Studies On Metabolic Changes Associated With Cotton Ovule Development In Vivo And In Vitro

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Chapter I

General Introduction

Cotton the ‘white gold’ has achieved a paramount status among all cash crops in the country being the principle raw material for a flourishing textile industry. Cotton provides livelihood to about sixty million people. India has the largest area under cotton (about 8.5 to 9.0 million hectares; Kaushik and Kapoor 2006) and is the only country growing cottons of all staples right from 15 mm to 40 mm length (Sreenivasan 2004). The country ranks third in production after China and USA (Annapurve et al. 2007). India accounts for approximately 21% of world total cotton area and 16% of global cotton production. Indian productivity hovers around 350-380 kg/h that is about 50% of the world’s average productivity of 600 kg/ha (Sivanappan 2004).

The genus *Gossypium* comprises of 41 species of which 37 occur in wild form (Patel and Patel 1999). The cultivated species comprises of *G. arboreum* and *G. herbaceum* (diploid) and *G. hirsutum* and *G. barbadense* (tetraploid). *G. arboreum* is a diploid species cultivated in India along with other species like *G. hirsutum* and *G. barbedense*. This species has multifarious figure as it responds to irrigation and withstand drought besides being tolerant to pests and leaf curl virus. *G. arboreum* adapted to wide climatic conditions from north eastern India to central and south India. Limitations exist with *G*. *arboreum* in respect to fiber properties as most of the released cultivars short and coarse (Kulkarni et al. 1999).

Hybrid cotton contributes lion’s share in cotton production of India and nearly 3.33 m ha has covered by hybrid cotton. Gujarat has the distinction of evolving the first hybrid cotton in the world unshared the “hybrid cotton era”. This intra-specific cross between two strains of *Gossypium hirsutum* was released in 1968 under the name Hybrid-4 and again a breakthrough has been achieved by the development of inter-specific hybrids in desi cotton, which suited the natural environmental conditions.

Cotton is an indeterminate plant in which vegetative and reproductive structures compete with each other for available photosynthates through development. Leaf area, rate of photosynthesis and number of competing sinks are the factors upon which amount of assimilates rely (Mauney 1986). Reproductive structures of cotton plant (boll) contain seeds and their epidermal trichome - fibers. Cotton fiber and cotton seed are economically important components whereas; boll wall and bracts are the protective structures, which provide protection to the flower bud and developing bolls, respectively. Cotton seed can be divided in to six-layered outer seed coat and seed kernel *i.e.* endosperm (Bhatt 1996). The specialized cells in the uppermost layer of the seed coat are the sources of lint and fuzz fibers. The endosperm is rich in carbohydrates, proteins and fats. Cotton seed contains 22-27% of oil, which is used as cooking oil in India. Oil crushing is an important industry in U.S.A and India is also slowly progressing in this direction. Cotton cake, a waste product of oil processing industry, contains as much as 40% of top quality protein.

In cotton, any increase in yield is possible by improving plants/unit land, bolls/plants, seeds/boll, or fiber/seed by one or all components (Lewis 1992, Seagull and Gialvalis 2004.). As a key material in textile industries, cotton fiber is extensively studied by numbers of the workers in India (Naithani et al. 1982, Rama Rao et al. 1982a, 1982b, Basra and Malik 1984, Thaker et al. 1989, Gokani and Thaker 2001, 2002, Barai et al. 2005, Dasani and Thaker 2006) and abroad (Ruska 1970, Trolinder et al. 1987, Haigler et al. 1991, Ruan and Chourey 1998, Kim and Triplett 2001, Ruan et al. 2003). In India, cotton contributing 85% of raw materials to textile industry. However, comparatively, little is known about the development of seeds and other parts of the boll (Rabadia et al.1999).

The productivity changed due to both the weight and the number of the seeds (Nefed'eva et al. 2005). Yield in cotton is generally associated with the number of bolls produced per unit area regardless of genotype and environment (Meredith and Wells 1989). Cotton crop produces more floral buds than mature bolls (Jenkins et al. 1990, Heitholt 1993). As abscission of fruiting forms is usual phenomena, fruiting forms of different age and at different positions on the sympodia are likely to compete for nutrition. Shortage in photosynthates supply has often been considered to be a major cause of abscission (Guinn 1985, Wullschleger and Oosteruis 1990). During the square period (from floral bud appearance to anthesis) and the boll period (from anthesis to boll opening), the susceptibility to abscission of the fruiting form is age-dependent (Hearn and Da Roza 1985, Leith et al. 1986, Constable 1991). Squares can abscise at any age but most do so during the first seven days after appearance. The most susceptible boll stage is during the week following anthesis (open flower stage); abscission decreases rapidly thereafter (Guinn 1982, Crozat et al.1999). Several factors could be involved in this decrease; increase in the sink size led to hormonal balance changes (Guinn 1998).

On the basis of growth analysis, cotton fiber development is divided into four distinct phases like initiation, elongation, secondary thickening and maturation (Jasdanwala et al.1980, Naithani et al. 1982, Thaker et al. 1986, 1989, Seagull and Gialvalis 2004). Using polynomial model, cotton seed development has been divided into four distinct phases (i) cell division (ii) cell elongation (iii) dry matter accumulation and (iv) maturation (Rabadia et al. 1999). However, growth phases are not worked out in seed parts *i.e.* kernel and seed coat. Further, developing cotton seeds are an excellent system to study the diverse patterns of carbon partitioning, including cellulose, starch, oil biosynthesis (Ruan et al. 1997, Ruan and Chourey 1998). By manipulation of physiological processes in cotton it may be possible to increase the yield in the existing germplasm.

Crop physiologists often speak about the sink organ “strength” and the photosynthetic organ “source”, which are important in the determination of crop yield. The problem is that all-growing organs are sink for assimilates initially but sinks like young leaves later become source that differ in size and distance from vascular connection to the sources (Evans 2003). Sink strength has been considered as a product of sink size and sink activity (Warren 1972). Sink size is defined as the physical constraint (cell number and cell size) and sink activity as the physiological constraint (rates and duration of assimilates, metabolic activities and storage capacity, Wareing and Patrick 1975). The former *i.e.* sink size can be determined by the numbers and size of the cells, while latter can be easily explained by the rate and duration of import of assimilates.

For maximum yield potential both, capacity of the seed to continue to utilize assimilates (sink capacity) and the ability of the maternal tissue to continue to supply assimilates are the major factors that could influence final seed weight. For maximum yield when photosynthates supply is not limiting, sink capacity plays very important role (Ho 1988, Thaker 1999). Increase in cell volume requires a net uptake of water by the cell dry matter accumulation without cell expansion, results in displacement of water and a decline in the water level of tissue (Cohen and Bandurski 1982). The water status of a developing seed is independent than the mother plant (Westgate and Thompson 1989).

The bracteoles as well as the carpellary wall (boll rind) have been found to play a key role in the transport of major nutrients. They act as the reservoir at the early stages of development for later use (beyond half boll maturation stage) by the seed and fiber components but also in channeling the nutrient flow from the source leaf and other sources. In general, hybrids were found to be more efficient in transporting nutrients into the bracteoles for transfer to the developing boll components as the parents (Bhatt 1996).

Investigating cotton fiber development in the field at the biochemical and molecular levels is not practical because of the limited growing season and non-repeatable natural temperature variations (Haigler et al. 1991). However, fibers growing *in vitro* according to the method of Beasley (1974) closely mimic the field responses in terms of fiber elongation and maturation. *In vitro* studies have revealed new information about temperature responses of fiber development that was not available from previous field studies (Roberts et al. 1992, Xie et al. 1993). Additionally, cotton ovule culture can help to understand the influence of hormones on other factors like dry weight and seed size etc. and so in the present study, *in vitro* experiments are also designed.

Hormones play an important role in plant development. Seed are rich source of plant hormones. Hormones do not act alone in isolated system but in an interrelated manner in the plant as a whole. Thus the proportions of various hormones present may vastly affect the growth rate of subsequent differentiation patterns of the tissue in the organism, while the presence of both promotive and inhibitory hormones permits a precise control of many developmental activities (Galston and Davis 1969). According to the revised hormone-balance hypothesis of seed dormancy proposed by Karseen and Lacka (1986), ABA and GA act at different times and sites during “seed life”, ABA induce dormancy during maturation and GA plays a key role in the promotion of germination. Seeds are the main storage of IAA (Krystyna 1999). It is also claimed that free and conjugated (ester linked IAA) levels are both relatively high in the early stages of seed development but drop during seed maturation. Cytokinins govern the cell cycle and many physiological processes are now well known (Mok and Mok 2001). And changes in ratio of cytokinins to auxin or visa versa may alter the morphogenesis in plants (Beemster and Baskin 2000). However, roles of PGRs (Plant growth regulators) during different seed development process are yet to reveal.

The present day knowledge of hormonal regulations is based on two experimental approaches (i) application of plant hormones and (ii) measurement of changes in endogenous hormonal levels. The first approach, often cynically termed “spray and pray”. Unfortunately, there is some uncertainty in this kind of experiment since applied hormones can sometimes cause responses, which are not apparently regulated by that hormone in normal development. If the applied substance significantly modifies the growth process, a possible regulatory role is implied for that hormone. The implication is only valid where the supply of a hormone limits the process and exogenous hormone corrects the deficiency. This problem can be answered by the second approach. To estimate endogenous levels of PGRs, techniques generally used are (i) bioassays, (ii) physicochemical detectors (iii) spectrometric methods like HPLC, GC, GC-MS, LC-MS, MS-MS, etc.

Many research workers have attempted to identify and measure hormonal levels in cotton plants by using various bioassays. Because plant hormones are present in microgram quantities (Shindy and Smith 1975) the usefulness of bioassays has been limited. Bioassays are moderately selective for classes of PGRs when compared with many physico-chemical assays; no known system is entirely free from interaction with extract impurities (Reeve and Crozier 1980). Since bioassay specificity cannot be altered the only way in which accuracy can be enhanced is purification of the extract.

