gradual increase with the seed age (Figure 19A). A peak value was observed at 42 d (8.12); at the later ages the value decreased and remained constant. Esterified pectic substances increased gradually, peaked at 42 d (11.49) while in later ages the value declined sharply (4.53), (Figure 19B).

The low molecular weight xyloglucans, remained very low up to 21 d (0.96), increased sharply up to 42 d (71.41, Figure 20A). At maturation its content declined and remained constant (46.14, 54 d). High molecular weight xyloglucans were very low during the initial seed ages; up to 24 days (3.06). The content thereafter increased sharply up to 39 d (37.75), declined (23.88) and remained constant at maturation (26.21), (Figure 20B).

DISCUSSION

Based on growth pattern seed development is divided into four distinct stages i.e. (i) Cell division (0-12 d V_1), (0-15 d V_2) (ii) Cell elongation (12-33 d V_1), (9-36 d V_2), (iii) Dry matter accumulation (27-42 d V_1) (18-39 d V_2),, (iv) Cell maturation (39-45 d V_1) (39-54 d V_2). Overlap between these phases was observed in both the varieties studied (Figure 17, 18). Similar growth pattern of seed development was reported earlier in cotton plant (Thaker, 1999; Rabadia et al., 1999). However Seijo and Ramos (1999) demonstrated three phases of seed development in *Pisum sativum*.

Dry weight and water content between these two varieties showed a significant difference ($P<0.05$) similarly rate of DMA and rate of water accumulation also showed a significant difference ($P<0.05$). It was hypothized that high rate of water uptake is required for elongation growth and higher level of water content supports elevated rate of dry matter accumulation (Rabadia et al., 1999). In this study, rate of DMA and rate of water accumulation remained double in V_1 compared to V_2 (Figure 18). Higher uptake of water content might be one of the factors for big size of V_1 seed. It has been suggested that seed water status play an important role in regulating its development (Egli, 1990). Kobata et al. (1992) found that a water stress imposed at anthesis, or over the first few days after anthesis, affects grain set and the grain filling rate, causing a reduction in yield components.

In developing seed cell expansion determines the sink capacity of a seed. Many studies have demonstrated that an extensive turnover of cell wall polysaccharides occur during cell elongation in higher plants. The complex process of cell elongation is mediated by a series of metabolic events coordinated with wall polymer synthesis and secretion. Pectins and xyloglucans are the two major polysaccharides that change during seed development.

In V_1 pectic polysaccharides remained higher throughout the seed development. In both seeds it was observed that non esterified pectic substances were significantly different ($P<0.05$). During cell division phase the amount of non esterified pectins were very low in V_1 and V_2. Both seeds showed same pattern of changes during cell elongation and dry matter accumulation phase where gradual rapid increase was observed, while during cell maturation stage value remained constant and stable. Stabilization of non esterified pectic substances at later stages of seed growth suggests the deposition of pectins as storage polysaccharides. In V_1 seed, as the cell elongation phase starts, significant increase in non esterified pectins was detected and remained double in content throughout the seed development.

Esterified pectic substances remained equal during the cell division phase in both the seeds. Whereas during cell elongation and dry matter accumulation phase remarkable increase in pectic substances was observed. Here also during these two phases value remained higher in V_1 seed, whereas at cell maturation phase straight decline was observed.

The DWt and WC data showed a significant correlation with esterified and non esterified pectins in both the seeds (Table 6). Changes in cell wall components play a determinative role in establishing the size of the cell. The pectic network is clearly involved in a range of functions relating to physiology, growth and development. The pectic matrix provides an environment for the deposition, slippage and extension of the cellulosic-glycan network. The change in cell wall porosity could be brought about by structural modifications of pectic molecules. Fujino and Itoh (1998) described a clear difference in cell wall architecture between elongating and non-elongating regions, suggesting a modification in the molecular form of pectin polysaccharides during the elongation of epidermal cells.

Takeda et al. (2002) found that the integration of whole xyloglucan suppressed cell elongation, whereas the integration of xyloglucan oligosaccharides promoted it. These authors proposed that xyloglucan fragments may loosen the cellulose–xyloglucan network by cleaving the xyloglucan tethers. However, even though cell wall expansion might depend on xyloglucan metabolism, in contrast to mechanical deformation or swelling, the secretion of biopolymers can not act as a driving force to cell expansion.

Low molecular weight xyloglucan remained negligible during cell division phase and early phase of cell elongation. At later days of cell elongation a marked difference in value was observed. During these days almost double value was observed in V_1 and at maturation it stabilized. During cell division and cell elongation phase the high molecular weight xyloglucan remained lower in quantity. However during these days value remained double in V_2. In V_1 seed, during dry matter accumulation phase the value rapidly increased, peaked at 36 d where rate of water uptake declined, while during maturation it decreased. In V_2, a gradual increase was observed during dry matter accumulation phase and peak was observed at 39 d where the rate of DMA was maximum and rate of water uptake declined. During cell maturation phase stabilization was observed. Most xyloglucans are subjected to turn over during growth (Terry et al., 1981). The partial breakdown of hemicellulose may be required in addition to the degradation of pectic materials to bring about the extensive expansion (Maclachan and Brady, 1994).

Xyloglucan metabolism is thought to have an important role in cell definition, cell expansion and regulation of plant growth and development (York et al., 1984; Augur et al., 1992). Disassembly of the cellulose-xyloglucan network is required for cell expansion and development. Enzymatic modification of the tethering xyloglucan

involves depolymerization and rejoining of xyloglucans chains (Fry et al., 1992; Nishitani and Tominaga, 1992), which may cause wall loosening both by enabling the integration of newly secreted xyloglucans into the wall and by restructuring existing wall bound xyloglucans (Thompson and Fry, 2001).

The results of the present study suggest that the cell wall metabolism changes from pectin synthesis to the synthesis of hemicellulose and cellulose when young tissue becomes mature. In this study pectic substances (esterified pectins) were higher during cell division phase as compared to xyloglucans which suggest that pectic polysaccharides are being synthesized during the early cell growth and transferred to newly growing cells. Xyloglucans were higher during cell expansion and dry matter accumulation stages. From these results it is suggested that pectic polysaccharides are necessary for early seed growth and xyloglucans are essential in cell wall loosening in developing *Cajanus cajan* seeds. Deposition of xyloglucans in the later stages of seed development may function as storage polysaccharides, which was observed in cotyledonous cells of *Hymenaea courbari* that accumulate large amount of storage xyloglucans in the wall (Tine et al., 2000; Santos et al., 2004). The reserve function of xyloglucan in cotyledons has been demonstrated in seeds of *Tamarindus indica* (Reis et al., 1987), *Copaifera langsdorffii* (Buckeridge et al., 1992). However, in this study, xyloglucans still remained higher and constant as the seed matured, which suggest that they may be responsible for mechanical strength of cell wall.

Table 6:

Correlation coefficient between wall components and dry weight seed^{-1} and water content seed^{-1}

	Dry weight		Water content	
	V_1	V_2	V_1	V_2
Non esterified pectins	0.97***	0.82***	0.90***	0.86***
Esterified pectins	0.80***	0.83***	0.84***	0.88***
Low molecular weight xyloglucans	0.91***	0.94***	0.88***	0.68**
High molecular weight xyloglucans	0.54**	0.90***	0.49*	0.74***

* Significant at $P<0.05$

** Significant at $P<0.01$

*** Significant at $P<0.001$

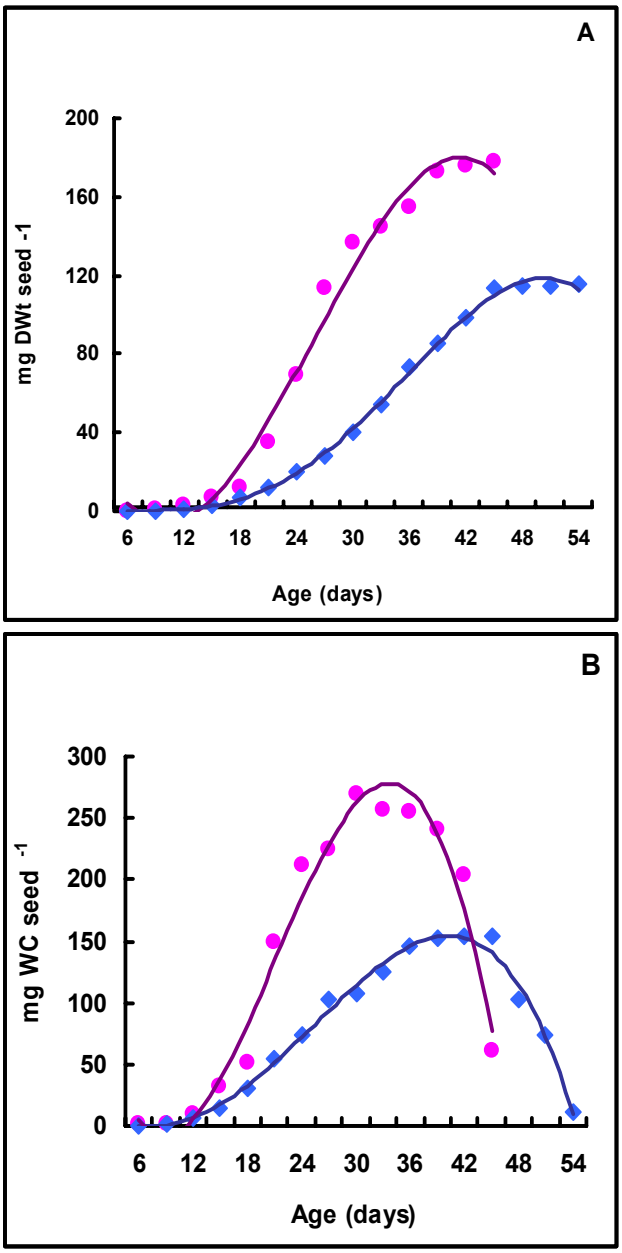


Figure 17:
Changes in Dry weight (A) and Water content (B) in developing seeds of V_1 (●) and V_2 (♦)

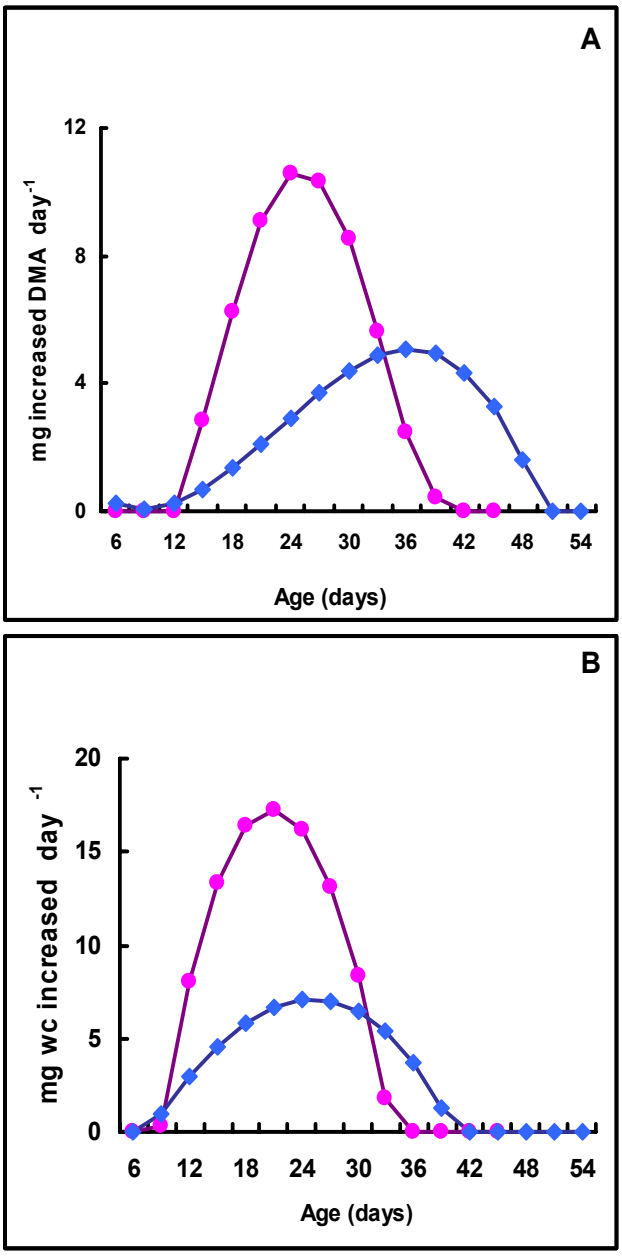


Figure 18:
Changes in rate of dry matter accumulation (DMA) (A) and rate of water accumulation (B) in developing seeds of V_1 (●) and V_2 (♦)

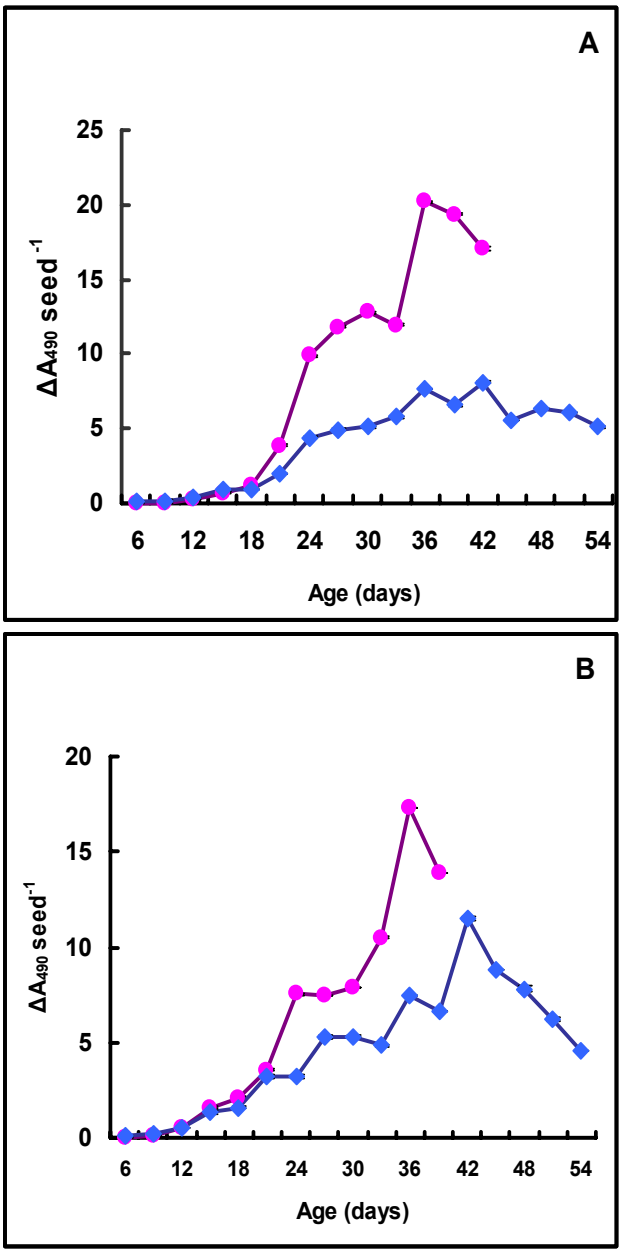


Figure 19:
Changes in non esterified pectins (A) and esterified pectins (B) in developing seeds of V_1 (●) and V_2 (♦)

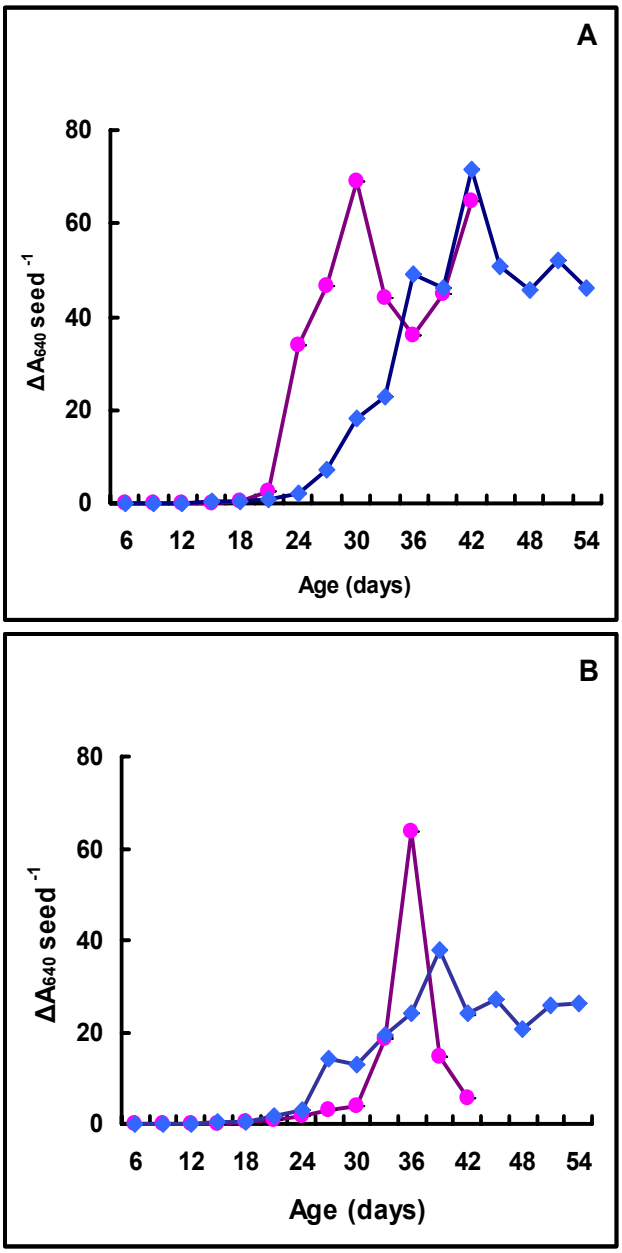


Figure 20:
Changes in low molecular weight xyloglucans (A) and high molecular weight xyloglucans (B) in developing seeds of V_1 (●) and V_2 (♦)

Chapter 6A

Influence of light intensities on elongation of seedlings, wall components and cell wall loosening enzymes

ABSTRACT

Seedlings of *Cajanus cajan* exposed to different light intensities showed significant difference in length. Maximum length was observed in green light followed by blue, red, yellow and white lights. Changes in wall components; pectins and xyloglucans were analyzed from the epicotyl growing under all light treatments. Non esterified pectins showed significant correlation with length in all lights, while esterified pectins showed correlation with red, blue and green lights. A significant correlation was observed with length and low molecular weight xyloglucans in all lights, while high molecular xyloglucans showed correlation in green light only. Wall loosening enzymes; glycosidases were estimated in response to difference in length. Cytoplasmic α-galactosidase showed significant correlation with length in all lights, β-galactosidase showed non significant correlation in red light while β-glucosidase was insignificantly correlated in all lights. Wall bound α-galactosidase showed correlation with length in all lights, β-galactosidase and β-glucosidase showed correlation only in blue and green light, respectively. Correlation between enzyme activity and wall components under all light treatments was also studied. Significant influence of light on enzyme activity is discussed.

INTRODUCTION

Light is a major environmental factor which regulates growth and development of the plant. Both quality (wavelength) and quantity (the intensity or concentration) of light are important criteria in the process (Begna et al., 2002). Plants utilize light as a source of energy as well as a major source of information from the environment. Presence or absence of light and its duration alters the growth pattern of plant.

Each wavelength of light in the visible spectrum (390 nm to 760 nm) reflects a different color. These wavelengths play an important role in seed germination and seedling establishment. It has been known that both red and blue light have an effect on stem elongation (Gaba and Black, 1983). Blue light is responsible primarily for vegetative (leaf) growth. Red light, when combined with blue light, encourages flowering. Monochromatic green light has been shown to act as a signal in regulating specific facets of plant physiology, inhibiting seedling mass, plant cell culture growth and light-induced gravitropic root elongation (Klein, 1992).

Dark grown (etiolated) seedlings display an apical hook, closed and unexpanded cotyledons and elongated hypocotyls. Upon light exposure, seedlings undergo de-etiolation: cotyledons open, expand and begin to photosynthesize, hypocotyl elongation is inhibited and cell differentiation is initiated in vegetative meristems. These events are known as photomorphogenesis and result largely from light-mediated alterations in gene expression (Ma et al., 2001; Tepperman et al., 2001; Schroeder et al., 2002). In the absence of light, sufficient energy is not available and then the seedlings growing under such conditions are usually devoid of energy. Consequently they lack sufficient food to sustain and therefore etiolate (Kiew, 1982).

Several photomorphogenic effects of blue light have been characterized in higher plants, including the suppression of epicotyl or hypocotyls elongation in dicots (Cosgrove, 1981; Baskin, 1986). The mechanism through which this suppression occurs is unknown, although it is probable that blue light affects the yielding properties of the cell wall and not the hydraulic conductivity of the growing tissue (Cosgrove, 1983). Similarly white light is inhibiting the length and growth of seedling (Miyamoto et al., 1992). These facts suggest that light changes the mechanical properties of the cell wall in the growing seedlings and decreases tissue stress, which is considered to be the driving force of shoot growth (Miyamoto et al., 1992). A long term light response helps to study the biochemical mechanisms connecting to light treatment.

Plant growth or plant cell enlargement results from a loosening of the plant cell wall and is turgor driven which involve the synthesis of new cell wall material and its integration (Taiz, 1984). The primary cell wall is also capable of expanding, indicating that the interactions between polymers can be modified to make walls extensible for elongation. The primary cell wall of plant is typically described as a complex network of cellulose microfibrils that are interwoven by two classes of matrix polymers, hemicelluloses and pectins, together with other less-abundant components such as structural proteins (Carpita and Gibeaut, 1993). The primary cell wall matrix plays a crucial role during expansive cell growth in the deposition of cell wall components and in allowing the load-bearing fibrils to slide apart (Cosgrove, 2000). The spacing between cellulose microfibrils was shown to remain constant during elongation. This may occur via weakening of the non-covalent bonding between polysaccharides, cleavage of the backbone of the major matrix polymers, and breakage of cross-links between matrix polymers (Cosgrove, 2001). Pectin is almost certain to have distinct roles during cell wall deposition and assembly and also subsequently during cell expansion. Modulation of pectin structure

within the cell walls may therefore reflect progressive changes in roles during cell development.

The major hemicellulose in dicotyledons is xyloglucan, which is thought to coat and tether the cellulose microfibrils together, forming an extensive cellulose-xyloglucan network (Hayashi and Maclachlan, 1984; McCann et al., 1990). Cellulose microfibrils are neither extensible nor degradable during cell elongation but they can move apart (Fry, 1989). This network is believed to represent a major constraint to turgor-driven cell expansion. Biochemical modifications of the cell wall, such as changes in the molecular size and quantities of cell wall polysaccharides, have been considered to be possibly involved in the regulation of cell wall extensibility (Sakurai, 1991; Kaku et al., 2002). Alteration of the molecular mass of the cell wall polysaccharides and mechanical extensibility of cell wall in response to various environmental stresses has been reported in various plant materials (Hoson, 1998).

Much evidence indicates that cell elongation and cell wall loosening are closely related to the metabolic turnover of specific polysaccharides in the cell wall. Plant cell walls play an important role in mediating physiological events in plant development and are specifically involved in the mechanism of cell elongation. The polysaccharide network of the wall contains bonds that are susceptible to a variety of hydrolytic enzymes (Murray and Bandurski, 1975). Numerous hydrolytic enzymes have been described in the cell wall of higher plants and the possibility that they might play some part in cell wall metabolism has also been considered. The presence of enzymes in the wall is of interest because of its possible role in cleaving cell wall polysaccharides. Glycosidases are the oligosaccharide hydrolyzing enzymes that are located in the cell wall. Glycoside hydrolases (glycosidases) are enzymes that catalyze hydrolytic cleavage of O-glycoside bond and are classed amongst enzymes of carbohydrate catabolism. Several studies have demonstrated that glycosidases are

present in cell walls and play a direct role in cell wall modification and cell expansion (Johanson et al., 1974; Murray and Bandurski, 1975).

In this study, seedlings grown in different light showed maximum length of seedling in green light followed by blue light. Expansive growth of plant cells require a weakening or loosening of the wall, yielding of the wall, and uptake of water. Thus it is assumed that the mechanism of cell elongation is determined by factors such as cell wall loosening, production of cell wall polysaccharides and osmotic pressure in presence of light. These facts suggest that light changes the mechanical properties of the cell wall in the growing seedlings. Earlier influence of light on growth was studied in etiolated seedling in number of plants by many workers. For de-etiolation, the elongation response has been studied most intensely. Fletcher et al. (1964) proposed that the difference in elongation of hypocotyls and petiole of bean seedlings grown in light of several spectral regions was more closely related to the extent of cell elongation than to an increase in cell number. However till to date no where influence of all light intensities on elongation is reported.

Considering the aforesaid, the main objective of the present work is to determine changes in wall components of epicotyl differing in their length with reference to changes in glycosidases (wall loosening enzymes) under various light quantities. Statistical analysis of data in terms of growth and wall components may help to interpret the results. In this study, different colored lights have been used to find out their effect on the growth of epicotyl.

MATERIALS AND METHODS

Plant Material and Light Sources

Seeds of *C. cajan* ($B.D.N_2$) were washed under running tap water and soaked in distilled water for 3 h. The imbibed seeds were kept on wet filter papers for germination and incubated in dark. After 48 h, the germinated seeds with an approximate root length of 1–1.5 cm were transferred on tray containing washed / sterile sea sand and distilled water. These trays were then kept under different lights i.e. white, yellow, red, blue and green. To study the influence of light intensity on growth (and development) of seedling, seeds were allowed to grow under continuous different colored lights. The experiment was conducted in a growth room at 25 ^{o}C under a continuous 24 h light. After 24 h, the equal sized seedlings from each light treatment were harvested for growth analysis, estimation of wall components and wall loosening enzymes.

Growth Measurements

For growth analysis, equal sized seedlings from each light treatment were harvested at every day initially up to five days and then at alternate days. Roots and epicotyls were separated; length and fresh weight were measured. For dry weight measurement, samples were oven dried at constant 80 ^{o}C for four days. Water content was determined by differences in fresh and dry weights. Data were taken in triplicates and the mean values were calculated with $\pm$ standard deviation.

Extraction and Estimation of Wall Components

The method described by Selvendran et al. (1985), was followed for preparation of cell wall material as well as further extraction of the native wall components, which is explained in detail in Chapter 5.

Preparation of the cytoplasmic enzyme extract

The seedlings from different age groups and from different treatments were weighed and crushed in 100 mM Na-acetate buffer (pH 4.5) using pre chilled pestle mortar at 0-4 ^{o}C in a cold room. Sterile sea sand was used to facilitate the homogenization. The homogenates were centrifuged at 10,000 g for 10 min at 4 ^{o}C. The supernatant was collected and pellet was washed twice with the same buffer for recovery of residual enzyme proteins to get maximum extraction. The pooled supernatants were considered as the source of the cytoplasmic enzymes. The required final volume of this extract was made using the same crushing buffer. To prevent any loss of the enzyme activity the extracts were kept on ice bath until assayed.

Preparation of ionically wall-bound enzyme extract

After the extraction of cytoplasmic enzymes, the pellet was washed repeatedly with distilled water. The washing was continued till the supernatants were found free of the marker cytoplasmic enzyme i.e. “peroxidase”. The pellets were then suspended in 1M sodium chloride (NaCl) solution. After an incubation of around 12 h at room temperature with intermittent stirring the tubes were centrifuged at 10,000 g for 10 min and the supernatant was collected. The pellets were washed twice with 1M NaCl and the pooled supernatants served as a source of ionically wall bound enzymes.

Estimation of the enzyme activities

Glycosidases assay

The activity of glycosidases was determined as described by Thaker et al. (1987). The reaction mixture consisted of equal volume of cytoplasmic and wall bound enzyme extract and respective substrate i.e., p-nitrophenyl β-D glucopyranoside for glucosidase and p-nitrophenyl α/β-D galactopyranoside for galactosidases. The concentration of the substrate was 500 µg/ml dissolved in Na-acetate buffer (100mM, pH 4.5). The reaction mixture was shaked thoroughly and incubated in dark at room temperature for 1 h. The enzyme reaction was terminated by addition of 1M Na_2CO_3 solution in double volume of the reaction mixture. The intensity of the yellow color developed was measured at 410 nm. The assay was performed in duplicates and mean values with $\pm$ standard deviation was calculated. Cytoplasmic and wall bound enzyme activity was expressed as mM pNP released epicotyl^{-1} h^{-1}.

The controls were prepared by adding Na_2CO_3 prior to enzyme in the reaction mixtures. The zero time incubated reactions were measured for the non-specific absorption and were used for correction of the reaction values.

Statistical analysis

Mean values are presented with standard deviation (SD). The statistical significance of the data on epicotyl growth, wall components and enzyme activities under different lights was analyzed using analysis of variance (ANOVA). Correlation between growth parameters, wall components and enzyme activities was determined for each light.

RESULTS

Growth analysis

Plant growth is controlled by numerous environmental stimuli that interact to regulate cell division and rate of cell expansion. Changes in growth pattern of seedling i.e. length (cm epicotyl^{-1}), dry weight and water content (mg epicotyl^{-1}) under different lights are presented in figure 21, 22, 23. Seedlings grown in different lights (white, yellow, red, blue and green) showed characteristic differences in length of epicotyls, dry weight, and water content. The gradual increase in dry weight and water content was observed in white, yellow, red, blue and green light, respectively.

Length of Epicotyl

The sequence of length of epicotyls from longest to shortest under different lights was green, followed by blue, red, yellow and white light. Initially, no clear difference in length of epicotyls was observed up to 24 h of seedling growth (Figure 21A). On first day the length of epicotyl was in a range of 1.27 cm to 1.57 cm in all light treatments thereafter up to 7th d a gradual increase was observed in white, yellow and red light. Maximum length of white, yellow and red light growing seedling was 9.58 cm, 12.62 cm and 13.66 cm, respectively on 7th d. In blue and green light a rapid increase in length of epicotyl started from the second day. Maximum length of epicotyl increased up to 11th d. The highest length of epicotyl was 20.0 cm and 24.0 cm in blue and green light growing seedlings, respectively. The double length of epicotyl was observed in blue and green light as compared to white, yellow and red lights.