Gas-liquid chromatography-mass spectrometry has been used for identification of hormones. Development of GLC or HPLC has provided a physical-chemical assay of ABA (Yarrow et al. 1988), IAA (Weeks and Lane 1967), cytokinins (Upper et al. 1970) and gibberellins (MacMillan 1970), demonstrating the feasibility for qualitative as well as quantitative measurements of naturally occurring plant hormones. Computer

controlled GLC-MS has been used for identification of phytohormones but there is no such physical-chemical assay available in which all hormones can be measured in a single extract. In addition, physico-chemical techniques require large sample size, extensive purification of samples, more sophisticated and expensive equipment along with bulk of chemicals. Physico-chemical assays are tedious, time consuming and expensive as they require many sophistications. For the rigid identification of PGR structures or elucidation of new structures, metabolic studies; the GC-MS analysis undoubtedly will remain the method of choice. The major analytical difficulties in physiological work can be answered with immunoassays.

The advantage of EIA (Enzyme Immuno Assay) for hormonal analysis is the ability to use the crude plant extract without scarifying sensitivity and selectivity. To amplify the reaction indirect ELISA was used by Gokani et al. 1998 for measurement of endogenous levels of phytohormones. Additionally, the production of PGRs and their action is part of a network of interacting processes (Trewavas 1991). Antibodies have long been recognized for their diagnostic and therapeutic purpose (Gomord et al. 2004). In present time, it has pave a way to an even greater demand because of its specific reaction, very accurate results and simple methodology as compared to tedious, complicated and costly biochemical reactions.

All PGRs are low molecular weight compounds, and therefore are not immunogenic. To make them immunogenic, synthesis of PGR-protein conjugates is necessary for an immune response. By appropriate choice of protein and binding site, antisera with predefined characteristics can be obtained. The potential of PGR immunoassay is based in its combination of intrinsic specificity; sensitivity and ease of operation, thus allowing a convenient analysis of rather impure samples for the

presence of specific PGRs. Problems of low recoveries during extensive purification of samples are largely or completely eliminated. Another part of the attractiveness of immunoassay is its large sample capacity (upto 300-500 extract can be preceded per day by one person) and the fact that a parallel analysis of several PGRs using the same basic protocol and equipment is easily possible.

Earlier work from our laboratory on cotton fiber development (Gokani and Thaker 2001, 2002, Dasani and Thaker 2006) with three different cotton cultivars (short, middle and long staple) explained that short staple cultivars are deficient in their capacity to synthesize the sufficient amount of promotory PGRs. However, there is no such report on regulation of hormonal metabolism in cotton ovule development with special reference to their source organs like boll wall and bract. Additionally, little is known about the endogenous control of seed coat development (Nooden et al. 1985). Therefore, in this thesis experiments are designed to evaluate the changes in endogenous hormonal levels in all components of seed *i.e*. kernel, seed coat and fiber as well as from protective parts *i.e.* boll wall and bract. Considerable evidences from the literature suggest that the peroxidase play an important role in regulation of endogenous IAA concentration and sink size development. An objective has been set to evaluate the changes in peroxidase activity in kernel and seed coat development.

Radiation induced mutations have been extensively used for the improvement of crop plants (Das et al. 2000), since last seven decades attempts were made to induce mutation by exposing seeds to the radiation. The number of induced mutants of crops has increased greatly over past years (Khalifa 1978, Saeed et al. 1982, Kurepin et al.1983). The heavy ion radiations, a high linear energy transfer (LET) are known to cause more localized, dense ionization within the cells compared to

low LET radiation (Kraft et al. 1992). Heavy ion irradiations modulate various physio-chemical and morphological processes in plants. Seed exposed to heavy ion during space flights showed growth inhibition, somatic mutations and tumor formation in seedlings (Peterson et al. 1977). Some cell lines, known to be radio resistant to X rays have exhibited great sensitivity to heavy ions. The heavy ions potential to penetrate precisely in tissues, suggested its possibility to be used as radio therapeutic tool, especially in case of cancer (Ando 2000), make sense to be examined in plants for various responses.

Some attempts were also made to induce variability by gamma radiation of cottonseeds (Constantin 1968, Sarkhanbejl and Yu 1984). Exploratory experiments just to see what may come out were later replaced by mutation breeding programmes with scientific, well-defined objectives. A large number of new cultivars have been developed in a variety of crop plants over the past three decades and mutation breeding is now accepted method for crop improvement programmes in India (Singh 2005). The biological effects of environmental stress and the physiological response of plants that allows their survival under stress conditions is a widely studied topic (Zaka et al. 2002). But effects of abiotic stress (radiation) on hormonal regulation are yet to evaluate. From the experimental data, it appears that the heavy ions possess deep deposition tendency and direct damaging action on biomolecules. Therefore, in the present study ^{7}Li ion radiation was selected to study its influence on endogenous hormonal level in cotton fiber.

Considering the aforesaid, following objectives has been set forth in the present study:

- Cultivation of different cotton genotypes varying widely in the staple length and seed index
- Growth analysis of two cotton genotypes *Gossypium hirsutum* L. (Hybrid 6) and *Gossypium arboreum* (Gujarat cotton-13) in terms of (a) kernel weight (b) seed coat weight and cumulatively (c) seed weight (d) fiber weight and their water content. And to find out the rate and duration of dry matter accumulation, water uptake
- Growth of (e) boll wall as well as (f) bracts; dry weight and water amunt
- Measurement of fiber length and to find out rate of elongation
- Determination of percent dry matter allocation and water amount partition in sink organs
- Raising of antibodies against PGR- protein conjugate
    - Preparation of different PGR-protein conjugates like PGR-BSA and PGR-Casein to obtain high molecular weight compound
    - Immunization –intramuscular route
    - Separation and purification of antiserum
    - Titer test and sensitivity test
- Preparation of samples (kernel, seed coat, fiber, boll wall and bracts) for immunoassay

- ELISA
  - Extraction and purification of endogenous hormones from cotton boll samples
  - Standardization of antigen antibody concentration for indirect ELISA
  - Preparation of calibration curves for all plant growth regulators (IAA, PAA, GA, ABA, zeatin)
  - Estimation of endogenous level of plant growth regulators from all boll components during entire period of cotton boll development
  - Estimation of conjugated hormonal levels from all boll components during entire period of cotton boll development
- Standardization of optimum media and hormonal concentration for *in vitro* studies
- Preparation and purification of materials for peroxidase enzyme assay
- Estimation of peroxidase from seed kernel and seed coat throughout the period of seed development
- Standardization of fiber samples for heavy ion radiation (^{7}Li) and estimation of endogenous contents of hormones

Chapter II
Growth Analysis

SUMMARY

Two cotton genotypes (*Gossypium hirsutum*) H-6 and (*Gossypium arboreum*) G. Cot varying widely in their seed index and fiber length were selected for growth analysis. The analysis was performed in terms of fresh weight, dry weight, water amount and fiber length throughout the developmental period. Cotton boll is made up of seed, fiber, boll wall along with septa and bracts. Further, seed is composed of kernel and outer coat with epidermal hairs. From the growth analysis kernel and seed coat were divided into four distinct growth phases (i) cell division (ii) cell elongation (iii) dry matter accumulation and (iv) maturation. Fiber development process was also followed by four different phases (i) initiation (ii) elongation (iii) secondary thickening and (iv) maturation. Both the genotypes followed the same developmental phases; rate and duration of dry matter accumulation was remarkably higher in H-6 as compared to G. Cot. Seed coat development in H-6 completed before kernel development started whereas in G. Cot higher degree of overlap between kernel and seed coat was observed which suggest that in small seeded genotype, the seed components compete for the available photosynthates. Apart from this, protective structures *i.e.* boll wall and bracts were also studied for their growth kinetics from the day of anthesis to maturation. Considerable overlap between elongation and dry matter accumulation was observed in seed components. Rate of dry matter accumulation and rate of fiber elongation showed close correlation with water amount suggesting an important role of water uptake in cell elongation as well as in dry matter accumulation. In general, initially 40-50% dry matter was stored in boll wall and 50-60% dry matter in bracts. At physiological maturity, it decreased up to 10-17% in boll wall and 1-1.5% in bracts. Similarity in results of percent water amount allocation was observed. The percent dry matter allocation and percent water amount allocation amongst boll components indicated that protective structures (boll wall with septa and bract) are participating in sink development and final yield.

Abbreviations: DPA- Day Post Anthesis, H- 6 - Hybrid-6, G. Cot– Gujarat Cotton-13, DMA- Dry Matter Accumulation

INTRODUCTION

Cotton being an indeterminate plant the vegetative and reproductive structures competes with each other for available photosynthates throughout the growing season. The branches on a cotton plant classified as either monopodial (vegetative branches) or sympodial (fruiting branches) that give rise to fruit. A mature cotton fruit can be dissected into seed, fibers, boll wall, septa and bracts. Leaf area, rate of photosynthesis and number of competing sinks are the factors upon which amount of assimilates rely (Mauney 1986). The developing fruiting bud defined as a square; develop at the main stem or at the subtending leaves decide the boll position. Several experiments on monitoring the movement of ^{14}C from leaves to boll suggest that boll position is an important feature for Carbon allocation (Ashley 1972, Benedict et al. 1973, Crozat et al. 1999). Cotton growth and development is time and temperature dependent regime (Roussopoulos et al. 1998). Recent evidences suggest that delayed fruiting reduce cotton seed yield and average boll weight at lower main stem nodes, than upper main stem nodes and thus show the shift in yield distribution as indicative of compensatory growth (Dumka et al. 2004). Several studies (Kletter and Wallach 1982, Jones et al. 1996a, Bednarz and Roberts 2001) have reported that cotton compensates for loss of early season fruiting forms through increased boll retention at upper main stem nodes. Although boll size, number of locule and number of seeds/boll are genetically controlled; environmental conditions play a major role in growth and development of the bolls in cotton plant (Singh 1986).

As bolls develop, they become much stronger sink (Constable and Rawson 1980) than the shoot and the root parts. Many workers have suggested that the cotton yield is primarily due the differences in reproductive sinks rather than photosynthetic capacity (Hearn 1969, Gandhi et al. 1999). Lalonde (2004) evolved that in most plants, organic assimilates (carbon and nitrogen

forms) are transported across multiple membranes on their way from source (production sites) to sink (storage locations). Transportation of assimilates play an important role in yield determination, which can be affected by (a) supply of photosynthates (Wullschleger and Oosterhuis 1990), (b) supply of nutrients (Bhatt 1996) and (c) environmental factors *i.e.* light (Singh 1986) and temperature (Thaker et al. 1989, Roussopoulos et al. 1998). In cotton any increase in yield is possible by improving plants/unit land, bolls/plants, seeds/boll or fiber/seed weight by one or all components (Lewis 1992).

Cotton seed is a major oil seed in domestic and international market (Kohel 1998). Developing cotton seeds are an excellent system to study diverse patterns of carbon partitioning, including cellulose, starch and oil biosynthesis (Ruan et al. 1997). For maximum yield potential, capacity of the seed to continue to utilize assimilates and the ability of the maternal tissue to supply assimilates are the important factors that influence final seed weight. Seed size is one of the important considerations of present day seed industries (Ramadevi and Ramarao 2005). A positive relationship was reported between size of planted seeds and seed yield, in cotton (Maiya et al. 2001), in groundnut (Borate et al. 1993) and in sunflower (Singh et al. 2003). This can be explained by (i) physical constrains (cell numbers and cell size) and (ii) physiological constrains (rate and duration of assimilates, metabolic activities and storage of assimilates) (Gipson and Ray 1969, Schubert et al. 1973). Review of literature supports the positive correlation between DMA and endosperm cell numbers (Cochrane and Duffus 1983), sink size (Chanda and Singh 1998, Rabadia et al. 1999, Thaker 1999); duration of DMA (Egli 1990) and rate of DMA (Gebeyehou et al. 1982, Smith and Nelson 1986).

A true seed can be defined as fertilized mature ovule consisting of embryo, stored material and protective coats. In cotton, seeds are covered with six-layered outer seed coat (Bhatt 1996), which is the main modulator of

interactions between the internal structures of the seed and the external environment (Francisco et al. 2001). Seed coat provides the integrity to the seed parts, protects the embryo against mechanical injuries and attacks of pests and diseases, regulates gaseous exchanges between the embryo and the external environment and also regulates imbibition (Peske and Pereira 1983, Swanson et al. 1985, Argel and Paton 1999).

The important events included in seed development and maturation are pollination, fertilization, and development of fertilized ovule. Earlier workers viz., Sofield et al. (1977) explained the seed development in two phases (i) initial cell division and (ii) rapid dry matter accumulation. Using polynomial model, cotton seed development have been divided into four distinct phases (i) cell division (ii) cell elongation (iii) dry matter accumulation and (iv) maturation (Rabadia et al. 1999).

There are only few cells in the plant kingdom that are as exaggerated in their size or composition as cotton fibers (Kim and Triplette 2001). Cotton fibers are single celled outer growth from individual epidermal cells on the outer integument of the ovule, which can undergo a striking amount of elongation during their development and can yield over 1000-3000 times longer than that of their diameter. It has been long recognized that although all epidermal cells are potential fibers, only about 30% of these cells actually differentiate into fibers (Basra and Malik 1984, Tiwari and Wilkins 1995). The mature cotton fiber used in textile processing is the end product of a series of developmental events in the life of ovule epidermal cell. Cotton fiber development is divided into four distinct phases initiation, elongation, secondary thickening and maturation phase (Jasdanwala et al. 1977, Naithani et al. 1982, Graves and Stewart 1988, Gokani et al. 1998). The recent evidences indicate that throughout the initiation and early elongation phases of development, cotton fiber expands primarily via diffuse growth method (Seagull 1995) and the secondary cell wall of cotton fibers is not rigid

and is capable of expansion (Seagull et al. 2000). Further, it has been shown by many workers that there is a considerable overlap between the elongation and secondary thickening phase (Meinert and Delmer 1977, Beasley 1979, Naithani et al. 1982).

Cotton fiber and cotton seed are economically important components whereas; boll wall and bracts are the protective structures that provide protection to the flower bud and also developing bolls. In addition, Morris (1965) and Bhatt (1988) have indicated that bracts and boll wall may also contribute to the production of photosynthates. As against 80% of the photosynthates supplied by the source leaf to the developing boll nearly 20% is contributed by the bracts (Benedict et al. 1973, Elmore 1973).

Earlier work on cotton fiber and seed development from our laboratory has suggested that rate of dry matter accumulation and water amount has close correlation in both seed and fiber (Naithani et al. 1982, Thaker et al. 1986, 1989, Gokani et al. 1998, Rabadia et al. 1999). Moreover, they have also recorded that fiber length is primarily governed by rate of water uptake during elongation phase. Roussopoulos and his co-workers also reported similar results (1998).

General growth and dry matter partitioning pattern of the different genotypes is the first step in identifying the possible cause in yield differences among them. With the increasing demand of industries, high oil content and long staple containing high yielding genotype is needed. Thus, cotton is an important cash crop, which provides five basic needs lint, linters, seed hull, oil and meal. It has been demonstrated that with the changes in environmental temperature; the rate and duration of different phases of cotton fiber and seed development is also altered (Thaker et al. 1989). Therefore it is inevitable to work out growth phases from all seed components with each experiment. Seagull and his coworkers (2000)

propose that drought conditions result in short fibers with increased diameter. In addition, Adams et al. (1980), Salado-Navarro et al. (1986) and Saab and Obendref (1989) proposed that seed growth rate could be affected under adverse environmental conditions.

Considering this, in the present study, two cotton genotypes were analyzed for their growth kinetics; *i.e.* boll growth in terms of fresh weight, dry weight and water amount of all the boll components (kernel, seed coat, fiber, boll wall and bracts) and their correlations with dry matter accumulation and rate of elongation were carried out throughout the total developmental period. Apart from this, dry matter allocation and water amount partitioning into different boll components was also observed.

MATERIALS AND METHODS

Two cotton genotypes *Gossypium hirsutum* (H-6) and *Gossypium arboreum* (G. Cot) differing in their seed index and fiber length were selected for their growth analysis. Certified seeds were purchased commercially from Rajkot. Seeds of cotton genotypes H-6 and G. Cot were grown during 2004 and 2005 in the botanical garden at Saurashtra University, Rajkot. Seeds were assorted for uniform size and soaked in water for 3 h, and 2-3 seeds were sown at the depth of about 1 cm in soil. Seedling growth was monitored and healthy plantlet with strong vigour was allowed to grow further and rests were removed. Culture practices including irrigation, application of fertilizers and insecticides etc. were conducted to optimize the yield.

Boll monitoring

On the day of anthesis, each flower was tagged and healthy bolls were harvested for growth analysis at a regular interval of three days. To minimize the effect of environmental variations, data for each set of analysis were collected from the flowers that bloomed on the same day during each stage of development.

Boll harvest and separation of boll components

Bolls of different ages ranging from 0-54 DPA were harvested. Freshly harvested bolls were dissected to separate all components *viz.*, seed, fiber, boll wall (along with septa) and bracts. Seeds were further separated in kernel and seed coat. Fibers were separated from the seeds manually.

Altogether different parameters like fresh weight, dry weight, water amount, fiber length, percent of dry matter allocation and percent water amount allocation etc. were studied to understand the growth of different parts of developing cotton fruit.

Measurement of fresh weight, dry weight and water amount

From three equal sized bolls; three locules were selected to measure the fresh weight and dry weight. It was difficult to separate the seed kernel in early stages of seed development and therefore, it was separated after 15 DPA in H-6 and after 21 DPA in G. Cot. Similarly, fibers were separated after 9 days from both the genotypes.

From freshly harvested boll components fresh weight was taken and they were oven dried at 80 ºC till constant weight was obtained. Water amount at each developing stage was determined as the difference in fresh weight and dry weight. The mean fresh weight, dry weight and water amount for each component with ±SD (standard deviation) was calculated.

Measurement of fiber length

Fiber length was determined by method described by Gipson and Ray (1969). Three locules from three different bolls were placed in boiling water (with few drops of 0.1N HCl) to allow seeds to separate from each other. Total fiber length was measured by spreading the ovule on a convex surface, using the stream of water. Length of the fibers was measured to nearest millimeter from chalazal end of the seed to the outermost fiber tip using a ruler. Without acid treatment, fibers were tightly packed and coiled, but after treatment; the jet of water easily straightened the fibers.

Data analysis

Sets of data on dry weight and water amount of all boll components were fitted to an appropriate polynomial equation by computer curvilinear method. Further, rate of dry matter accumulation, rate of water uptake, rate of fiber elongation were obtained. Apart from this, percent dry matter allocation and percent water amount allocation were also calculated for all boll components

for both the genotypes. Correlation coefficients between the rate of dry matter accumulation and water amount along with rate of dry matter accumulation and rate of water amount in all boll components were calculated. ANOVA was performed amongst boll components of both the genotypes.

RESULTS

Two cotton genotypes H-6 and G. Cot were selected for the study because of wide differences in their seed index (148.25 mg/seed, 94 mg/seed at maturity, respectively) and staple length (33 mm and 19 mm, respectively). Data of fiber length, dry weight and water amount of boll components were fitted to polynomial equations of different degrees and 'best fit' equation was determined statistically for different R^2 values. Fourth degree fitting was found adequate.

Dry weight

Dry weight of all parts of the cotton boll *i.e.* seed kernel, seed coat, fiber, boll wall with septa and bracts was estimated in both the genotypes.

Kernel

In H-6, kernel dry weight was initially low up to 21 DPA (7.37 mg seed kernel^{-1}), increased up to 48 DPA (57.35 mg seed kernel^{-1}) and declined thereafter slightly (Fig. 2.5a). In G. Cot, similar trend was observed; dry weight remained low up to 30 DPA (5 mg seed kernel^{-1}), increased up to 45 DPA (34.5 mg seed kernel^{-1}) and declined at maturity (Fig. 2.7a). Maximum rate of dry matter accumulation in H-6 and G. Cot was achieved on 33 DPA (3.11 mg seed kernel^{-1} day^{-1}) and on 36 DPA (2.56 mg seed kernel^{-1} day^{-1}) respectively. However, the time taken by the kernel to attain its maximum dry weight (duration of dry matter accumulation) and rate of dry matter accumulation varied in both the genotypes (Table-3). In H-6, maximum dry weight recorded at 48 DPA (57.35 mg seed kernel^{-1}), whereas in G. Cot it was recorded at 45 DPA (34.5 mg seed kernel^{-1}).