In all lights, maximum rate of epicotyl length (cm increased day^{-1}) was observed on 3rd d, remained stable up to 4th d (green, blue and white) and declined gradually with growth. Maximum increase in rate of length observed was 5.32 cm in green light followed by 5.19 in blue, 3.59 in red, 3.32 in yellow and 2.25 in white light (Figure 21B). Here red and yellow lights showed parallelism in increase in length. Similarly green and blue lights also showed this parallelism but their rate was double compared to white light.

Dry weight of Epicotyl

In all lights, after 24 h (1st d) the dry weight (DWt) was in a range of 3.46 to 3.63 mg epicotyl^{-1} (Figure 22A). On second day a distinct difference in DWt was observed in all lights. Seedlings growing under white light had a dry weight of 3.83 mg on 2nd d. Afterwards a gradual increase in DWt was observed. Maximum dry weight (11.0 mg) was observed on 7th d. Under yellow light, dry weight of epicotyl was 3.68 mg at 2nd d and maximum DWt (13.75) was observed on 9th d. Red light showed slight higher DWt compared to white and yellow lights. Maximum DWt was observed on 9th d (15.37 mg). Blue and green lights showed the parallel growth pattern. In both lights, epicotyls weight was increased up to 9th d and maximum dry weight was 20.7 and 25 mg in blue and green lights, respectively. Analysis of variance showed significant difference ($P<0.05$) in dry weight of epicotyl under all light treatments.

Maximum rate of dry matter accumulation was observed on 4th d in white, yellow, blue and green lights and on 5th d in red light (Figure 22B). The maximum value of rate of DMA was 1.73 (white), 2.41 (yellow), 1.93 (red), 4.07 (blue) and 4.04 (green). In white, blue and green lights it remained stable up to 5th d and declined later on.

Water content of Epicotyl

Water content showed a highly significant difference ($P<0.001$) amongst all lights. After 24 h (1st d) little difference was observed in water content of all seedlings (Figure 23A). In white, yellow and red lights a gradual increase in water content was started from the 2nd d to 7th d. Maximum water content, 63.5 mg, 77 mg and 85.75 mg was observed on 7th d in white, yellow and red lights, respectively. Blue and green lights showed highest water content on 11th d. In blue light maximum water content was 138.35 mg and in green light the value was 157 mg. Double value of water content was observed in blue light and in green light.

The rate of water accumulation (mg increased day^{-1}) was maximum in green light at 4th d (29.9), while in white, red and blue lights at 3rd d (10.9, 16.5 and 27.8, respectively). In yellow light the maximum value was 11.3 on 2nd d. In green light, the rate of water accumulation was approximately three times higher compared to white light (Figure 23B).

Wall components

Changes in wall components of epicotyls growing under different lights are presented in Figure 24, 25.

Non esterified pectins

Figure 24A shows changes in non esterified pectins (ΔA_{490} epicotyl^{-1}) under all lights. In white light non esterified pectic substances per epicotyl were increased gradually up to 7th d and stabilized afterwards. Maximum value was observed at 11th d (2.45) old seedling. In yellow light, non-esterified pectic substances increased up to 2nd d then value remained lower up to 4th d. Next peak was observed at 5th and 7th d and later little decrease was observed. Maximum value at 5th and 7th d was 1.95 and 1.92, respectively. In later days it showed stabilization however

the value remained lower than white light. In red light, non esterified pectic substances increased gradually and peaked at 11th d (3.59). In blue light, non esterified pectic substances increased with the epicotyl growth and showed two peak values. A first peak value (2.639) was at 5th d and second was 4.589 at 11th d. In green light non esterified pectic substances increased gradually up to 11th d (4.770).

Esterified pectins

In all light treatments esterified pectins (ΔA_{490} epicotyl^{-1}) remained lower as compared to non esterified pectins (Figure 24B). In white light esterified pectic substances increased up to 5th d (0.86) and declined with age. In white light, initially up to three days, esterified pectins were higher; while from the 4th d non-esterified pectins were more than double in value. In yellow and red lights esterified pectic substances increased up to 5th and 7th d, respectively while blue and green lights showed similar pattern and increased up to 5th d. A peak value was 0.73, 0.76, 1.41 and 1.78 in yellow, red, blue and green lights, respectively. Green light showed maximum value of esterified pectins throughout the seedling growth.

Low molecular weight xyloglucans

Changes in low molecular weight xyloglucans (ΔA_{640} epicotyl^{-1}) are presented in figure 25A. In white and yellow lights low molecular weight xyloglucans increased up to 4th d and 9th d, respectively. In white light, the maximum value was 0.184 while little changes in value were observed later on. In yellow light, maximum value was 0.415 at 9th d and later on it decreased gradually. In red, blue and green lights low molecular weight xyloglucans increased up to 5th d there after declined and showed stability later on. A peak value was 0.31, 0.40 and 0.39 in red, blue and green lights, respectively.

High molecular weight xyloglucans

Figure 25B shows changes in high molecular weight xyloglucans (ΔA_{640} epicotyl^{-1}) under all light treatments. In white light, high molecular weight xyloglucans increased up to 3rd d, declined and peaked at 7th d (0.32). In yellow light, maximum value was observed on 2nd d (0.33) and later on no clear trend was there. In red and blue light, xyloglucans increased with similar pattern and peaked at 11th d (0.51 - red and 0.36 - blue). In green light xyloglucans increased gradually up to 9th d (0.58) and subsequently it declined.

α-galactosidase activity

Cytoplasmic α-galactosidase activity was ten times higher than the wall bound enzyme activity (Figure 26A, B). In cytoplasmic and wall bound enzyme activity, a distinct difference was observed at later days under all light treatments but initially (1 to 3 d) cytoplasmic activity was lower and equal in all lights. From the 4th d, with increasing age, the activity increased in all lights. In white light, maximum activity was observed at 4th d and up to 9th d it remained stable and then decreased gradually during later stages. While in yellow, red, blue and green lights a peak value was observed at 5th d and later on declined gradually.

Wall bound enzyme activity remained low up to four days and increased afterwards (Figure 26B). In white, red and blue light treatments, a peak value was observed at 5th d and in yellow and green lights peak was observed at 9th d. Maximum value was in blue light followed by green, red, white and yellow light. In later stages of seedling growth, the activity decreased with the age but remained higher in green light.

β-galactosidase activity

Cytoplasmic β-galactosidase activity was lower at initial three days and later on showed a significant difference in all light treatments (Figure 27A). In all lights the activity increased up to 5th d, remained stable up to 11th d and then declined. Maximum activity was observed in green light followed by red, blue, yellow and white light. Throughout the seedling growth, the cytoplasmic β-galactosidase activity remained higher in green light.

Wall bound enzyme activity was lower than the cytoplasmic (Figure 27B). Overlapping in activity was observed in all light treatments; however the activity increased gradually with the age. In red and blue light parallel pattern of activity was observed and peak value was obtained at 5th d. In white, yellow and green lights a peak value was observed at 7th d, afterwards it declined. Highest activity observed was in green light followed by yellow, blue, white and red light.

β-glucosidase activity

As compared to cytoplasmic galactosidase activity, glucosidase activity remained many times low (Figure 28A). In white and yellow lights no clear trend was observed with the growth of seedling. In white light maximum activity was observed at 11th d. In yellow light highest activity was observed at 9th d and later on negligible activity was present. In red light, activity remained negligible up to 4th d and peaked at 5th d and declined gradually with age. In blue light peak was observed at 2nd d and later on no clear trend was observed. In green light the activity increased with age and peak was observed on 9th d.

Figure 28B represents changes in wall bound β-glucosidase activity. In white light, wall bound activity increased up to 5th d and declined at later ages. In yellow light, the activity peaked at 3rd d and 7th d and then decreased gradually. In red light, activity peaked at 3rd d while in blue light activity increased gradually up to 5th d and decreased with the age. In green light, the activity increased steadily up to 3rd d, remained stable up to 7th d and peaked at 9th d.

DISCUSSION

Results of growth showed the significant effects of spectral quality during seedling elongation (Figure 21A). This showed the first indication that light intensity affects growing seedlings. ANOVA confirmed that dry weight ($P<0.05$) and water content ($P<0.01$) of epicotyl under different light treatments were also significantly different. Length of epicotyls showed distinct difference ($P<0.05$) under all light treatments (Table 7).

Further, correlation with different growth parameters i.e. (a) length with DWt (b) length with WC (c) dry weight with water content showed statistically highly significant values ($P<0.001$). It is well documented that steady state cell expansion is achieved when the rate of water uptake equals the rate of wall yielding (Lockhart, 1965; Cosgrove, 1981). A close positive correlation with final fiber length and water content has been observed with different varieties of developing cotton fiber. The long staple cultivar has more water uptake followed by middle and short staple cultivar (Rabadia et al., 1999). Thus in the present study, the relationship with water uptake and length in respective wavelength suggest an important role of water content in epicotyl length.

Red and blue lights showed correlation only with water content while green light showed significant correlation with dry weight and water content both (Table 8). Significant correlation of dry weight of epicotyl suggests the synthesis of pectic polysaccharides (Table 9A). A significant correlation of pectins with DWt and WC in green light showed that it might stimulate uptake of water. Sale and Vince (1959) found that while short term, red light exposures were more effective than blue light in inhibiting stem elongation in etiolated pea seedlings. Pea epicotyl is more sensitive to blue light for inhibition of elongation (Baskin, 1986; Laskowski and Briggs, 1989).

In addition, blue light induced rapid inhibition of stem elongation with 15 to 150 s in etiolated seedlings of *Sinapsis, Cucumis, Pisum, Cucurbita, Helianthus and Phaseolus species* (Cosgrove, 1981, 1982). Liscum et al. (1992) showed that blue light induces inhibition of hypocotyl elongation through genetically distinct pathway.

The synthesis of both the cellulosic and the matrix polysaccharides is essential for the hypocotyl elongation (Kawamura et al., 1976). Polysaccharides synthesis plays two important roles in elongation. It keeps the cell wall in a "loosened" condition by producing new extensible cell walls, while its other role is probably related to the fixation or extension of polymers already present in the cell wall (Hoson et al., 1991). In the present study, in all light treatments, it was observed that the amount of pectins and xyloglucans seemed to increase as the epicotyls elongated. Both the polysaccharides estimated from different light treatments showed correlation with dry weight and water content (Table 9A, 9B).

Non esterified pectins showed high significant correlation ($P<0.001$) with length in all light treatments. Dry weight and water content of epicotyl also showed high significant relation with non esterified pectic substances ($P<0.001$). In contrast, esterified pectic substances were significantly different amongst the epicotyl growing under different lights ($P< 0.05$). With increase in length, esterified pectins showed a significant correlation ($P<0.01$) in red, blue and green lights. However the relation was not statistically significant in white and yellow light.

Parvez and co-workers (1996) have observed that white light inhibits elongation of maize coleoptiles by modifying both the mechanical properties of the cell walls and cellular osmotic potential, which control the rate of water uptake. Inhibition of epicotyl elongation under white light suggests less uptake of water content. Reports showed that when seedlings grown under white light, low intensities of this light are responsible for inhibition of seedling elongation (Elliott and Miller, 1974).

It was also proposed that xyloglucan metabolism controls plant cell elongation (Yaoi and Mitsuishi, 2002). In the growing plant cell wall, xyloglucan oligosaccharides may provide positive or negative feedback control during cell elongation. Consequently, compositional analysis of oligosaccharide units in xyloglucan polymers is very important. In this study, low molecular weight xyloglucans showed a highly significant correlation with length and water content in green light ($P<0.001$) followed by blue, white, yellow and red lights, respectively (Table 8, 9B). This showed that increase in water uptake has increased the length of epicotyl. Positive and significant correlations with low molecular weight xyloglucans in all lights suggest the critical role of xyloglucans in cell wall construction. White and yellow light showed less significant level with DWt compared to green light, while, red light showed no clear correlation.

High molecular weight xyloglucans showed significant correlation with DWt and WC in green light ($P<0.001$). In blue and white light, the level of significance decreased ($P<0.05$). In red light no significant correlation was observed while yellow light showed negative correlation (Table 9A, 9B). This suggests that red light may influence the synthesis of xyloglucans. High molecular weight xyloglucans were significantly present only in green light while in other lights it showed no correlation with length. These results showed continuous de polymerization of high molecular weight xyloglucans to low molecules under all light treatments.

In the present study, correlation between water content and wall components suggest that the expansion of cell wall is due to water uptake. Correlation between dry weight and wall components suggest the synthesis of new cell wall polysaccharides. In green light, increase in length of epicotyl showed increase in wall components as compared to other light treatments. Close correlation of dry weight and water content with wall components; pectic substances and xyloglucans of green light epicotyl suggest that expansive growth requires a weakening or loosening of the wall, yielding of the wall and uptake of water.

Much direct and indirect evidence suggests that cell wall-degrading enzymes such as glycosidases play an important role in cell wall loosening (Asamizu et al., 1981). Close correlations between the activities of wall glycosidic enzymes and the rate of cell elongation have been reported in different systems (Tanimoto, 1985). It is assumed (from many reports in the literature) that the mechanism of cell elongation is determined by factors such as cell wall loosening, production of cell wall polysaccharides and osmotic pressure (Cleland, 1977). The presence of glucosidase in plant cell walls has been demonstrated in various plant organs, which seem to be important for cell wall metabolism during growth (Fry, 1988). Gomez et al., (1995) proposed a relationship between β-glucosidase activity and bean hypocotyl growth.

In the present study, glycosidases activities as influenced by light treatments are presented in figure 26, 27 and 28. In cytoplasmic fraction α-galactosidase showed significant relationship ($P<0.01$) with length in all lights (Table 10A). In blue light, the relationship was significant at $p<0.001$. β-galactosidase was more significant in blue and green light ($P<0.01$) followed by white and yellow light ($P<0.05$) but no such significance was observed in red light (Table 10A). β-glucosidase activity in cytoplasmic fraction showed no correlation with any light treatment studied (Table 10A). Similarly changes in wall bound glycosidases are presented in figure 26B, 27B and 28B. Like that of cytoplasmic fraction, wall bound α-galactosidase showed significant correlation with length in all light treatments (Table 10B). The correlation was highly significant in green light ($P<0.001$) and less significant ($P<0.01$) in white light. Wall bound β-galactosidase was correlated with length in only blue light while in other light treatments it was statistically non-significant. Wall bound β-glucosidase activity was statistically significant only in green light.

Plant cell walls possess various combinations of matrix materials, although the basic components are common (Albersheim, 1976). Thus expression of different glycosidases with elongation growth of plant is common observation. However, in this study expression of different glycosidases with different light intensities suggest a probable role of light in enzyme expression. It is observed from this study that blue light expressed more wall bound β-galactosidase than any other light intensities. Similarly green light expressed more wall bound β-glucosidase activity.

Correlation worked out with enzyme activity and wall components under different light treatments (Table 11A,11B) revealed that cytoplasmic α-galactosidase activity of blue light had a close correlation ($P<0.001$) with esterified and non esterified pectins and low molecular weight xyloglucans suggesting continuous synthesis of wall components during elongation. Even in wall bound fraction α-galactosidase showed clear correlation with length. This correlation was higher in green light ($P<0.001$) than all other light treatments ($P<0.01$). However cytoplasmic and wall bound α-galactosidase of green light was not correlating with non-esterified pectins. Cytoplasmic β-galactosidase showed less correlation with length as compared to α-galactosidase (Table 10A) significantly higher in blue light followed by green, white, and yellow while red light showed poor correlation. Also cytoplasmic β-galactosidase activity under blue light influence illustrated direct correlation with pectins and low molecular weight xyloglucans (Table 11A). Wall bound β-galactosidase showed significant correlation with the length of blue light growing epicotyl ($P<0.001$). No significant activity was expressed in other light treatments.

Similarly wall bound β-galactosidase proved no significant correlation with wall components except blue light. In blue light it had correlation with esterified pectins and low molecular weight xyloglucans. β-glucosidase activity was poor in cytoplasmic fraction of all light treatments and showed negative correlation with length in white light. Cytoplasmic β-glucosidase activity of yellow and red light showed correlation with low molecular weight xyloglucans. Green light showed correlation only with non esterified pectins while blue light showed no correlation with wall components.

In general it was observed that in blue light and in green light length of the epicotyl was significantly higher compared to other light treatments. Similarly enzyme activity and wall components were also significantly higher in these light treatments. It is interesting to note that β-galactosidase and β-glucosidase activity in cytoplasmic and wall bound fractions was also higher in blue light treatment than any other light studied. These results suggest that light intensity has also influenced the expression of enzyme activities. Significant correlations of length and wall components with blue light enzyme activities suggest the importance of blue light in elongation.

Several reports have showed that blue light inhibits the growth. The effect of blue irradiation is more complex. Blue light given alone promotes elongation but given with light of 600 nm, it inhibits (Elliott and Miller, 1974). A study on cucumber showed that blue light retarded stem elongation in cucumber by inhibiting wall yielding, yet it had negligible effect on wall mechanical properties (Cosgrove, 1988). From the research on influence of blue light on elongation shows that its effect on growth may be plant specific; in some case it inhibits the growth, while several reports suggests enhancement of growth by blue light. Even in legume seeds the response of blue light is varies. Irradiation with blue light causes a rapid decrease in stem elongation in *Pisum sativum* (Laskowski and Briggs, 1989).

Previously it was shown that stem elongation depends upon the light conditions under which the plants are grown (Elliott and Miller, 1974). Folta (2004) has observed that green light treatment causes the hypocotyl to grow more rapidly compared to irradiation with all other light qualities studied. This indicates that a green light-activated light sensor promotes early stem elongation that antagonizes growth inhibition (Folta, 2004).

CONCLUSION

Positive and significant correlations with low molecular weight xyloglucans in all lights suggest the critical role of xyloglucans in cell wall construction. While a significant correlation of pectins with DWt and WC in green light showed that it might be stimulating uptake of water. α-galactosidase activity was predominant at all stages of development followed by β-galactosidase and it increased steadily, exhibiting a rise during elongation phase (4^{th} d to 11^{th} d). The data presented in this study provide strong evidence for the involvement of galactosidases in the modification of cell wall components during the epicotyl elongation. From the correlation of enzyme activity with length suggest that expression of cytoplasmic α-galactosidase activity in blue light and wall bound α-galactosidase activity in green light is involved in elongation of epicotyl.

Table 7:

Analysis of Variance between means of growth parameters of epicotyls growing under different light intensities

	DF	F-value
Length epicotyl^{-1}	4,35	2.91*
DWt epicotyl^{-1}	4,35	2.87*
WC epicotyl $^{-1}$	4,35	6.12***
Non esterified pectins epicotyl $^{-1}$	4,40	0.52
Esterified pectins epicotyl $^{-1}$	4,40	2.99*
Low molecular weight xyloglucans epicotyl $^{-1}$	4,40	0.37
High molecular weight xyloglucans epicotyl $^{-1}$	4,40	0.20

***** Significant at $P<0.05$

****** Significant at $P<0.01$

******* Significant at $P<0.001$

Table 8:
Correlation coefficient between length of epicotyl and growth parameters, wall components of epicotyl growing under different light intensities

	White	Yellow	Red	Blue	Green
Dry weight	0.95***	0.96***	0.95***	0.98***	0.98***
Water content	0.81***	0.96***	0.95***	0.99***	0.99***
Non esterified pectins	0.93***	0.79***	0.81***	0.71***	0.85***
Esterified pectins	0.15	0.27	0.53*	0.44	0.60**
Low molecular weight xyloglucans	0.60**	0.47*	0.49*	0.68**	0.70***
High molecular weight xyloglucans	0.29	-0.23	0.41	0.46	0.78***

* Significant at $P<0.05$

** Significant at $P<0.01$

*** Significant at $P<0.001$

Table 9A:
Correlation coefficient between dry weight epicotyl^{-1} and wall components epicotyl $^{-1}$ under different light intensities

Dry weight					
	White	**Yellow**	**Red**	**Blue**	**Green**
Non-esterified pectins	0.92***	0.83***	0.79***	0.71***	0.85***
Esterified pectins	0.19	0.15	0.38	0.36	0.58**
Low molecular weight xyloglucans	0.46	0.57*	0.34	0.57*	0.63**
High molecular weight xyloglucans	0.48*	- 0.16	0.36	0.53*	0.85***

Table 9B:
Correlation coefficient between water content epicotyl^{-1} and wall components epicotyl $^{-1}$ under different light intensities

Water content					
	White	**Yellow**	**Green**	**Blue**	**Red**
Non-esterified pectins	0.70***	0.87***	0.98***	0.75***	0.79***
Esterified pectins	0.43	0.31	0.64**	0.47*	0.61**
Low molecular weight xyloglucans	0.55*	0.55*	0.54*	0.69**	0.70***
High molecular weight xyloglucans	0.53*	- 0.10	0.81***	0.52*	0.40

* Significant at $P<0.05$

** Significant at $P<0.01$

*** Significant at $P<0.001$

Table 10A:
Correlation coefficient between length epicotyl^{-1} and cytoplasmic enzymes epicotyl $^{-1}$ under different light intensities

Length					
Cytoplasmic enzyme activity	**White**	**Yellow**	**Red**	**Blue**	**Green**
α-galactosidase	0.64**	0.50**	0.65**	0.77***	0.62**
β-galactosidase	0.49*	0.49*	0.45	0.68**	0.58**
β-glucosidase	-0.05	0.25	0.37	-0.05	0.14

Table 10B:
Correlation coefficient between length epicotyl^{-1} and wall bound enzyme epicotyl $^{-1}$ under different light intensities

Length					
Wall bound enzyme activity	**White**	**Yellow**	**Red**	**Blue**	**Green**
α-galactosidase	0.52*	0.54*	0.57*	0.63**	0.72***
β-galactosidase	0.17	0.19	0.49*	0.70***	0.28
β-glucosidase	0.25	0.32	0.14	0.29	0.58**

* Significant at *P*<0.05

** Significant at *P*<0.01

*** Significant at *P*<0.001

Table 11A:
Correlation coefficient between cytoplasmic enzyme activity and wall components epicotyl $^{-1}$ under different light intensities

	White	Yellow	Red	Blue	Green
α-galactosidase					
Non-esterified pectins	0.51*	0.28	0.53*	0.76***	0.46
Esterified pectins	0.36	0.65**	0.31	0.73***	0.73***
Low molecular weight xyloglucans	0.66**	0.11	0.80***	0.84***	0.81***
High molecular weight xyloglucans	0.06	-0.47	-0.02	0.07	0.55*
β-galactosidase					
Non-esterified pectins	0.42	0.42	0.37	0.68**	0.56*
Esterified pectins	0.41	0.84***	0.38	0.76***	0.84***
Low molecular weight xyloglucans	0.62**	0.13	0.78***	0.88***	0.75***
High molecular weight xyloglucans	0.49*	-0.23	-0.01	0.07	0.66**
β-glucosidase					
Non-esterified pectins	0.16	0.30	0.29	0.28	0.52*
Esterified pectins	-0.33	-0.19	0.27	0.29	0.24
Low molecular weight xyloglucans	-0.009	0.93***	0.62**	0.34	0.06
High molecular weight xyloglucans	0.56*	-0.25	-0.03	0.24	0.50*

* Significant at $P<0.05$

** Significant at $P<0.01$

*** Significant at $P<0.001$

Table 11B:
Correlation coefficient between wall bound enzyme activity and wall components epicotyl $^{-1}$ under different light intensities

	White	Yellow	Red	Blue	Green
α-galactosidase					
Non-esterified pectins	0.23	0.54*	0.38	0.47	0.40
Esterified pectins	0.85***	0.06	0.35	0.91***	0.83***
Low molecular weight xyloglucans	0.08	0.85***	0.59**	0.85***	0.66**
High molecular weight xyloglucans	-0.23	-0.22	-0.003	-0.07	0.76***
β-galactosidase					
Non-esterified pectins	0.22	-0.16	0.19	0.42	0.32
Esterified pectins	-0.23	-0.27	0.48*	0.55*	0.42
Low molecular weight xyloglucans	0.39	0.01	0.83***	0.87***	0.16
High molecular weight xyloglucans	0.43	-0.25	-0.09	-0.04	0.32
β-glucosidase					
Non-esterified pectins	-0.01	0.27	0.005	0.38	0.44
Esterified pectins	0.73***	-0.22	-0.15	0.82***	0.34
Low molecular weight xyloglucans	0.01	0.48*	-0.008	0.46*	0.21
High molecular weight xyloglucans	0.03	0.04	-0.05	-0.01	0.79***

* Significant at $P<0.05$

** Significant at $P<0.01$

*** Significant at $P<0.001$

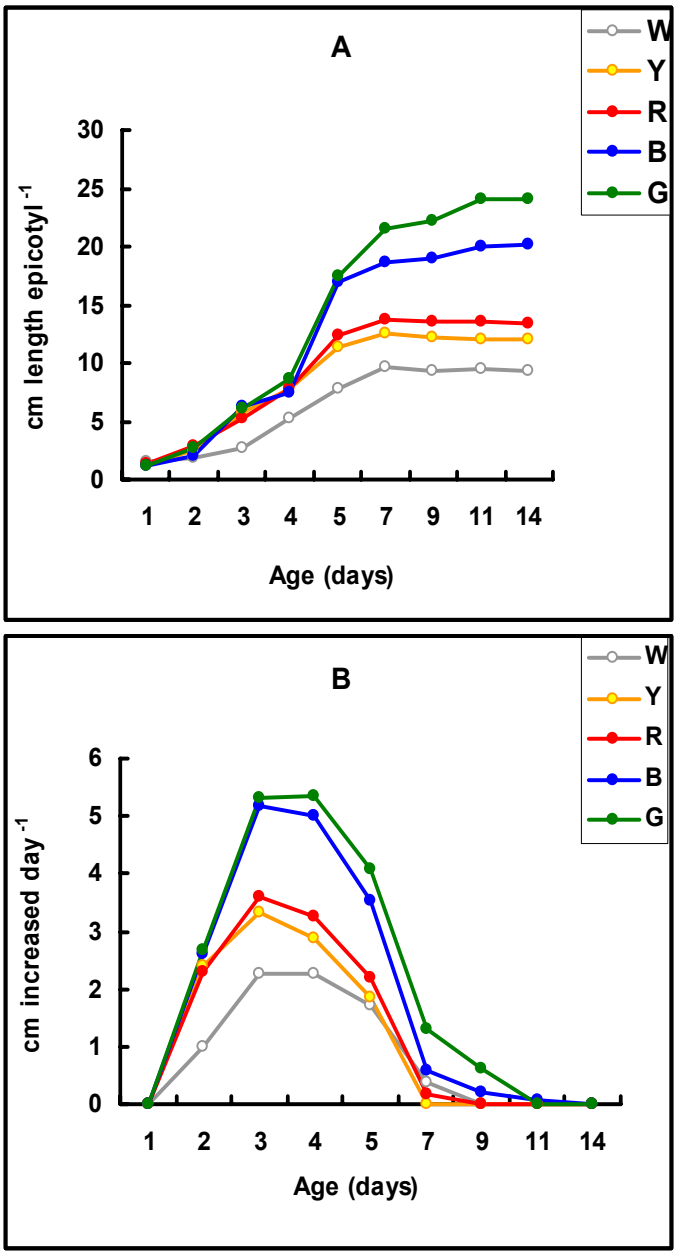


Figure 21:
Changes in length (A) and rate of length (B) of epicotyls growing under different light (W; white, Y; yellow, R; red, B; blue and G; green) intensities

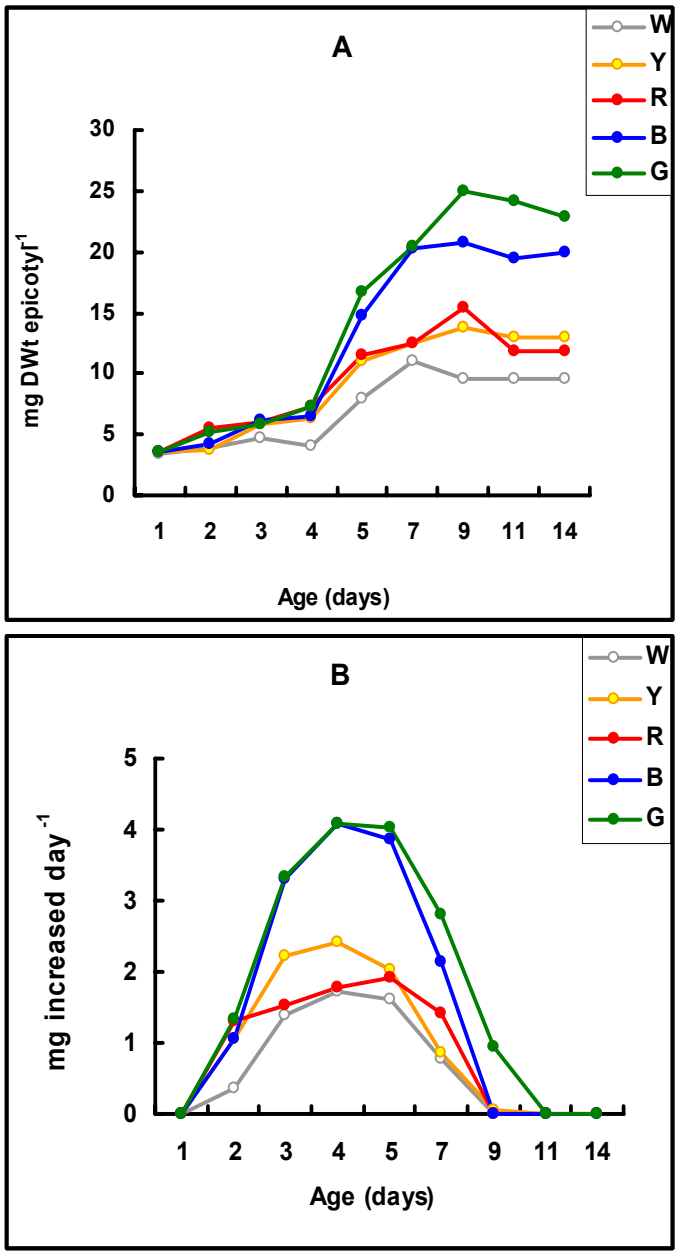


Figure 22:
Changes in dry weight (A) and rate of dry matter accumulation (DMA) (B) of epicotyls growing under different light (W; white, Y; yellow, R; red, B; blue and G; green) intensities