Seed coat

In H-6 seed coat, dry weight increased slowly up to 27 DPA then slight fall was observed, again increased and achieved peak on 48 DPA (33.6 mg seed coat^{-1}) (Fig. 2.5b) while, in G. Cot, dry weight increased up to 42 DPA

(31 mg seed coat), slightly decreased and stabilized in maturation phase (Fig. 2.7b). Maximum rate of dry matter accumulation was recorded on 9 DPA (2.08 mg seed coat^{-1} day^{-1}) and 15 DPA (1.80 mg seed coat^{-1} day^{-1}) in H-6 and G. Cot, respectively. Rate of DMA was many times higher in H-6 than G. Cot (Table-3).

Fiber

Similar sigmoidal pattern observed for fiber dry matter accumulation as it was obtained in kernel and seed coat. In H-6 fiber, dry weight remained low up to 15 DPA (3.99 mg), increased slowly, achieved a peak on 36 DPA (60 mg seed fiber^{-1}) and declined thereafter (Fig. 2.5c), correlatively rate of dry matter accumulation was higher up to 24 DPA, maximum rate was found on 21 DPA (3.88 mg seed fiber^{-1} day^{-1}, Fig. 2.9c) and duration for dry matter accumulation was restricted up to 36 DPA. In G. Cot, maximum dry weight was obtained on 39 DPA (34 mg seed fiber^{-1}, Fig. 2.7c) and maximum rate of DMA at 24 DPA (1.70 mg seed fiber^{-1} day^{-1}, Fig. 2.9f).

Boll wall

Dry weight and rate of dry matter accumulation was recorded approximately double in boll wall of H-6 as compared to G. Cot (Table-3). In H-6 boll wall, maximum dry weight was 1.28 g boll^{-1} and in G. Cot maximum dry weight reached up to 0.62 g boll^{-1} (Figs.2.6a, 2.8a). Rate of dry matter accumulation in H-6 boll was 69 mg boll^{-1} day^{-1} (15 DPA), while in G. Cot it was 35 mg boll^{-1} day^{-1} (12 DPA) (Fig. 2.13a, c respectively).

Bract

In H-6 bract, maximum dry weight was achieved on 15 DPA (85 mg boll bract^{-1}) whereas in G. Cot maximum dry weight was recorded on 24 DPA (70 mg boll bract^{-1}, Figs. 2.6b, 2.8b). Like dry weight, rate of dry matter accumulation was also higher in H-6 bract (8.21 mg dry weight boll^{-1} day^{-1} on 3 DPA) than G. Cot (1.99 mg dry weight boll^{-1} day^{-1} on 12 DPA), though duration of DMA was noted for longer period in G. Cot (Table-3, Fig. 2.10b, d).

Water amount

Kernel

After an initial lag period of 15 days in H-6 and 21 days in G. Cot, kernel water amount continued to increase, attained a peak when rate of dry matter accumulation was maximum, then declined gradually and reached to negligible level at maturity. In H-6 and G. Cot water amount increased up to 36 DPA (29 mg) and 39 DPA (26 mg), respectively (Figs. 2.5a, 2.7a). Maximum rate of water uptake in these genotypes was recorded at 15 DPA (4.56 mg seed kernel^{-1} day^{-1}) and 33 DPA (2.59 mg seed kernel^{-1} day^{-1}) respectively (Fig. 2.12a, d, Table-3).

Seed coat

In H-6 seed coat, initial lag phase was recorded only up to 3 days, increased steadily up to 18 DPA (84.4 mg seed coat^{-1}), then declined gradually and reached to negligible level at maturity (Fig. 2.5b). Similarly, in G. Cot initial lag phase was up to 6 days, water amount increased gradually, attained a peak at 27 DPA (65 mg seed coat^{-1}) and declined thereafter (Fig. 2.7b). Maximum rate of water uptake in these two genotypes was recorded at 9 DPA (6.80 mg seed coat^{-1} day^{-1}) and 15 DPA (4.43 mg seed coat^{-1} day^{-1}) respectively (Fig. 2.12b, e).

Fiber

Maximum water amount was recorded in H-6 fiber at 30 DPA (101 mg seed fiber^{-1}) and in G. Cot it was achieved on 21 DPA (51 mg seed fiber^{-1}) (Figs. 2.5c, 2.7c). Maximum rate of water amount uptake in these two genotypes was recorded at 12 DPA (8.79 mg seed fiber^{-1} day^{-1}) and 18 DPA (4.25 mg seed fiber^{-1} day^{-1}) respectively (Fig. 2.12c, f).

Boll wall

Maximum water amount in boll wall of both the genotypes H-6 and G. Cot was recorded at 18 DPA (5.83 g boll^{-1}) and 21 DPA (2.20 g boll^{-1}),

respectively (Figs. 2.6a, 2.8a). Maximum rate of water uptake in these genotypes was recorded at 9 DPA (366 mg boll^{-1} day^{-1}) and 18 DPA (115 mg boll^{-1} day^{-1}) respectively (Fig. 2.13a, c, Table-3).

Bract

Whereas, in bracts of both the genotypes H-6 and G. Cot, maximum water was 274 mg boll bract^{-1} (9 DPA) and 182.5 mg boll bract^{-1} (21 DPA) respectively (Figs. 2.6b, 2.8b). Rate of water uptake in bracts was 6.18 mg boll bract^{-1} day^{-1} in H-6 while in G. Cot, it was 15.5 mg boll bract^{-1} day^{-1} (Fig. 2.13b, d, Table-3).

Fiber length

In H-6, maximum staple length was measured on 33 DPA (33.41 mm) and in case of G. Cot maximum fiber length was observed on 30 DPA (19 mm) (Fig. 2.11a, e). Maximum rate of elongation in H-6 was achieved on 12 DPA (1.98 mm day^{-1}). Similarly, in G. Cot maximum rate of elongation was achieved on 12 DPA but it was less as compared to H-6 (1.07 mm day^{-1}, Fig. 2.12c, f).

Dry matter and water amount allocation

In H-6, total dry matter accumulation in boll was 5.32 g boll^{-1} at 48 DPA, whereas, in G. Cot it was observed 1.96 g boll^{-1} at 39 DPA (Fig. 2.1a, b). Similarly, in both the genotypes water amount was recorded as 11.17 g boll^{-1} (18 DPA) in H-6 and 4.460 g boll^{-1} (21 DPA) in G. Cot (Table-1b). Total water amount in H-6 boll was near about three times higher than G. Cot (Fig. 2.1a, Table-1b). In addition, percent dry matter allocation amongst all boll components was recorded (Fig. 2.2). In general, initially 40-50% dry matter was stored in boll wall and 50-60% dry matter with bracts that showed fast reduction as boll age increases and reached up to 10-17% in boll wall and just 1-1.5% in bract at physiological maturity. Contrary to this,

total reverse pattern of dry matter allocation was observed in the seed components. In seed coat of both the genotypes, rapid increase in dry matter allocation was observed and reached to maximum 38% dry matter at 12 DPA in H-6 and 40% dry matter at 24 DPA in G. Cot. Similarly, seed kernel and fiber of both genotypes have shown a gap of approximately 20 DPA and 9 DPA, respectively. In H-6, kernel and fiber showed steady increase in dry matter allocation that attained maximum value as 31% allocation at 42 DPA and 41% allocation at 33 DPA respectively, which stabilized thereafter (Fig. 2.2a). Further, in G. Cot kernel dry matter partitioned after 21 DPA and reached to maximum level at 42 DPA (33%). While in case of fiber, dry matter allocation was observed after 9 DPA, attained maximum 30% allocation at 39 DPA and slightly declined thereafter (Fig. 2.2b).

Likewise dry matter allocation, initially percent water amount allocation was very high in bract of the genotypes, 57% in H-6 and 88% in G. Cot (Fig. 2.3a, b). Initially, increase in percent of water amount partition was observed in boll wall of both the genotypes (approximately 60%) that stabilized after 12 DPA; however in H-6 slight fall was noted after 42 DPA.

As shown in figure 2.3a, in H-6 seed coat water amount partition was recorded about 40-50% up to 9 DPA than first fall was recorded, at the same time increase in water amount in fiber was observed and after 24 DPA another fall was noted when kernel tissue initiated to get water amount, with that constant increase in percent water amount allocation till maturity. In G. Cot seed coat, increase in water amount allocation was noted up to 9 DPA and stabilized in subsequent ages and declined after 42 DPA. Whereas, in fiber water amount was partitioned after 9 DPA stabilized during 20-36 DPA and reached up to negligible level at physiological maturity. Similar trend was followed by G. Cot kernel where water amount was allocated after 21 DPA, increased up to 45 DPA and declined thereafter (Fig. 2.3b).

DISCUSSION

After fertilization, the boll begins to develop and continue up to 7-9 weeks, depending upon the genotype (genetic factor) and environmental influence (Guinn 1982). Cotton boll is a strong carbohydrate sink; known to accumulate photosynthates from the vegetative part in various boll components (Constable and Rawson 1980). The cotton boll is made up of boll wall, bracts; seed contain kernel and outer coat with epidermal hairs. Distribution of photosynthates in these parts of boll determined the commercial value of cotton variety. In the present study, final yield in terms of seed weight, boll weight, seeds/plant, bolls/plants, yield/ha was remarkably different in both the genotypes studied (Tables-1a, b).

In the present study, remarkable changes in partitioning of photosynthates in to various boll parts in both the genotypes were observed. During initial period 80-90% dry matter was allocated to boll wall and bract whereas only 10-20% dry matter was allocated to seed coat. However, kernel and fiber showed initial lag period. With increase in age, higher amount of dry matter as well as water amount was partitioned to ovules (kernel + seed coat) and fibers and rapid decrease in percent dry matter and water amount from boll wall and bract was observed (Figs. 2.2, 2.3). This suggests that boll wall and bracts are participating in photosynthates transportation and hence play an important role in seed development (Bhatt 1988). Reduction in boll wall and bract dry matter coincided with increase in other three boll components *i.e.* kernel, seed coat and fibers. In high yielding genotype H-6, more dry matter was allocated into kernel and fiber as compared to low yield genotype G. Cot, where more percent of dry matter was partitioned into seed coat than kernel and fiber (Fig. 2.2). Moreover, in G. Cot dry matter partition in kernel and fiber increased during later phases of development. These results suggest that the duration of dry matter partition is an important criteria for final yield determination.