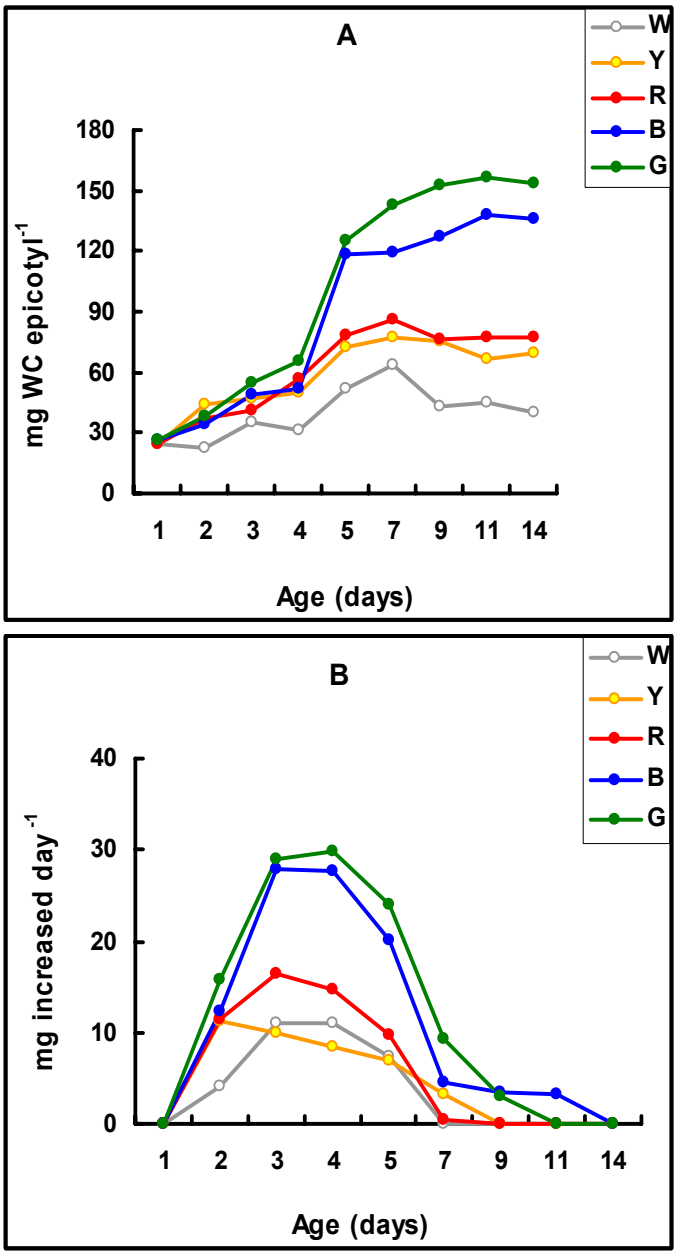


Figure 23:
Changes in water content (A) and rate of water accumulation (B) of epicotyls growing under different light (W; white, Y; yellow, R; red, B; blue and G; green) intensities

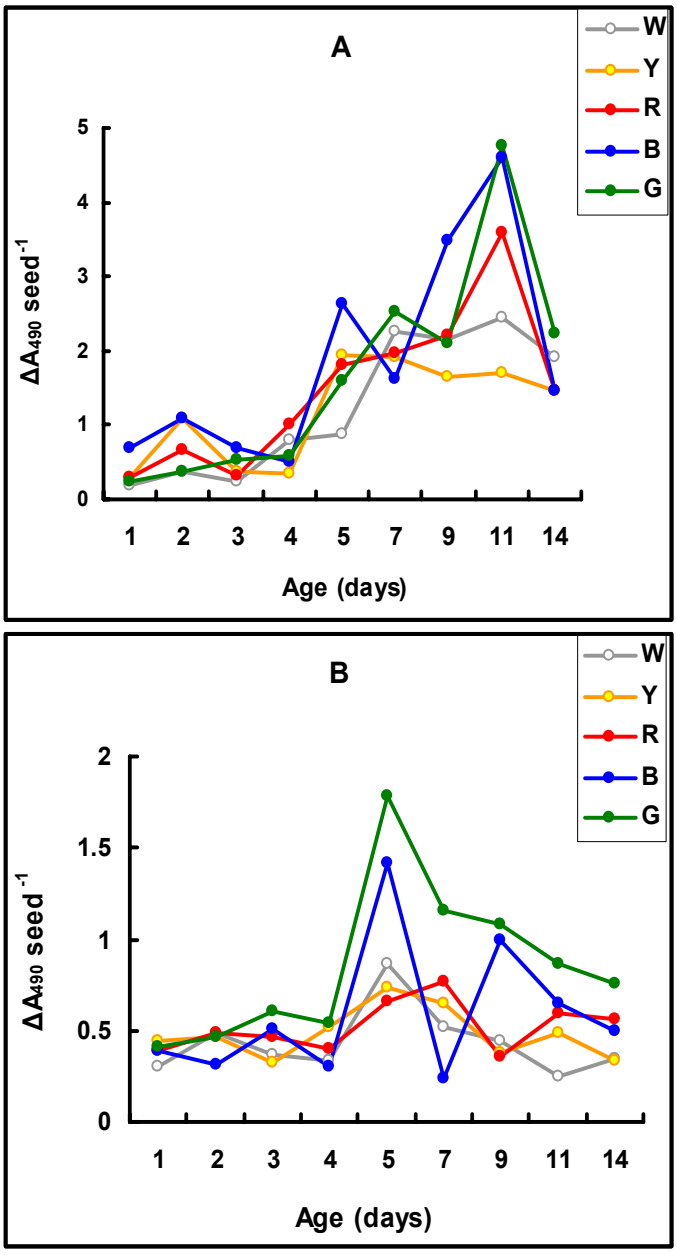


Figure 24:
Changes in non esterified pectins (A) and esterified pectins (B) of epicotyls growing under different light (W; white, Y; yellow, R; red, B; blue and G; green) intensities

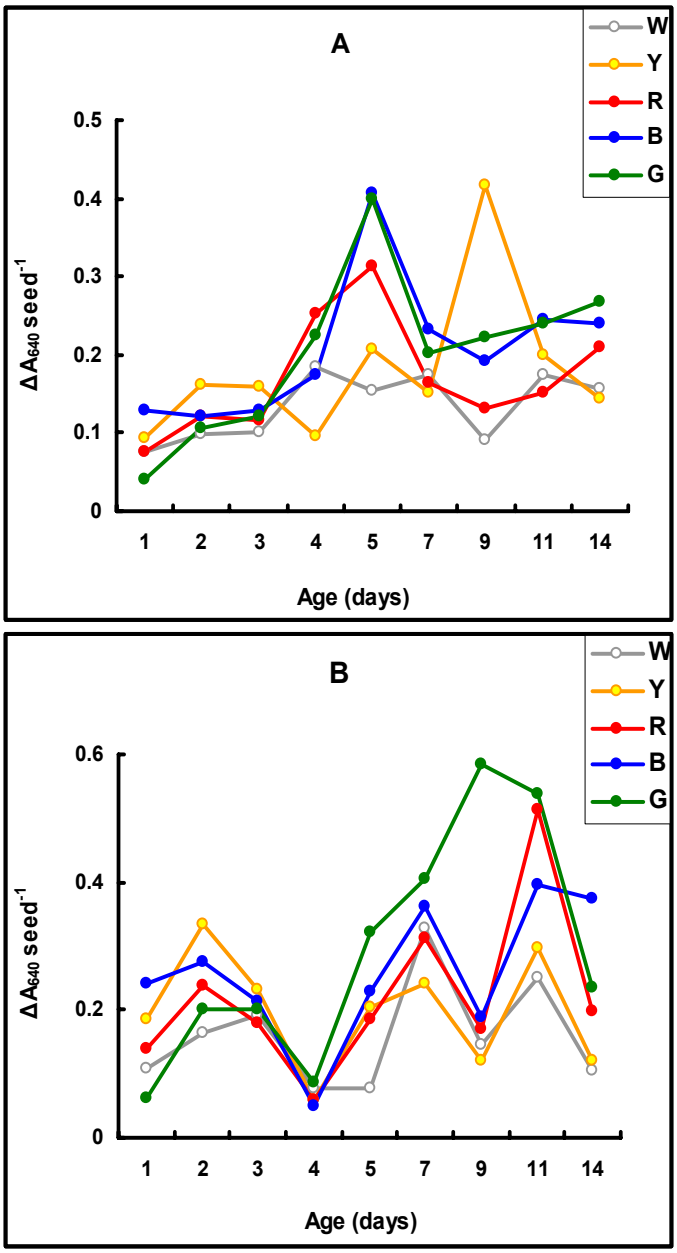


Figure 25:
Changes in low molecular weight xyloglucans (A) and high molecular weight xyloglucans (B) of epicotyls growing under different light (W; white, Y; yellow, R; red, B; blue and G; green) intensities

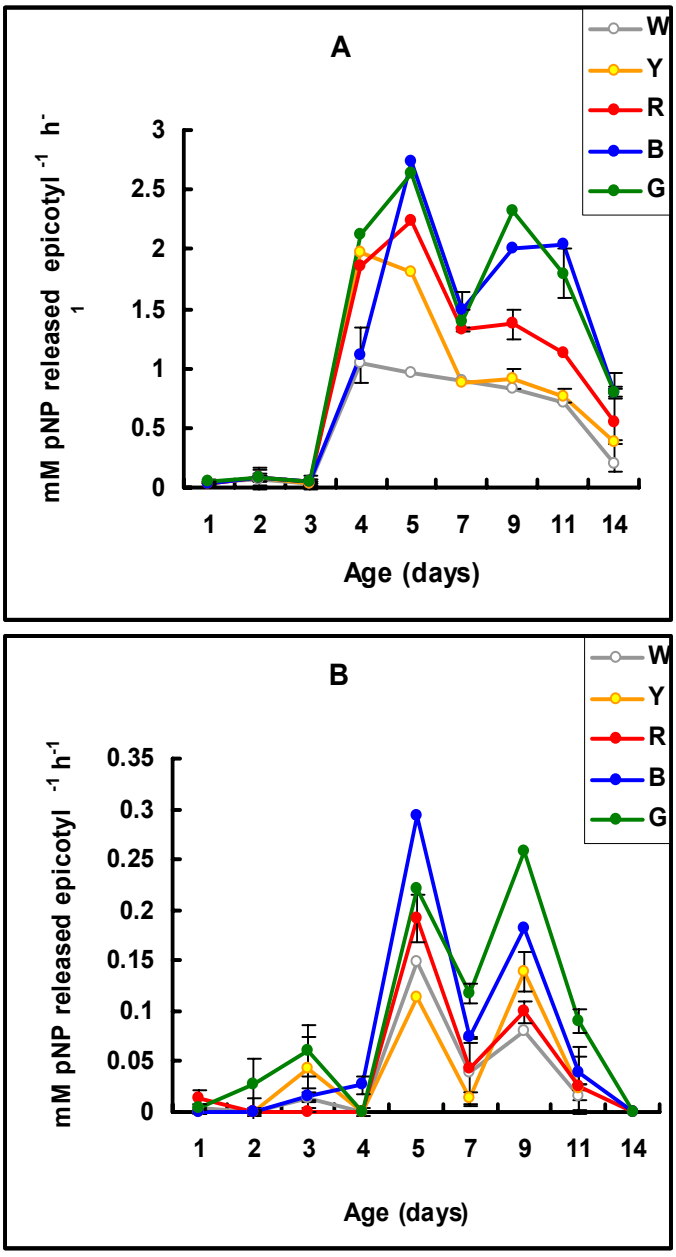
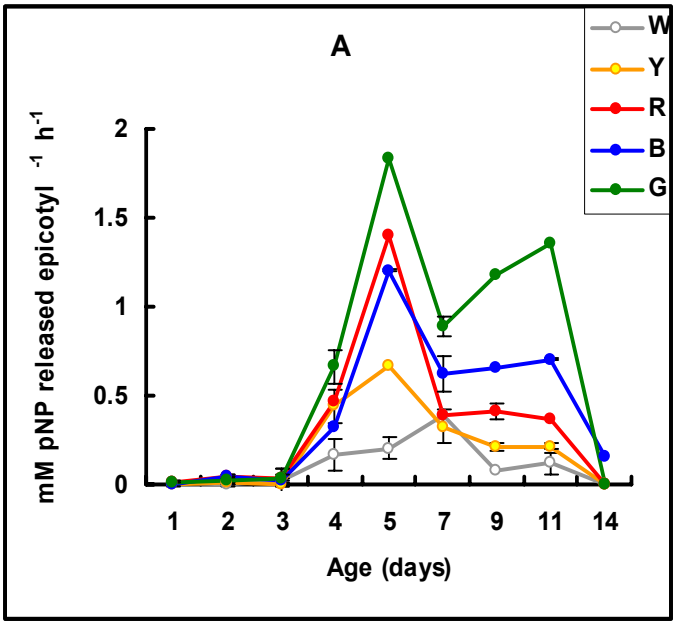


Figure 26:
Changes in cytoplasmic α-galactosidase activity (A) and wall bound α-galactosidase activity (B) of epicotyls growing under different light (W; white, Y; yellow, R; red, B; blue and G; green) intensities



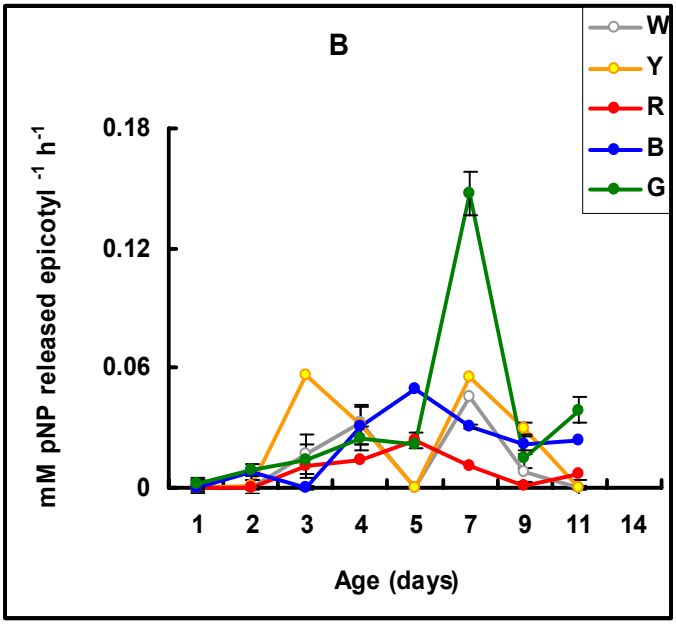


Figure 27:
Changes in cytoplasmic β-galactosidase activity (A) and wall bound β-galactosidase activity (B) of epicotyls growing under different light (W; white, Y; yellow, R; red, B; blue and G; green) intensities

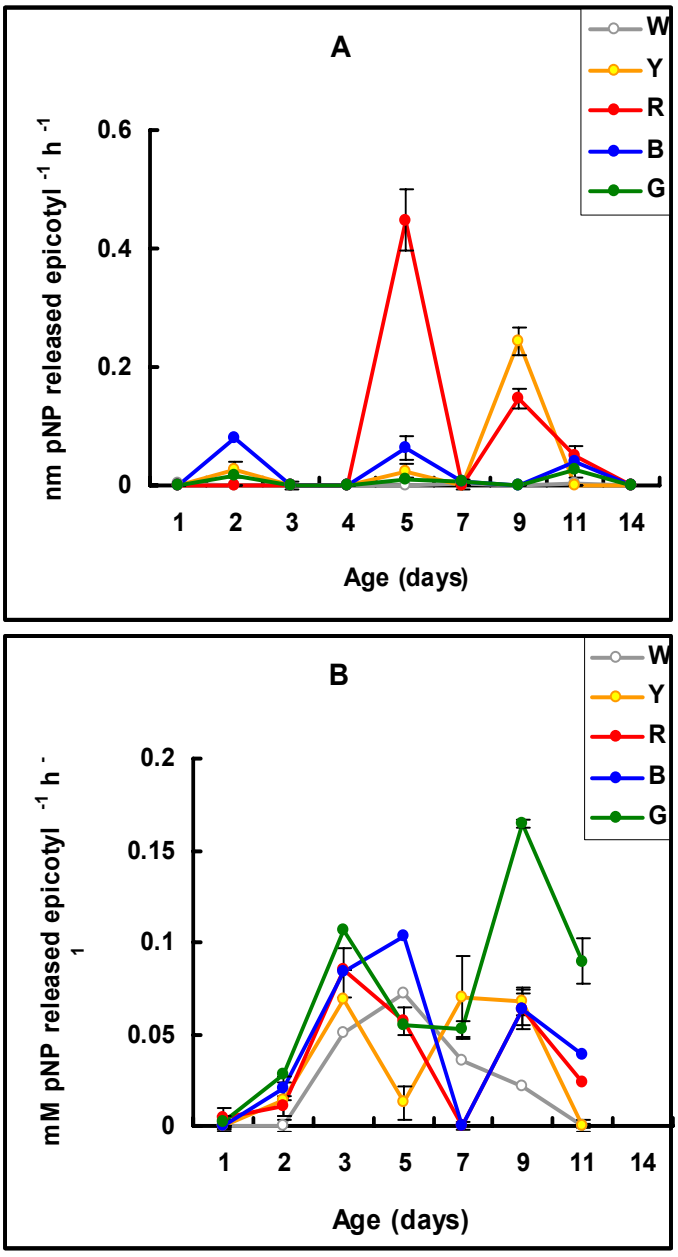


Figure 28:
Changes in cytoplasmic β-glucosidase activity (A) and wall bound β-glucoosidase activity (B) of epicotyls growing under different light (W; white, Y; yellow, R; red, B; blue and G; green) intensities

Chapter 6B

Influence of light intensities on acid and alkaline phosphatase activities

ABSTRACT

Cytoplasmic and wall bound acid and alkaline phosphatase activity was measured from the epicotyl growing under different light intensities. Cytoplasmic acid phosphatase showed significant difference among the all lights. Cytoplasmic acid phosphatase showed positive correlation with length and water content of epicotyl in white, blue and green light. Wall bound acid phosphatase showed insignificant correlation with length in all lights. Cytoplasmic alkaline phosphatase showed negative correlation with length in all light treatments, while wall bound alkaline phosphatase showed positive correlation with length except yellow light. Influence of different lights on enzyme activity is discussed.

INTRODUCTION

Phosphatases have been traditionally classified as being acid or alkaline phosphatase (Vincent et al., 1992). Acid phosphatases are a group of enzymes that nonspecifically catalyze the hydrolysis of a variety of phosphate esters in an acid environment and they are widely distributed in nature (Duff et al., 1994). Phosphorous not only plays a vital role in energy transfer and in metabolic regulation (Haran et al., 2000) but is also an important macromolecular constituent, such as in phospholipids, proteins and nucleic acids. The growth and development of plants is particularly dependent upon the availability of phosphate, under conditions of phosphate limitation (Lefebvre et al., 1990). The hydrolysis of phosphate esters is an important process in energy metabolism, metabolic regulation and a wide variety of cellular signal transduction pathways of the plant cells (Vincent et al., 1992). The demand of phosphorous increased dramatically during period of rapid cell division and growth in cotyledons of germinating soybean seedlings (Hegeman and Grabau, 2001). In plants acid phosphatase activity increases with seed germination and seedling growth and is thought to play an important role in phosphate mobilization contributing to the growth of embryonic axes (Bewley and Black, 1985).

Alkaline phosphatase are widely distributed; their function has been studied in microorganisms but little data exist about their role in higher plants, and despite having broad specificity (Kaneko et al., 1990), relatively little is about their physiological roles.

MATERIALS AND METHOD

Preparation of the enzyme extract

The cytoplasmic and wall bound enzyme extracts were prepared as described in Chapter 6A.

Estimation of the enzyme activities

Acid phosphatase assay

The acid phosphatase activity was determined as described by Thaker et al. (1996). The reaction mixture consisted of equal volume of 5 mM p-nitrophenyl phosphate (pNP) substrate (in Na-acetate buffer, 100 mM, pH 4.5), and enzyme in a final volume of 1 mL was incubated for 1 h in dark at room temperature. The reaction was terminated by adding 2 mL of 1 M Na_2CO_3 solution. To correct the non-specific color development, the control was prepared by terminating the reactions without any incubation. Calibration was done using 5-25 µg p-nitrophenol and the enzyme activity was expressed as mM p-nitrophenol released epicotyl^{-1} h^{-1}. The assay was performed in triplicate and the mean values with $\pm$ standard deviations were considered.

Alkaline phosphatase assay

The alkaline phosphatase activity was determined as described by Thaker et al. (1996). The reaction mixture consisted of equal volumes of 5 mM p-nitrophenyl phosphate (pNP) substrate (in glycine-NaOH buffer, 100 mM, pH 10.4) and enzyme. The zero minute reading served as the control. The reaction mixture incubated in dark for 1 h at room temperature and the optical density was read at 410 nm. The calibration was done using 5-25 µg pNP and the enzyme activity was expressed as mM pNP released epicotyl^{-1} h^{-1}. The mean value of three replicates was calculated with $\pm$ standard deviation.

RESULTS

Acid phosphatase

Cytoplasmic acid phosphatase activity was higher than the wall bound acid phosphatase (Figure 29). Cytoplasmic enzyme activity was lower at initial three days of seedling age. Maximum activity was observed at 4th d in red light followed by green yellow, blue and white light (Figure 29A). During later stages of seedling growth, the activity decreased gradually with increasing the age. However, in later stages activity remained higher in green light followed by blue, red, yellow and white light, respectively.

Wall bound acid phosphatase activity remained low up to two days and increased with the age of seedling (Figure 29B). In white, red and blue lights, a peak was observed at 4th d. In green and yellow light, a peak was observed at 7th d. At later ages, the activity was declined in all lights but remained higher in green light.

Alkaline phosphatase

In all light-growing epicotyl, cytoplasmic enzyme activity was higher at initial days and then decreased gradually. In all lights except red, a peak value was observed at 2nd d and maximum activity was observed in green light followed blue, white, and yellow light whereas in red light peak was observed at 5th d (Figure 30A).

Wall bound activity remained low at initial 4th days in all light treatments. In blue light a peak was observed at 5th d while in green, white, red and yellow light a peak was observed at 7th d. Maximum activity was recorded in blue and green light than the white, red and yellow light, respectively (Figure 30B).

DISCUSSION

Phosphatases are widely found in plants having intracellular and extracellular activities (Sharma et al., 2004). From seeds and seedlings, the physiological function of the acid phosphatases is to provide inorganic phosphate to the growing plant during germination and many different phosphate esters of sugars and substrates stored in the seed and seedling need to be hydrolyzed during germination and growth (Akiyama and Suzuki, 1981; Schultz and Jensen, 1981; Hoehamer et al., 2005). Phosphatases are believed to be important for phosphorous scavenging and remobilization in plants, but its role in elongation under different light intensities at germination level has not been evaluated. To examine how epicotyls different in length in respond to light influence, in the presented work acid and alkaline phosphatase activities in cytoplasmic and wall bound fractions was determined. The cytoplasmic acid phosphatase showed a significant difference ($P<0.05$) among the all lights, suggesting thereby the involvement of individual light in expression of enzyme activity. A positive correlation was observed between length of epicotyl and cytoplasmic acid phosphatase activity in white, blue and green lights (Table 12A). Similarly water content of white, blue and green lights showed significant correlation with cytoplasmic acid phosphatase (Table 12B). Contrary to this wall bound fraction showed insignificant correlation with length in all lights. Likewise water content of epicotyl also showed non significant correlation with wall bound enzyme activities under all lights. These results suggest that wall bound acid phosphatase has no role in elongation in all light treatments.

Many plants including rice, wheat, and tomato secrete increased amounts of acid phosphatase from the roots in response to phosphorus stress (Tadano et al., 1993). Acid phosphatases are constitutively expressed in seed during germination and their activities increased with germination to release the reserve material for the growing embryo (Biswas and Cundiff, 1991; Thomas, 1993).

With increase in the length under all lights cytoplasmic alkaline phosphatase activity decreased and showed negative correlation with length (Figure 30A, Table 12A). Likewise water content under all light intensities showed negative correlation with enzyme activity (Table 12B). While wall bound alkaline phosphatase showed significant correlation with length in all lights applied except yellow light. Water content of epicotyl also showed positive correlation in all lights except yellow light (Table 12B). This correlation was highly significant in white, blue and green lights ($P<0.001$).

Cytoplasmic alkaline phosphatase activity remained hundred times lower than acid phosphatase activity in all light treatments. Earlier, Thaker et al. (1996) reported low levels of alkaline phosphatase in cotton fiber and suggested that alkaline phosphatase may not have any significant role in phosphate metabolism of the tissue. However wall bound alkaline phosphatase showed correlation with length suggest that wall bound activity may have role in elongation. Here insignificant correlations of wall bound alkaline phosphatase with water content and length in yellow light provides evidence that yellow light might have suppressed the activity.

Induction of phosphatase activity in response to phosphate starvation is a common phenomenon among organisms acquiring phosphorous from the environment (Juan Carlos del Pozo et al., 1999). These enzymes have been successfully used as markers to investigate the molecular mechanisms underlying the adaptive responses to phosphate starvation of bacteria and fungi (Lenburg and O'Shea, 1996; Torriani, 1990; Vogel and Hinnen, 1990). However, in addition to understanding the production of phosphatases enzymes during germination, it would be necessary to know more about the genetic expression under light influence in response to elongation. This directs us in future to identify phosphatase gene expression in response to light influence.

Table 12A:
Correlations between length and acid, alkaline phosphatase activity of epicotyl under different light intensities

Length					
	White	**Yellow**	**Red**	**Blue**	**Green**
Cytoplasmic acid phosphatase	0.55*	0.40	0.38	0.56*	0.59*
Wall bound acid phosphatase	0.18	0.29	0.003	0.07	0.21
Cytoplasmic alkaline phosphatase	-0.81	-0.87	-0.16	-0.67	-.0.70
Wall bound alkaline phosphatase	0.59*	0.40	0.71***	0.47*	0.75***

Table 12B:
Correlations between water content and acid, alkaline phosphatase activity of epicotyl under different light intensities

Water content					
	White	**Yellow**	**Red**	**Blue**	**Green**
Cytoplasmic acid phosphatase	0.48*	0.30	0.42	0.52*	0.59**
Wall bound acid phosphatase	0.46	0.29	0.04	-0.01	0.19
Cytoplasmic alkaline phosphatase	-0.68	-0.75	-0.07	-0.65	-0.70
Wall bound alkaline phosphatase	0.88***	0.39	0.75***	0.53*	0.74***

* Significant at $P<0.05$

** Significant at $P<0.01$

*** Significant at $P<0.001$

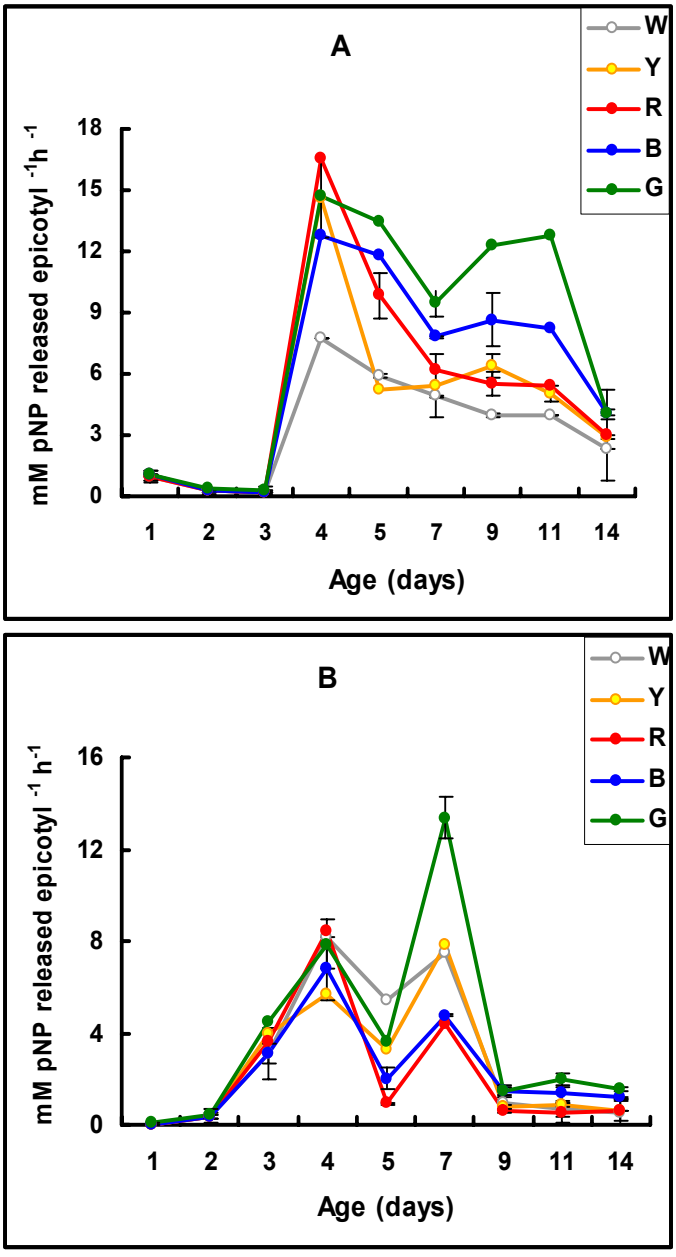


Figure 29:
Changes in cytoplasmic acid phosphatase activity (A) and wall bound acid phosphatase activity (B) of epicotyls growing under different light (W; white, Y; yellow, R; red, B; blue and G; green) intensities