Further, total boll development can be explained by correlation amongst all the boll components. As shown in Table-2, negative correlation between boll wall and seed components and similarly bract and seed components for percent dry matter partition obtained that confirmed the role of boll wall and bract in photosynthates transportation to seed. In addition, boll wall had high negative correlation with kernel and fiber but not with percent dry matter allocated to seed coat that indicated the important role of boll wall in development of kernel and fiber (Table-2).

Bract has high negative correlation with fiber in both the genotypes. It is interesting to note that in G. Cot percent dry matter allocation between bract and seed coat had close negative correlation than kernel (Table-2) and this might have restricted the kernel growth in G. Cot.

Percent water amount in bract was recorded highest on the day of anthesis in both the genotypes (in H-6 about 60% and in G. Cot > 80%) after that it continuously decreased. Contrary to the result of percent dry matter allocation in boll wall, the percent water amount in it increased up to 9 DPA and then stabilized. In addition, when water amount in bract reached to negligible level rapid increase in water amount partition in kernel was observed (21 DPA on wards) when percent water amount was either stabilized or slightly decreased in seed coat and fibers. Thus, duration of translocation of water amount play an important role in organ development as well as in boll development.

It is reported that seed growth curve has sigmoidal pattern (Chanda and Singh 1998, Thaker 1998, Rabadia et al. 2006). In the present study, on the basis of dry weight typical curve pattern was observed for all seed components (kernel, seed coat, fibers) of both the genotypes. Using polynomial model, cotton seed development have been divided into four distinct phases (i) cell division (ii) cell elongation (iii) dry matter accumulation and (iv) maturation. Although, both cotton genotypes showed

similar growth pattern but duration of developmental phases were different amongst the genotypes studied. Similar results were also recorded by Rabadia et al. (1999).

In the present study, in H-6 polynomial fitting of dry matter accumulation of seed showed an initial lag up to 9 DPA, thought to be the cell division phase and then it entered into a linear phase of DMA up to 48 DPA, following this maturation phase was observed (Fig. 2.4a). Data on fourth degree polynomial fitting of water amount explained cell elongation phase up to 27 DPA in H-6 (Fig. 2.4a). Similarly, in G. Cot seed initial lag period extended up to 12 DPA, elongation phase recorded up to 27 DPA, dry matter accumulation observed up to 45 DPA and than it entered into maturation phase (Fig. 2.4b).

Cotton seed can be divided into kernel, seed coat and epidermal hairs – fibers and so individual seed component for their growth was studied. In seed kernel of both genotypes, initial lag period considered as cell division phase (15 DPA in H-6 and 24 DPA in G. Cot), elongation phase (33 DPA in H-6 and 36 DPA in G. Cot), dry matter accumulation phase (in H-6, 45 DPA and in G. Cot, 42 DPA) was followed by maturation phase till boll opened (Fig. 2.5).

Seed coat data also fitted to polynomial equation to understand the growth pattern in both the genotypes. From DMA and water amount, seed coat development also divided into four distinct phases (i) cell division (in H-6 3 DPA and in G. Cot up to 9 DPA) (ii) cell elongation (in H-6 21 DPA and in G. Cot 24 DPA) (iii) dry matter accumulation (in H-6 45 DPA and in G. Cot 48 DPA) and (iv) maturation (in H-6 45 DPA onwards and in G. Cot 48 DPA onwards) (Figs. 2.5b, 2.7b). It is interesting to note that in H-6, seed coat showed higher rate of dry matter accumulation as well as rate of water uptake than the G. Cot (Table-3). However, in H-6 seed coat completed dry matter accumulation at the time (24 DPA) when seed kernel has started

DMA (18 DPA, Fig. 2.9a, b). While in case of G. Cot, higher degree of overlapping between dry matter accumulation phase of kernel (21 - 42 DPA) and seed coat (3 - 30 DPA) was observed (Fig. 2.9d, e). Thus, in G. Cot both kernel and seed coat compete for the available photosynthates. Other workers (Calero et al. 1981) indicated that the relative weight proportion represented by the seed coat decreases as seed weight increases in soybean. Egli (1990) showed that testa influences cotyledon growth rate and ultimately seed size in legume species.

Close correlation was obtained between rate of dry matter accumulation and water amount from seed kernel, seed coat (Fig. 2.9, Table - 4a), suggested that regulation of water uptake by the seed organs is important determinant of seed weight and hence seed development. Presumably, this process is driven by osmotic gradients (Egli and Tekrony 1997, Smart et al. 1998, Boyer and Westgate 2004). Similar conclusions derived for maize (Borras et al. 2003) and soybean embryo (Swank et al. 1987) indicates that dry matter accumulation follows a predictable pattern associated with internal changes in kernel water relations.

On the basis of earlier work from our laboratory (Jasdanwala et al. 1977, Naithani et al. 1982, Thaker et al. 1986, Rabadia et al. 1999, 2006), cotton fiber development is divided into four distinct phases *i.e.* initiation, elongation, secondary thickening and maturation. This work is also supported by other workers *viz.*, Graves and Stewart (1988), Seagull and Gialvalis (2004). Like, seed kernel and seed coat; fiber dry weight data also showed a sigmoidal curve pattern in both genotypes studied.

In the present study, both genotypes showed initial lag period of 9 DPA in H-6 and 15 DPA in G. Cot, entered in to elongation phase up to 33 and 36 DPA in H-6 and G. Cot fibers, respectively, followed by DMA phase and then entered into maturation phase (45 onwards). Correlation coefficient between rate of fiber elongation and rate of water amount was highly

significant in both the genotypes H-6 ($p \leq 0.001$) and G. Cot ($p \leq 0.001$) (Table-4a), which supports the earlier work of our laboratory. As shown in figure 2.13 and Table-3, long staple genotype (33 mm length) has shown higher rate of water uptake and dry matter accumulation as compared to the short staple genotype (19 mm length). The data supports our earlier hypothesis that rate and/or duration of fiber elongation showed greater influence on the final fiber length. Further, fiber dry weight to ovule dry weight ratio was significantly higher in H-6 than G. Cot ($p < 0.01$). Genotypic variation in duration and elongation rate in cotton fiber has been reported (Roussopoulos et al. 1998).

Rate of DMA and rate of fiber elongation showed clear overlap (Fig. 2.11b, f) but rate of DMA achieved peak when rate of elongation started to decline. Cell elongation requires two major processes, (i) uptake of water and (ii) cell wall turnover (Taiz 1984, Cosgrove 2001). Parallelism in rate of water uptake with rate of elongation (Fig. 2.11c, g) suggests that it is required for generation of osmotic force and ultimately cause fiber length elongation. Role of turgor pressure in cell expansion was studied by many workers (Cosgrove 1993, 1999, 2001, Egli and Tekrony 1997, Smart et al. 1998). Cotton fiber length and its dry weight per seed are the two important parameters of commercial importance. In the present study, fiber to ovule dry weight ratio was distinctly higher in H-6 than G. Cot (Fig. 2.11d, h).

Data presented in this study (Figs. 2.9 to 2.13), and earlier work of our laboratory and elsewhere support the view that water uptake can be considered as a marker for cell elongation in the developing tissue. Generally, developing sink or a plant part is complex mixture of cells and hence simultaneous elongation in these cells is discernible, it is difficult to conclude for the cell elongation phase. In fact, cell elongation of a sink cannot be ruled out as a one of the important determinant for yield potential. In this study, single cell model system of cotton fiber suggests solution to this conundrum.

Data on DMA of boll components showed significant variation amongst these genotypes (Table-5). As discussed in this chapter, cotton yield is primarily due to the differences in reproductive sink rather than photosynthetic capacity (Hearn 1969, Gandhi et al. 1999). Therefore, variation in seed size or seed weight between these two genotypes is the cause of physical and physiological constrains.

Boll wall and bract did not follow the typical sigmoidal growth curve as it was observed for seed components, because at time of anthesis bract was fully developed and expanded; it did not follow the growth phases as described for seed or fiber. In contrast, to sink organs (seed and fiber), source organs (bracts and boll wall) showed close correlation with rate of DMA and rate of water amount (Table-4b). Thus, relationship can be explained with the percent partitioning of dry matter accumulation in source organs. Drying of boll wall and bracts at later stages might have contributed reserved material to developing sink. Bhatt et al. (1984) suggested that removal of bract reduce the weight of seed and also affect the fiber property in a mature boll.

Major findings:

- Duration of total seed and fiber development was almost equal in both the genotypes studied
- Cotton seed development is divided in to four distinct phases *i.e.* cell division, cell elongation, DMA and maturation
- Cotton fiber development is also divided into four distinct phases *i.e.* initiation, elongation, secondary thickening and maturation. There is a considerable overlap between elongation and secondary thickening phases
- Final dry weight of both seed tissues *i.e.* kernel and seed coat as well as of fiber and other parts of boll like boll wall and bract was higher in H-6 than G. Cot

- Further water amount in all boll components was higher in H-6 than G. Cot
- Close correlation between fiber elongation and rate of water uptake was observed in both the genotypes studied indicates the role of water uptake as a driving force for elongation
- Rate of DMA was higher in long staple (H-6) than short staple (G. Cot) genotype
- Close correlation was observed between rate of dry matter accumulation and water amount in sink organs (seed components) indicates the importance of water logging in dry matter accumulation, whereas in source organs *i.e.* boll wall and bract close correlation was observed between rate of dry matter accumulation and rate of water amount uptake
- Fiber dry weight to ovule dry weight ratio remains low during elongation phase, increases during dry matter accumulation and again decreases during maturation phase (Fig. 2.11d, h). H-6 showed high ratio (>0.8) whereas, G. Cot showed low ratio (<0.6)