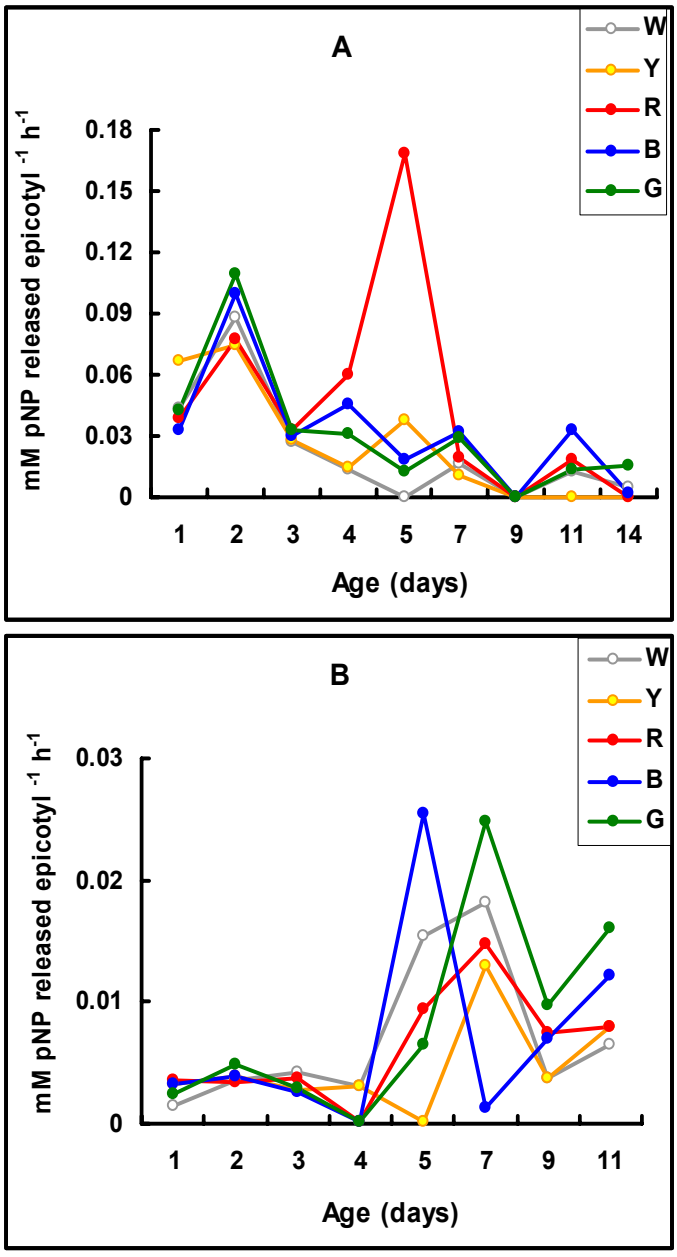


Figure 30:
Changes in cytoplasmic alkaline phosphatase activity (A) and wall bound alkaline phosphatase activity (B) of epicotyls growing under different light (W; white, Y; yellow, R; red, B; blue and G; green) intensities

Chapter 7

General Discussion

The research work presented in this thesis is divided in two parts; in the first part seed and pod development was studied for growth analysis, changes in endogenous hormonal levels and role of wall components in the sink size development in two distinct varieties of *C. cajan* varying in their photoperiod. In second approach, different monochromatic lights were given to the germinating seedlings. Growth and elongation physiology was studied with reference to wall loosening enzymes and cell wall components, to evaluate role of light intensities in the process of plant growth and development.

Light has a profound influence on virtually all aspects of plant growth and development, including seed germination, seedling development, morphology and physiology of the vegetative stage, and flowering (Kim et al., 2002; Nemhauser and Chory, 2002). It is one of the most important abiotic factor affecting growth, development and competition of plants as it is directly concerned with production of growth and biomass. The intensity and duration of light requirement vary from plant to plant. Growing habits of many plants are directly affected by day length; this phenomenon is known as photoperiodism. Photoperiod is one of the major environmental factors that influence the adaptation of crops through their effects on days to flowering (Chauhan et al., 2002).

Long photoperiod increase crop growth rate and decrease partitioning of photosynthate to pods and the duration of effective pod filling phase (Bagnall and King, 1991; Nigam et al., 1994 and 1998). Decrease in light intensity has diverse effect on leaf area development, plant growth and yield. Decreased partitioning to grain competitively favors partitioning towards organs that continue vegetative growth, thereby increasing dry matter production and leaf area (Wallace and Yan, 1998). Temperature

also has a significant influence on plant and pod growth rates in peanut (Cox, 1979; Leong and Ong, 1983; Bagnall and King, 1991; Nigam et al., 1994).

Cajanus cajan is an important legume crop in the dry land agricultural production systems, mainly because of its ability to produce large biomass and protein-rich seeds. India is the largest producer of Pigeon pea, accounting for over 80% of the world production (46,000 km^2) of which 82% is grown in India. In present work two varieties of *C. cajan* i.e., Black seeded SP-25 (V_1) and B.D.N_2 (V_2) differing in their growth rate were selected, among them V_1 is big seeded and V_2 is small seeded variety. Photoperiod intensity plays a significant role in adaptation of crop genotypes and it has effect during post flowering reproductive development and affects photosynthates partitioning. In this study, it was observed that V_2 required long photoperiod duration and flowering started one month later compared to V_1 (Figure 1). The number of flowers and the length of pod and weight of seed and pod remained higher in V_1. On the basis of research with *Phaseolus vulgaris,* Wallace and co-workers (1993) and Wallace and Yan (1998) suggested that long photoperiod caused inhibition of allocation of assimilate to seeds and increased allocation to vegetative organs.

For study of influence of light on development of seed and pod, changes in endogenous levels of hormones were estimated from these two varieties of *C. cajan*. Data on growth analysis i.e. dry weight (DWt) and water content (WC) of seed and pod of both the varieties and pod length were fitted to the polynomial equation by computer curvilinear method. The rate of dry matter accumulation (DMA) and water content obtained from the curve. Based on growth analysis seed development in two varieties of *C. cajan* was divided into four distinct stages i.e. (i) Cell division; initial lag phase of DMA (0-15 d), (ii) Cell elongation; rapid water uptake phase (12-36 d in V_1 and 9-36 d in V_2), (iii) Dry matter accumulation; rapid rate of DMA phase (21-42 d in V_1 and 18-39 d in

V_2), (iv) Cell maturation; stabilization of DMA phase (42-54 d in V_1 and 39-54 d in V_2).

Since these phases continued for stipulated time period, a marked overlapped in these phases were observed in both the varieties studied (Figure 2, 3). Similar overlap in growth phases of seed development were reported in cotton (Rabadia et al., 1999; Thaker, 1999). Using molecular and histological methodologies, cell division and cell enlargement phase of fruit development were found to overlap in early pea fruit development (Ozga et al., 2002).

DWt, WC, DMA, and rate of water accumulation were higher in V_1 compared to V_2. In both seeds, maximum DWt and WC was achieved on the same day but near to double value observed in V_1. The maximum rate of DMA was 9.3 and 5.07 in V_1 and V_2, respectively. The maximum rate of water accumulation was 12.99 in V_1 and 7.011 in V_2, respectively. During cell division phase and elongation phase DWt and WC of seed in both varieties were not statistically significant. Whereas during DMA and maturation phases a significant difference ($P<0.05$) was observed. Additionally the rate of DMA and rate of water accumulation in seeds also showed significant differences (Table 1).

A close correlation was observed between water content and the rate of DMA in both the varieties, suggesting the role of water content in DMA. These results suggest that high rate of water accumulation has increased the rate of DMA in seeds and hence both the seeds were different in weight. In developing seed water status play an important role in dry matter partitioning. The seed water may be characterized by an energy status and is likely to be determined through the potential energy. An important role of water content was also reported in various plant species. Villela (1998) suggested that during seed development, the water content after fertilization is typically high and decreases as the

physiological maturity occurs. In legume seed development due to higher water uptake, transgenic cotyledons take up more amino acids, leading to higher protein content (Borisjuk et al., 2003).
Water stress during vegetative or early productive growth of soybean usually reduces yield by reducing the number of seeds per unit area (Egli et al., 1983; Korte et al., 1983; Boonjung and Fukai, 1996; Zhang et al., 1998) while stress during seed filling reduces seed size (weight per seed) (Vieira et al., 1992; de Souza et al., 1997).

During the development of reproductive structures, assimilates, mineral nutrients and water are translocated from source to sink. Legume seeds develop within the confines of an ovary derived pod whose walls provide numerous functions for the seeds. Plant hormones play a significant role in the process that lead to mature fruit and viable mature seeds. Pod can produce photosynthates (Willmer and Johnston, 1976; Atkins et al., 1977) and it is part of the source–sink pathway that transports nutrients to the seed (Harvey, 1973; Setia et al., 1987). PGRs are considered as key substances which may stimulate fruit growth and modify the sink strength (Brenner and Cheikh, 1995). To determine the sink size variation in seeds and pods during development, changes in endogenous levels of hormones viz. IAA, PAA, GA and ABA were estimated by a comparatively more sensitive and specific technique i.e. indirect ELISA. Since phytohormones are synthesized in small quantities a sensitive and accurate method of an enzyme immunoassay was developed for hormonal analysis in plant tissue.

Past studies on exogenous hormone application and analysis of endogenous changes have supported the hypothesis that fruit development is regulated by hormonal interaction (Ozga and Reinecke, 2003). To understand interaction of hormones during different stages of fruit development, it is necessary to separate fruit development into several phases (Ozga and Reinecke, 2003). In this study, endogenous level of IAA, PAA, GA and ABA in developing seeds was explained on basis of growth

phases. IAA, PAA and ABA levels were not significantly different among the two varieties of seeds, while GA level was significantly different ($P<0.05$).

The phytohormone auxin plays a central role in the control of cell growth. It can stimulate or inhibit cell expansion, stimulate cell division, promote differentiation of vascular tissues (Casimiro et al., 2001; Marchant et al., 2002; Aloni et al., 2003). In both the varieties, the free IAA increased gradually with the seed age; and during cell division phase in V_2 seed, IAA level showed close parallelism. A steady increase was observed during the cell elongation to DMA phase (Figure 7); but value remained near to double in bigger seed. Studies on auxin during embryogenesis have been established in various seeds (Fisher-Iglesias et al., 2001).

It is well established that IAA can occur either as the hormonally active free acid or in bound forms in which the carboxyl group is conjugated to sugars and *myo*-inositol via ester linkages or to amino acids or peptides via amide linkages (Cohen and Bandurski, 1982; Bartel et al., 2001; Ljung et al., 2002). Free forms are utilized during elongation while conjugated IAA is a storage form that can release free IAA on cellular or metabolic demand. The level of conjugated IAA was more in V_1 compared to V_2 but the time of accumulation was different in both varieties which showed statistically significant difference ($P<0.01$).

In V_2 seed, conjugated IAA accumulated up to cell elongation phase while in V_1, it was present during DMA phase. The ratio of conjugated to free IAA from the cell division to maturation phase decreased gradually in V_1 (Figure 8A) while in V_2 it increased gradually and declined at later stages (Figure 8B). This result suggests that during cell division and cell elongation phase, cells in V_1 seed have hydrolyzed conjugated IAA to free forms; while in V_2, cells may not have sufficient IAA during cell division and cell elongation phases. Less available free IAA during cell

elongation phase of V_2 might have restricted the size of seed. Higher accumulation of conjugated IAA at later stage in big seed may be because of higher amount of storage protein accumulation in the seed. High concentrations of IAA conjugates in plant tissues suggest that these compounds may play an important role in the metabolism of IAA. High IAA conjugate in cotyledons of mature seeds is considered as source of IAA required for the growth of seedlings (Bialek et al., 1992).

Phenyl-acetic acid (PAA) is known to play an auxinomimetic role in plant growth regulation (Fries, 1977). In V_1 seed the level of PAA was almost absent during cell division phase and accumulation was observed at later days of seed growth (Figure 9A). In V_2 PAA level increased gradually and decreased during seed maturation (Figure 9B) and showed similar trend like IAA. Similar to IAA, this compound is able to stimulate cell enlargement (Milborrow et al., 1975). Accumulation of PAA at later stage of seed suggests that when IAA is conjugated, cells may utilize or synthesized PAA as an alternative source of auxin.

Gibberellins have been suggested to be involved in early stages (cell division) of seed development and even play important role during cell elongation phase in fruit development (Zhang et al., 2005). On the basis of seed growth phases, in V_1 seed the endogenous GA level increased rapidly during cell elongation phase till the maximum value for DMA and WC was achieved. Similarly in V_2, the level of GA increased gradually till DMA phase where there was maximum level of water content (Figure 13A).

Kato et al. (1993) reported that ABA content in large-sized grains was higher than that in small-sized grains during rice grain filling. In this study, it was observed that though the growth rate was higher in V_1, no significant difference in ABA content was observed. In both varieties the physiological age of ABA accumulation was different and this difference was remarkable in DMA phase. During cell division phase it remained low in both the

varieties, increased at early elongation phase in smaller seed (V_2) while at later days of cell elongation and DMA phase in bigger seed (V_1) (Figure 14).

Thus cell elongation phase of V_2 seed was remarkably affected by ABA (Figure 14B). Inhibitory effect of ABA in cell elongation of cotton fiber and seed development is well known. Dasani and Thaker (2006) observed that exogenous application of ABA inhibits fiber elongation and showed negative correlation with length.

Conjugated ABA was accumulated up to cell elongation phase in V_1 and during cell division phase in V_2, suggesting its regulatory role in cell division and cell elongation phases. In V_2 seed, four times higher ABA accumulated at cell division phase and decreased gradually from cell elongation to maturation phase and value remained almost equal (Figure 15). In V_2 seed, as the conjugated ABA declined, rapid increase in free ABA started (Figure 14B, 15B). In general no statistical significant correlation was observed with growth parameters and conjugated ABA level.

In V_1 seed, IAA, PAA, GA and ABA showed correlation with dry weight and water content. IAA and PAA showed insignificant correlation with rate of DMA, while GA ($P<0.001$) and ABA ($P<0.001$) showed significant correlation with rate of DMA. Close correlation of all endogenous hormones with dry weight and water content in V_1 suggest cumulative role of phytohormones in cell elongation and DMA.

In V_2 seed, IAA and GA showed correlation with DWt while PAA and ABA showed insignificant correlation with DWt. IAA, PAA and GA showed correlation with WC while ABA showed insignificant correlation. PAA ($P<0.01$), GA ($P<0.001$) and ABA ($P<0.05$) showed significant

correlation with rate of DMA. Highly significant correlation of GA suggests its important role in V_2 seed development.

In legume plants, pod development is characterized by active cell division in the young ovule and is marked by rapid pod expansion; both processes are very sensitive to water uptake. Pods are part of source-sink pathway that can produce photosynthates and deliver nutrients to the seeds. The availability of assimilate and changes in the endogenous concentrations of the phytohormones are the physiological factors that control the pod growth by regulating cell division and expansion within the ovaries (Liu et al., 2004).

In this study, pods are bigger in V_1 (11-12 cm) than V_2 (5-6 cm). The number of seeds per pod was 6-7 in V_1 and 4-5 in V_2. Further, length of pod in both varieties showed significant difference ($P<0.01$). The maximum rate of dry matter accumulation of pod was 28.84 mg in V_1 and 6.9 mg in V_2. The maximum rate of water accumulation was 77.15 mg in V_1 and 21.3 mg in V_2. Statistical analysis of these two varieties showed high significant difference in pod DWt and WC ($P<0.001$) (Table 1). Rate of pod length showed close correlation with rate of WC. This suggests that increase in length was due to higher water uptake; and enhanced the accumulation of dry matter. Earlier work from our laboratory provided evidences for important role of water content in cell elongation and DMA. It was observed in cotton fiber that high rate of water uptake increased the length of fiber elongation (Rabadia et al., 1999; Gokani and Thaker, 2002).

Level of IAA in pod was not significantly different in both the varieties. In V_1, no clear trend in IAA level was observed. Maximum amount of IAA was present only at early days which declined later on. In contrast to V_2 the IAA level remained lower during initial days, accumulated maximum during elongation period and declined at maturation. Water content of V_2

pod showed significant correlation with IAA while rate of DMA and WC showed negative correlation (Table 2B), suggesting the important role of IAA in water uptake. Auxin-induced elongation has been extensively studied in excised stem and coleoptile segments. Evidences suggest that auxin is capable of promoting cell elongation in intact plants (Yang et al., 1993; Romano et al., 1995; Gray et al., 1998).