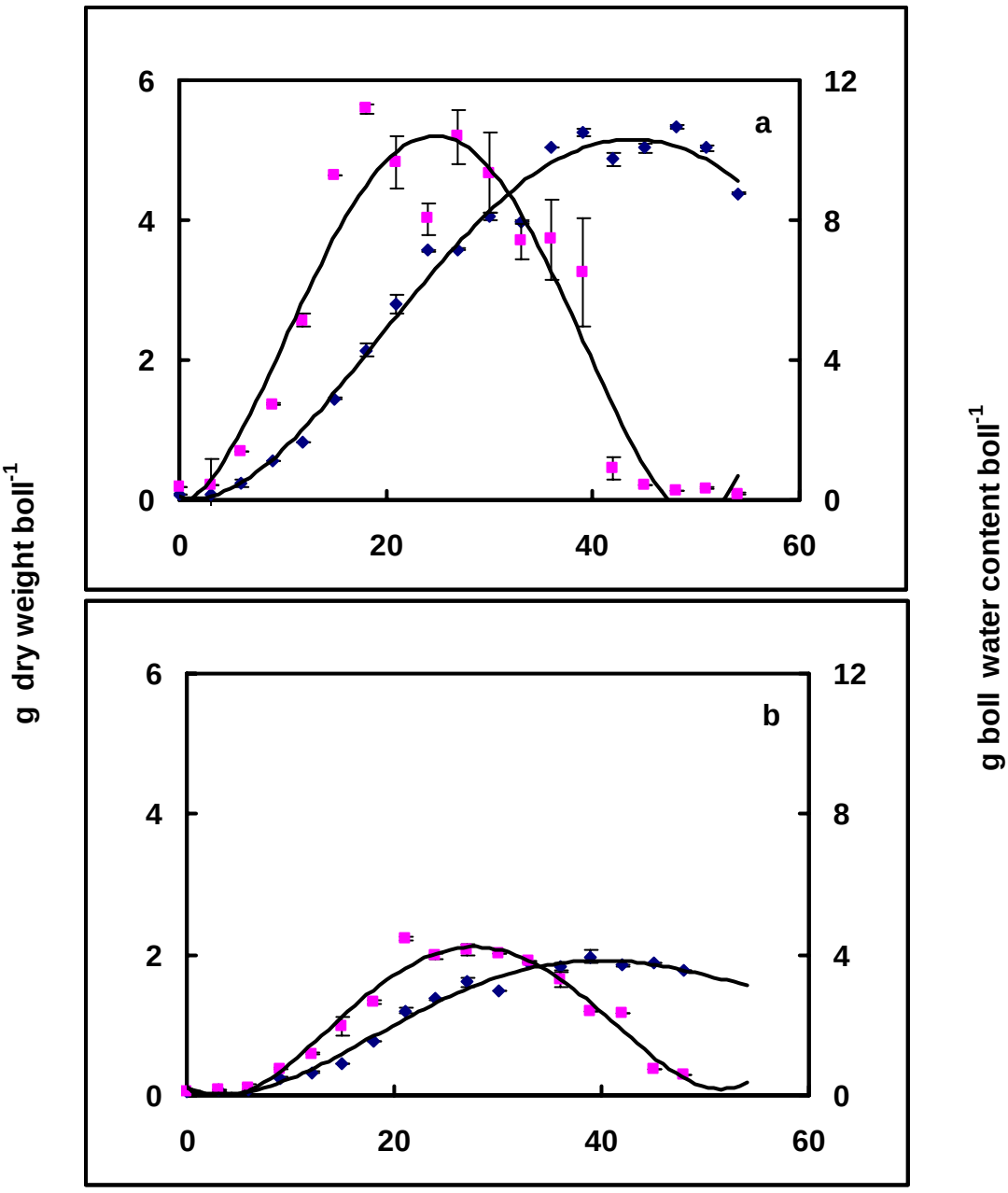


Fig-1: Boll dry weight ( ) and water content ( ) against boll age in two different cultivar H-6 (a) and G-cot (b). Vertical bars represent ±SD or are within the symbol

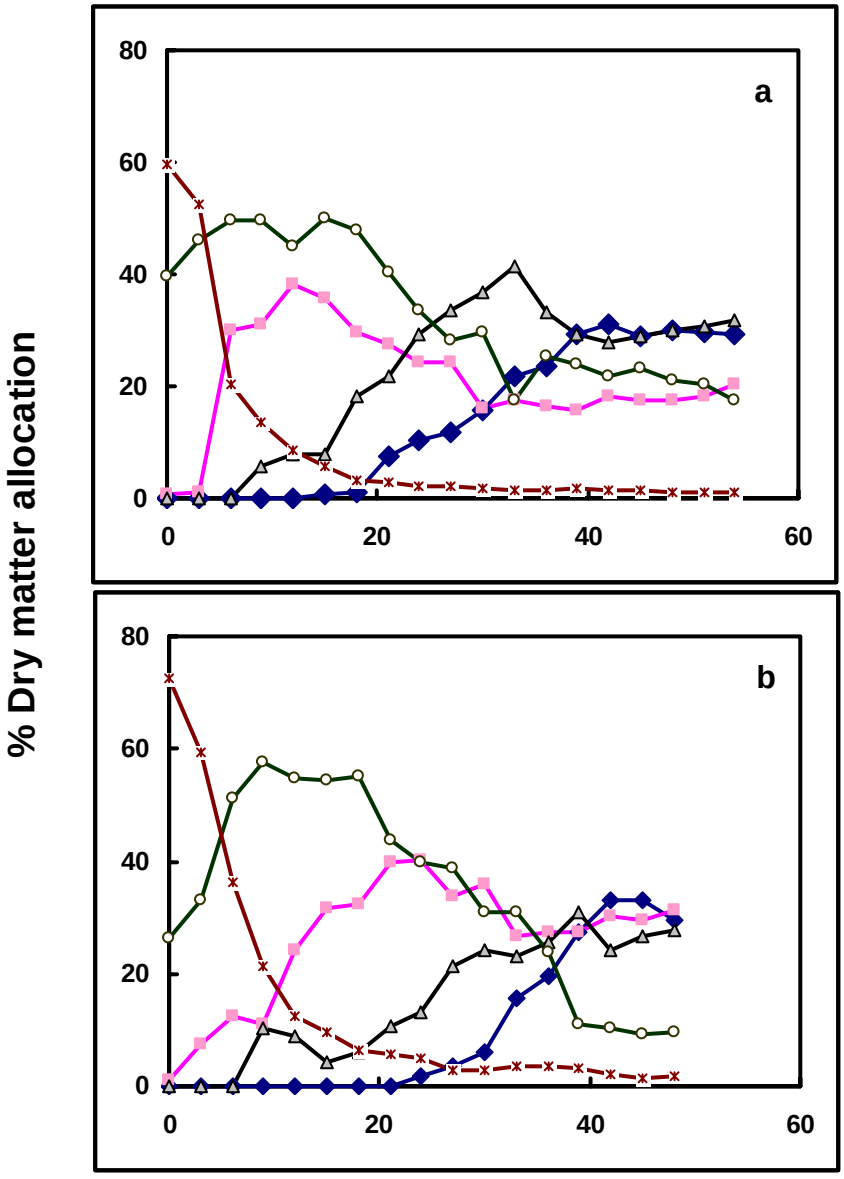


Fig- 2: Percent dry matter allocation in kernel( ), seed coat( ), fiber ( ), boll wall ( ), bract (x-- x) against boll age in two different cultivars (a) H-6 and (b) G. Cot-13.

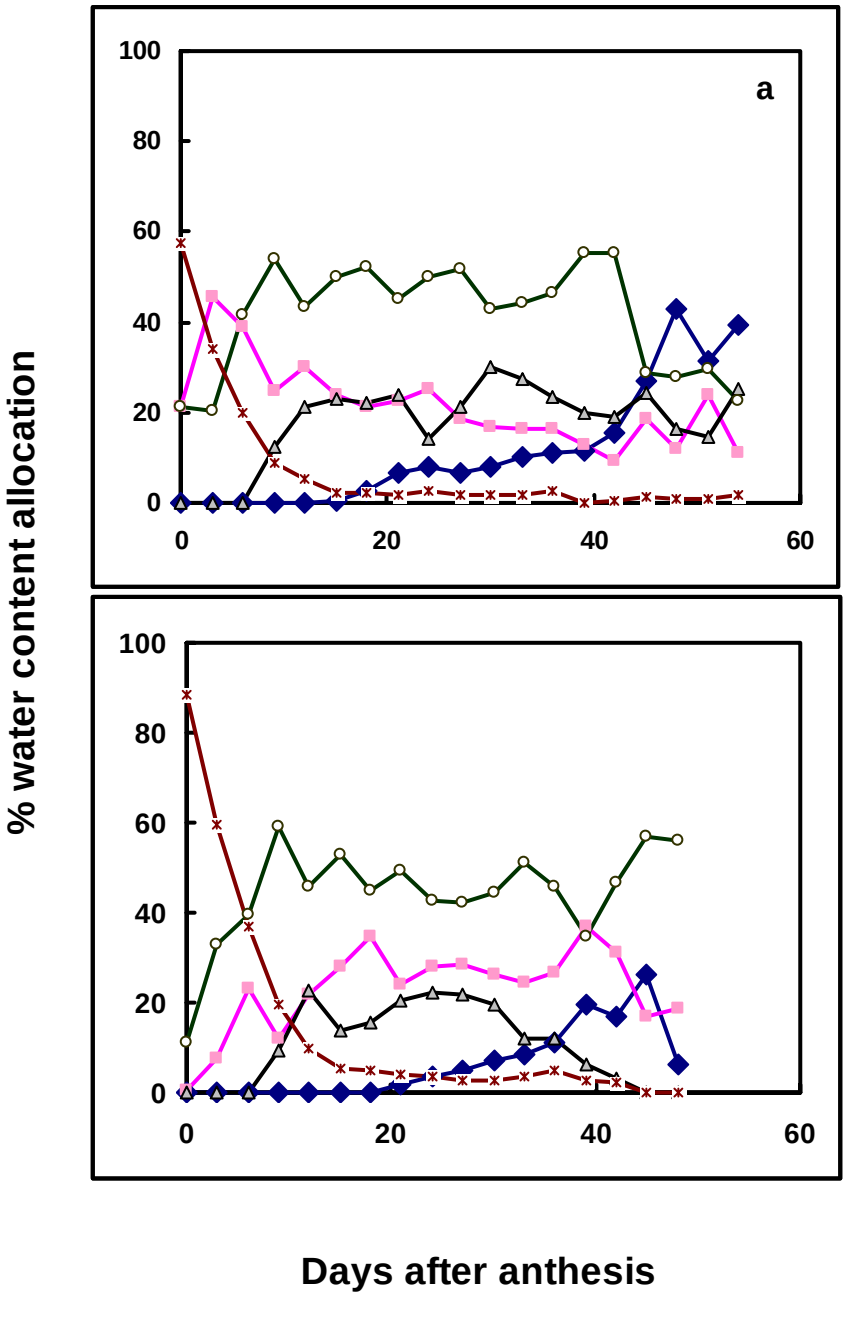


Fig- 3: Percent water amount allocation in kernel( ), seed coat( ), fiber ( ), boll wall (o---o), bract (x-- x) against boll age in two different cultivars (a) H-6 and (b) G. Cot-13.