PAA levels showed significant difference and showed same trend in both the varieties but it remained five times higher in V_1. In both pods it remained lower initially, increased gradually till maximum DMA and declined later on. Only water content of V_1 pod showed significant correlation with PAA while rate of DMA and rate of WC showed negative correlation. PAA also showed auxin like activity by stimulating elongation of wheat coleoptiles and hypocotyls of sugar beet seedlings (Wheeler 1977). Earlier, Gokani and Thaker (2002) have also found a close relation between PAA and cotton fiber elongation.

Endogenous GA per pod showed a significant difference ($P<0.05$) between these varieties. In V_1 pod, the endogenous level of GA increased gradually with pod age. WC showed significant correlation with endogenous GA level in both the pods but its level remained lower in V_2 suggesting thereby that low GA may lower the water uptake during the pod development. Gibberellins enhance longitudinal growth in shoot and increase water uptake (Banyal and Rai, 1983). Similarly difference in rate of fiber length and water content was observed in three cotton cultivars (Rabadia et al., 1999). Here, water content of pod showed significant correlation with GA, thus suggesting the important role of GA in pod development. This is mainly due to GA mediated increase in cell expansion.

No significant difference of free ABA level was observed in developing pod between two varieties. In V_1 higher accumulation of ABA was

observed during early days of pod development decreased gradually and again accumulated at maturation (Figure 16). Similarly negative correlation of DWt and WC with ABA was observed. 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. Significant positive correlation of WC and DMA with ABA during the pod development in V_2 suggests its role in growth and development.

In V_1 seed presence of GA at cell elongation phase and accumulation of ABA at later stage of cell elongation suggest the important role of GA in elongation and ABA in increase in DMA of seed. Presence of conjugated IAA and free ABA during cell elongation phase of V_2 seed suggests that less available free IAA and presence of ABA are responsible for inhibition of cell size. Thus the data collected in this experiment lead to the conclusion that ABA has important role in inhibition of cell elongation and promotion in DMA in seeds. In general IAA, PAA and GA level showed close correlation with water uptake suggesting interacting role of IAA, PAA and GA in DMA and sink size development.

Regulation of fruit size is of major importance in higher plant development (Gillaspy et al., 1993) and an economical factor for many crops. In V_1 pod, instead of IAA it may synthesize PAA as an alternative source of auxin as it shows correlation with water content. Higher amount of GA in bigger pod may increase uptake of water in total fruit. It is proposed that endogenous GA and water content of pod play an important role in increasing fruit size of *C. cajan*. As the production of high quality seed is essential to crop production in agriculture, exogenous application of GA may help in improvement of seed index and so final yield in legume seed. Cumulative results of this presented work suggest the individual role of endogenous hormones; IAA, PAA,

GA and ABA and its interaction for assimilate mobilization in developing seed and pod.

Cell division and cell expansion are the two fundamental processes governing plant size and shape. Structural modification of the cell wall is important considering regulation of cell growth (Jan et al., 2004). Parameters determining final cell size are water uptake, solute accumulation and cell wall extensibility (Lockhart, 1965). It has been suggested that seed water status play an important role in regulating seed development (Egli, 1990). Cell enlargement is the first physiological process to be affected by water (Nonami and Boyer, 1990). A change in cell wall polysaccharides is an essential feature of cell wall loosening (Catala et al., 2000). Pectins and hemicelluloses are the major polysaccharide complex of the cell wall embedded in cellulose network. This cell wall network is believed to represent a major constraint to turgor driven cell expansion (Whitney et al., 1999). In developing seed, cell expansion determines the sink capacity of a seed (Thaker, 1998). Many studies have demonstrated that an extensive turnover of cell wall polysaccharides occur during cell elongation in higher plants. Changes in cell wall components, esterified and non-esterified pectic substances along with low and high molecular weight xyloglucans in relation to water uptake and cell elongation were studied.

In this study, pectic polysaccharides remained higher in V_1 throughout the seed development. In both seeds it was observed that non esterified pectic substances were significantly different ($P<0.05$). Esterified and non esterified pectic substances remained equal during the cell division phase in both the seeds. Whereas during cell elongation and dry matter accumulation phases remarkable increase in pectic substances was observed. In V_1 seed as the cell elongation phase started a significant increase in esterified and non esterified pectins was detected and remained double in content throughout the seed development. The dry

weight and water content data showed a significant correlation with esterified and non esterified pectins in both the seeds which suggests that increase in dry weight is due to increase in rate of water uptake.

Low molecular weight xyloglucan remained negligible during cell division phase and earlier days of cell elongation. At later days of cell elongation a marked difference in value of xyloglucans was observed. During these days almost double value was there in V_1 which stabilized at cell maturation phase. High molecular weight xyloglucan remained lower in quantity during cell division and cell elongation phases. However, during these days its value remained double in V_2. In V_1 seed, during dry matter accumulation phase a rapid increase in xyloglucans was observed till 36 d where rate of water accumulation declined. In V_2, a gradual increase was observed during DMA phase and peak was observed at 39 d where the rate of DMA was maximum and rate of water accumulation declined. Dry weight and water content of V_1 showed high significant correlation with low molecular weight xyloglucans while it was less significant with high molecular weight xyloglucans. This suggests that there might be continuous depolymerization of high molecular weight xyloglucans during cell elongation. Decrease in molecular mass of xyloglucans in cell walls during the elongation stage of cotton fiber suggests the possible involvement of xyloglucan metabolism in regulation of elongation (Tokumoto et al., 2003). In V_2 seed, both the xyloglucans showed close correlation with dry weight and water content. This suggests that at maturation phase high molecular weight xyloglucans may participate in secondary wall formation.

The results of the present study suggest that the cell wall metabolism changes from pectin synthesis to the synthesis of hemicellulose and cellulose when young tissue becomes mature. From these results it can be suggested that pectic polysaccharides are necessary for early seed growth and xyloglucans are essential in cell wall loosening in developing

C. cajan seeds. However, xyloglucans still remained higher and constant as the seed matured (in V_2), which suggest that they may be responsible for mechanical strength of cell wall.

In V_1 seed, IAA showed close correlation ($P<0.001$) with both the pectins and high molecular weight xyloglucans. PAA showed high significant correlation with non esterified pectins while less significance with low molecular weight xyloglucans. GA showed high significant correlation ($P<0.001$) with non esterified and low molecular weight xyloglucans while less significance with esterified pectins ($P<0.05$).

In V_2 seed, IAA showed significant correlation with esterified and low molecular weight xyloglucans ($P<0.01$) and less significance with non esterified and high molecular weight xyloglucans ($P<0.05$). PAA showed significant correlation with both the pectins (0.01) while less significance with both the xyloglucans ($P<0.05$). GA showed close correction with both types of pectins ($P<0.001$) and xyloglucans ($P<0.01$).

The role of hormones in release of pectic polysaccharides and xyloglucans is well documented (Patel and Thaker, 2004). In dicotyledonous plants, the metabolism of xyloglucan appears to be central mechanism of auxin-induced wall loosening (Labavitch and Ray, 1974; Hoson et al., 1991). Sakurai et al. (1979) observed that auxin treatment causes a decrease in the molecular weight of hemicellulose within 30 min of treatment. GA_3 appeared to promote the hydrolysis of pectic polysaccharides (Bornman et al., 1969). GA increases cell expansion by modifying cell wall properties (Cosgrove, 1993) where GA increases water absorption, wall yielding properties and thereby promote cell expansion (Kamisaka et al., 1972). Several workers (Potter and Fry, 1993, 1994; Smith et al., 1996) provided evidences that GA treatment induces the elongation of leaves and stems in several plant species by increasing XTH (xyloglucan endotransglucosylases/ hydrolases) activity

and showed a strong positive correlation with GA_3-enhanced length in pea internodes.

In this study, free IAA, PAA, GA and wall polysaccharides showed close correlations. In bigger seed, IAA, PAA, GA and ABA were more than smaller seed. Bigger seed has greater ratio for promotory to inhibitory hormones than smaller seed. Thus release of higher wall polysaccharides during the developmental period in bigger seed supports the fact that hormones play an important role in sink development.

Some researchers conducted seedling assays in light, while others did them in darkness. Arnim and Deng (1996) found that hypocotyl growth is negatively associated with light intensity and is affected by light quality. Thus, light may alter the relative growth of the hypocotyl. In second part, the influence of light intensity on elongation of seedling of *C. cajan* was studied. In this study, different colored lights were used to find out the effect of light on epicotyl's growth. It is known that plants utilize light as a source of energy and as a major source of information from the environment. Presence or absence of lights and its duration alters the growth pattern of plant. Seedling growth under different lights showed a considerable difference in length. Maximum length was observed in green followed by blue, red, yellow, and white lights. Further, correlation with different growth parameters i.e. (a) length with DWt (b) length with WC (c) DWt with water content showed statistically highly significant values ($P<0.001$).

Cell elongation is determined by cell wall loosening and production of cell wall polysaccharides. In this study, changes in wall components of epicotyl differing in their length were estimated. In all light treatments, it was observed that the amount of pectins and xyloglucans increased as the epicotyls elongated. In all light treatments non esterified pectins showed significant correlation with length ($P<0.001$). Esterified pectins

were significant in red, blue and green light only. DWt and WC showed significant correlation with non esterified pectins in all lights ($P<0.001$), (Table 9A).

In red, blue and green lights esterified pectins showed correlation with water content while in green light it showed correlation with DWt only (Table 9B). Difference in esterified pectins may be one of the factors for changes in elongation in all lights. A significant correlation of pectins with DWt and WC in green light showed that it might be stimulating uptake of water.

Low molecular weight xyloglucans showed correlation with length in all light treatments while high molecular weight xyloglucans showed correlation in green light only (Table 8). Low molecular weight xyloglucans showed correlation with water content in all lights; similarly dry weight also showed correlation with all lights except red (Table 9A, 9B). High molecular weight xyloglucans showed significant correlation with DWt and WC in green light ($P<0.001$). In blue and white lights, the level of significance decreased ($P<0.05$). In red light no significant correlation was observed while yellow light showed negative correlation. This suggests that red light may influence the synthesis of xyloglucans.

Several photomorphogenic effects of light intensity have been characterized in higher plants, including the suppression of epicotyl or hypocotyls elongation in dicots (Beggs et al., 1980; Warpeha and Kaufman, 1989, 1990; Kigel and Cosgrove, 1991). However data on changes in wall polysaccharides with elongation with different lights are limited. In the present study, correlation between water content and wall components suggests expansion of cell wall due to water uptake whereas correlation between dry weight and wall components suggests synthesis of new cell wall polysaccharides. In general, it was observed that in blue and green lights length of the epicotyl was significantly

higher compared to other light treatments. Positive and significant correlations with low molecular weight xyloglucans in all lights suggest the critical role of xyloglucans in cell wall construction.

Furthermore, many cellular metabolic and regulatory pathways are coordinately regulated by light. Some (including all photosynthetic genes) are activated by light, whereas others such as cell-wall-loosening enzymes and water-channel proteins are repressed by light (Ma et al., 2001). It is considered that structural changes in the cell wall network are regulated by enzymatic modification, and therefore wall-modifying (loosening) enzymes would be expected to play an important role during cell elongation.

To examine how epicotyls differ in length in response to light influence, changes in glycosidases (wall loosening enzymes) activity under various studied light intensities were measured. Cell wall hydrolases cooperate in cell wall modification to achieve specific developmental results (Rose and Bennett, 1999; Catala et al., 2000). In this study, expression of different glycosidases activity with different light intensities suggests a probable role of light in enzyme expression.

In cytoplasmic fraction α-galactosidase showed significant relationship ($P<0.01$) with length in all lights (Table 10A). In blue light the relationship was highly significant ($P<0.001$). β-galactosidase was more significant in blue and green light ($P<0.01$) followed by white and yellow light ($P<0.05$) but no such significance was observed in red light (Table 10A). β-glucosidase activity in cytoplasmic fraction showed no correlation with any light treatments studied (Table 10A). This suggests that cytoplasmic β-glucosidase may be inhibited by all lights and has no role in elongation of epicotyl. Jabeen and co-workers (2006) observed in maize coleoptile that blue light induce the β-glucosidase activity within illuminated halves.

Similar to cytoplasmic fraction wall bound α-galactosidase showed significant correlation with length in all light treatments (Table 10B). The correlation was highly significant in green light ($P<0.001$) and less significant ($P<0.01$) in white light. Wall bound β-galactosidase was

correlated with length in only blue light while other light treatments were statistically non significant. Wall bound β-glucosidase activity showed correlation with length only in green light suggesting its role in elongation.

Even in wall bound fraction α-galactosidase showed clear correlation with length. This correlation was higher in green light ($P<0.001$) than all other light treatments ($P<0.01$). Cytoplasmic β-galactosidase showed less correlation as compared to α-galactosidase and significantly higher in blue light followed by green, white, and yellow lights while red light showed poor correlation. Similarly wall bound β-galactosidase exhibited no significant correlation with wall components except in blue light. β-glucosidase activity was poor in cytoplasmic fraction of all light treatments but showed negative correlation with white light. Wall bound β-glucosidase showed significant correlation with esterified pectins in blue and white lights ($P<0.001$).

Correlation worked out with enzyme activity and wall components under different light treatments (Table 11A) revealed that cytoplasmic α-galactosidase and β-galactosidase activity in blue light showed a close correlation ($P<0.001$) with esterified and non esterified pectins and low molecular weight xyloglucans suggesting continuous synthesis of wall components during elongation.

Several light induced changes in seedling growth supports the light-mediated alterations in gene expression (Ma et al., 2001; Tepperman et al., 2001; Schroeder et al., 2002). None of the reports are supporting the view about expression of glycosidase genes in plants growing under different intensities. Significant correlation of length and wall components with enzyme activity in blue light suggests importance of blue light in elongation. Cytoplasmic α-galactosidase activity was predominant at all stages of development followed by β-galactosidase and it increased steadily, exhibiting a rise during elongation phase. The data presented in this study provides strong evidence for the involvement of galactosidases in the modification of cell wall components during the epicotyl elongation. Thus blue and green light exposure may induce the expression of galactosidase

genes. Significant correlation of enzyme activity with length suggest that expression of cytoplasmic α-galactosidase activity in blue light and wall bound α-galactosidase activity in green light is involved in elongation of epicotyl.

Table 13:

Correlation coefficient between endogenous IAA, PAA and GA seed^{-1} and wall components in two varieties of *Cajanus cajan*

	IAA seed^{-1}		PAA seed^{-1}		GA seed^{-1}	
	V_1	V_2	V_1	V_2	V_1	V_2
Non esterified pectins	0.78***	0.46*	0.79***	0.60**	0.93***	0.74***
Esterified pectins	0.80***	0.63**	0.52*	0.68**	0.56*	0.78***
Low molecular weight xyloglucans	0.41	0.60**	0.53*	0.52*	0.85***	0.61**
High molecular weight xyloglucans	0.82***	0.52*	0.51*	0.53*	0.51*	0.69**

***** Significant at $P<0.05$

****** Significant at $P<0.01$

******* Significant at $P<0.001$

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