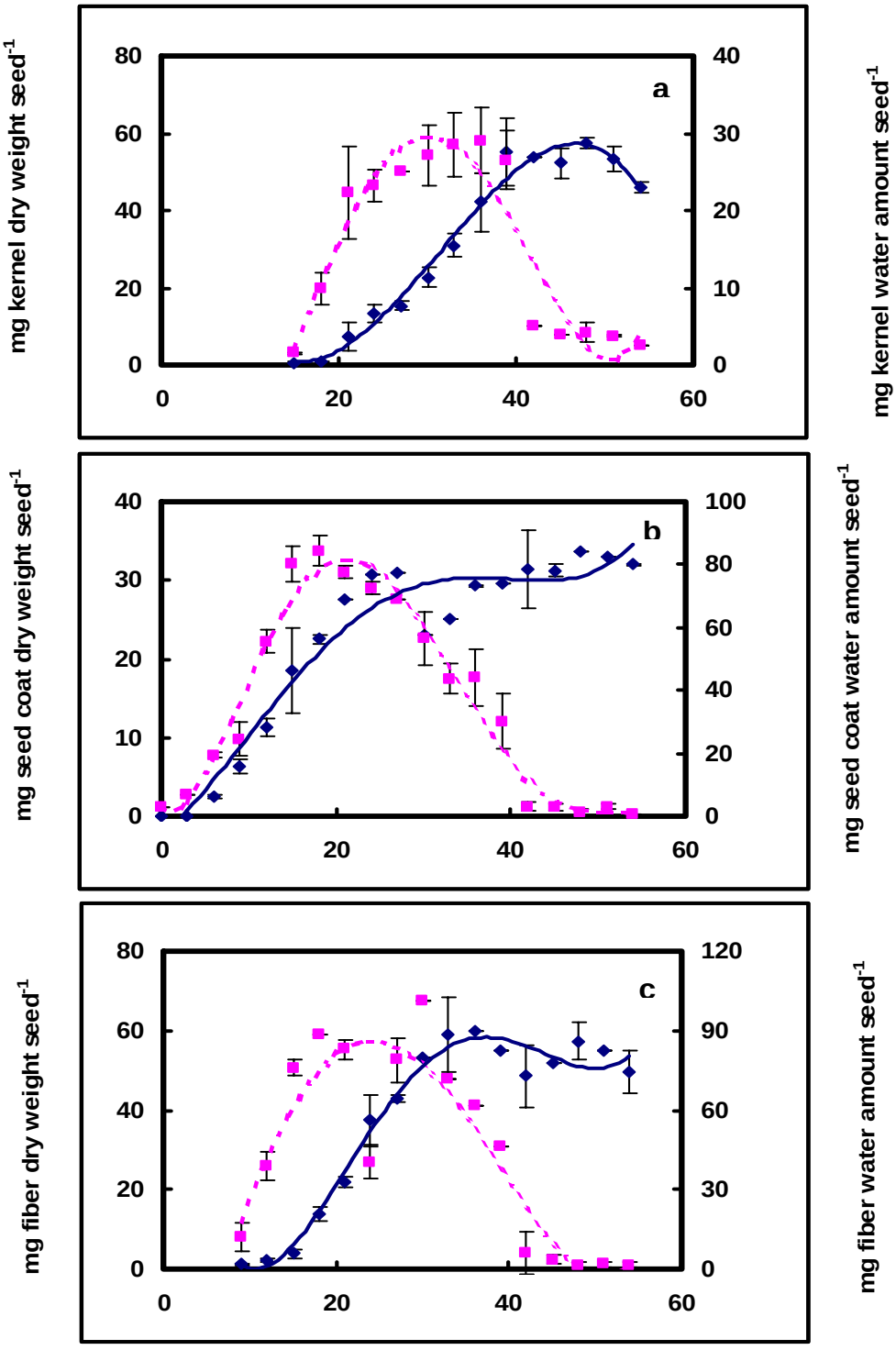
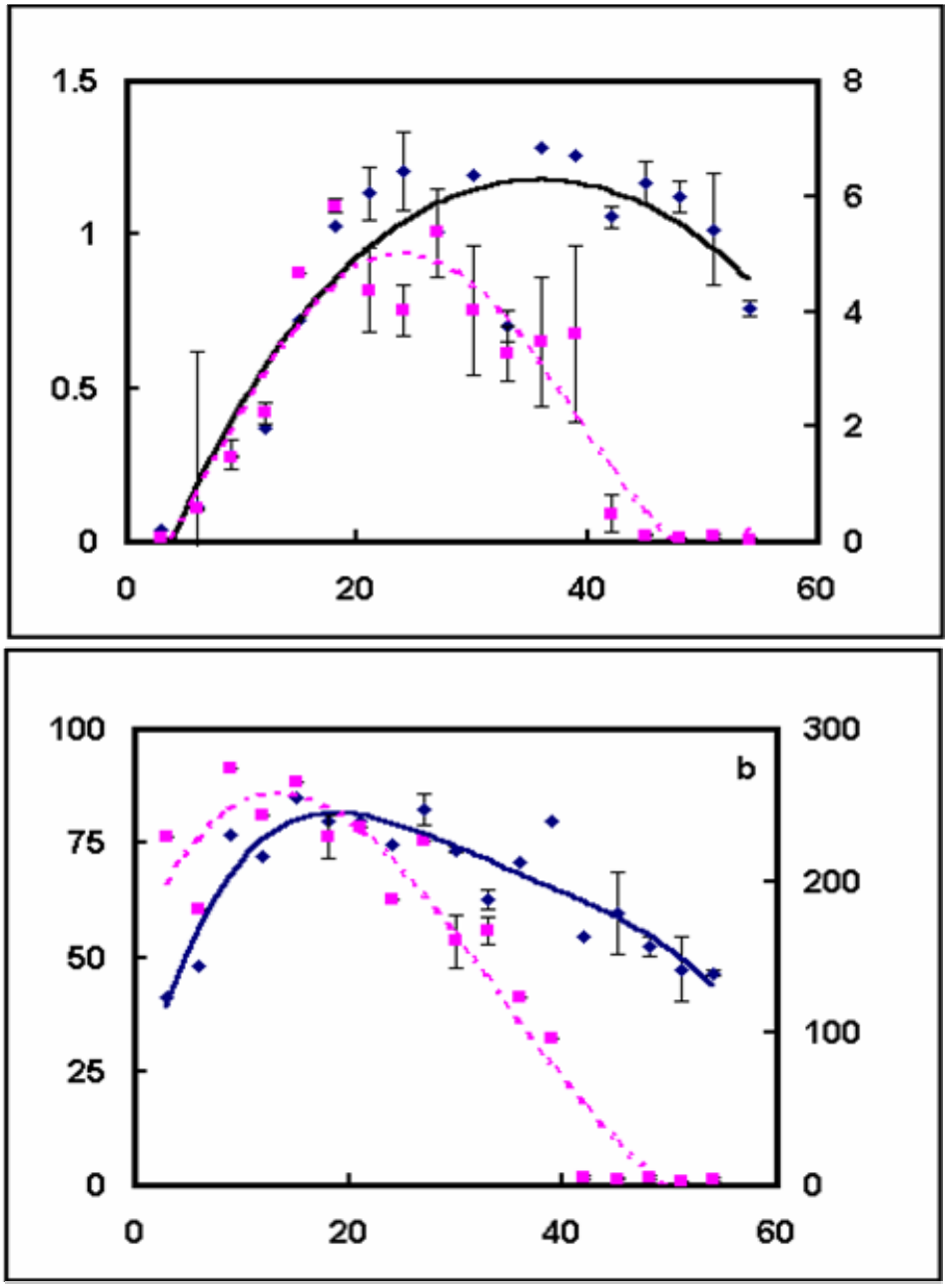


Fig-5: H-6 seed kernel (a), seed coat (b) and fiber (C)- dry weight (◆) and water content( ) against boll age.Vertical bars represent ±SD or are within the symbol



Days after anthesis

Fig-6: H-6 boll wall (a), bracts (b) - dry weight(♦) and water content( ) against boll age.Vertical bars represent ±SD or are within the symbol

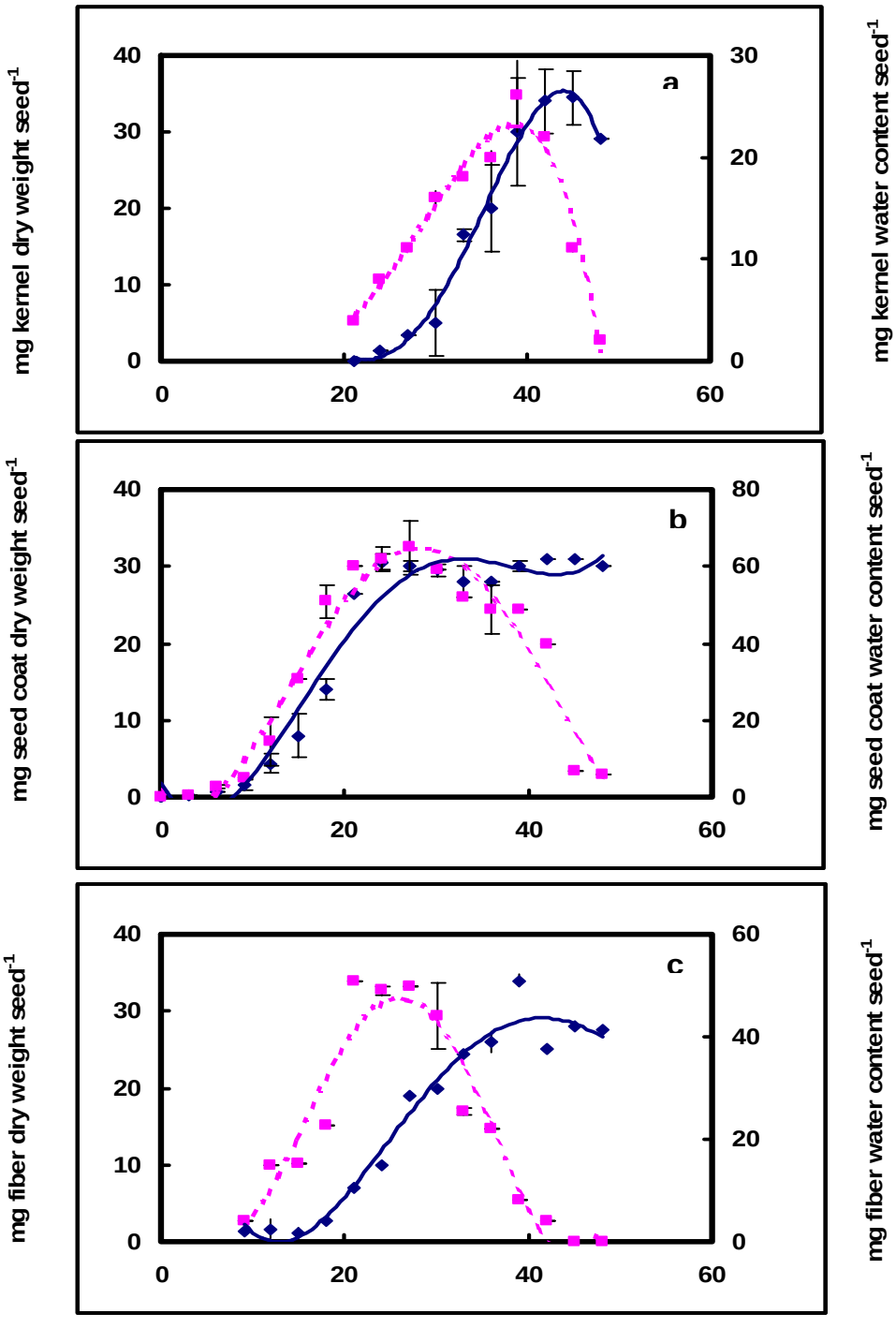


Fig-7: G-cot seed kernel (a), seed coat (b) and fiber (C)- dry weight(◆) and water content(■) against boll age.Vertical bars represent ±SD or are within the symbol

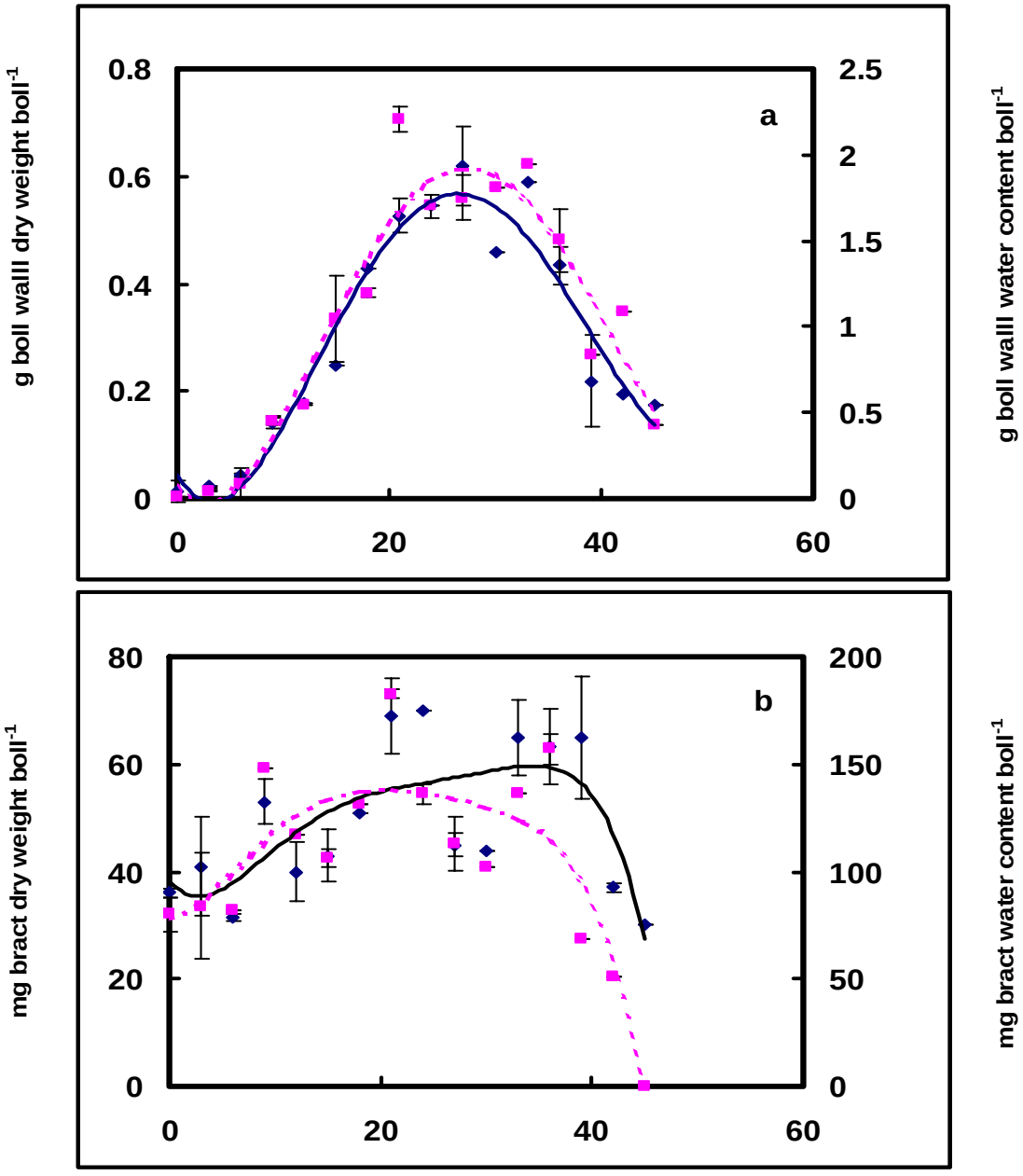


Fig-8: G-cot boll wall (a), bracts (b) - dry weight( ) and water content ( ) against boll age. Vertical bars represent ±SD or are within the symbol

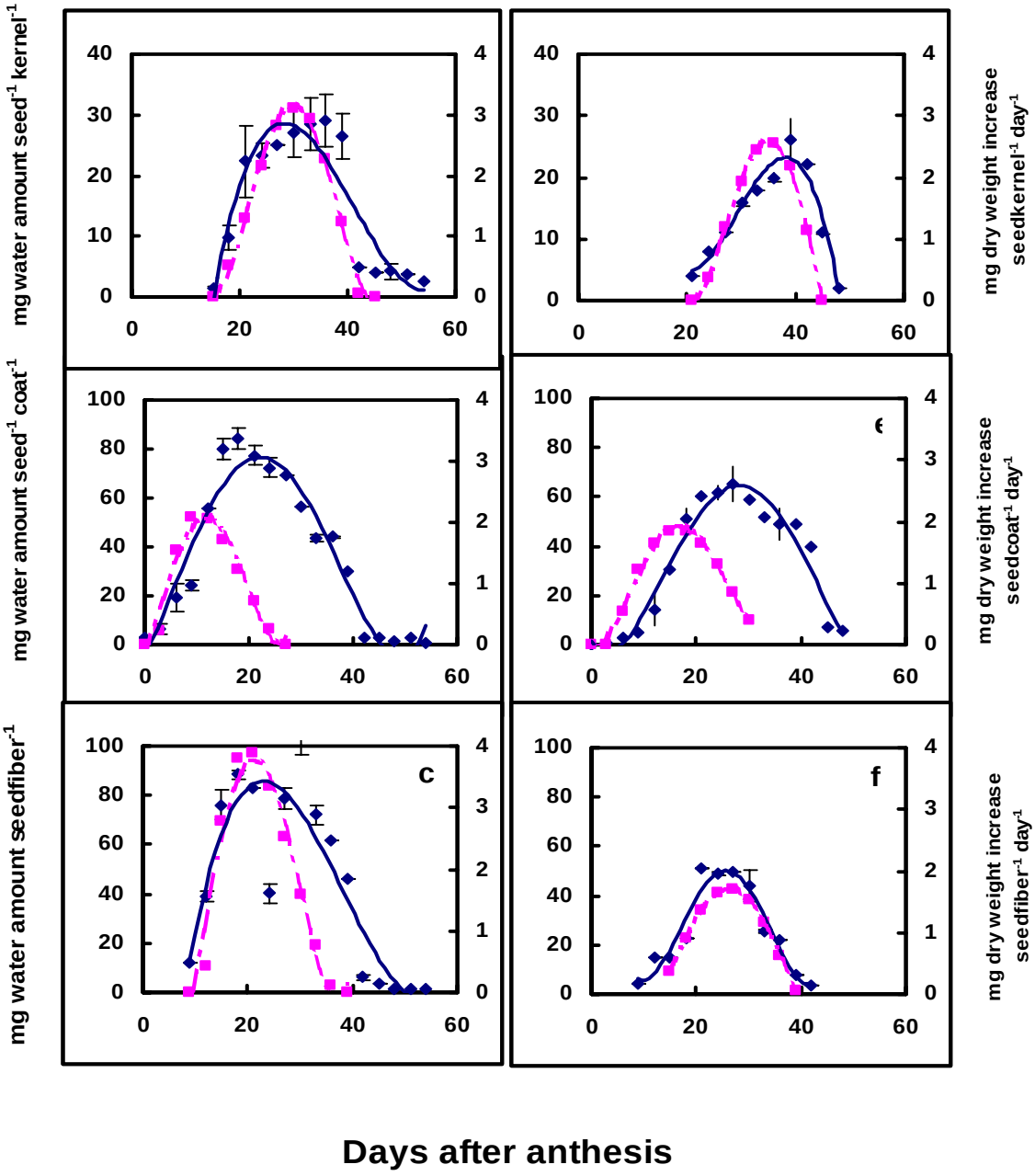


Fig- 9: Rate of dry matter acumuation ( )and water content ( ) against boll age in seed kernel (a,d), seed coat (b,e) and seed fiber (d,f) in two different cultivar H- 6 (a, b, c) and G-cot (d, e, f). Vertical bars represent ±SD or are within the symbol.

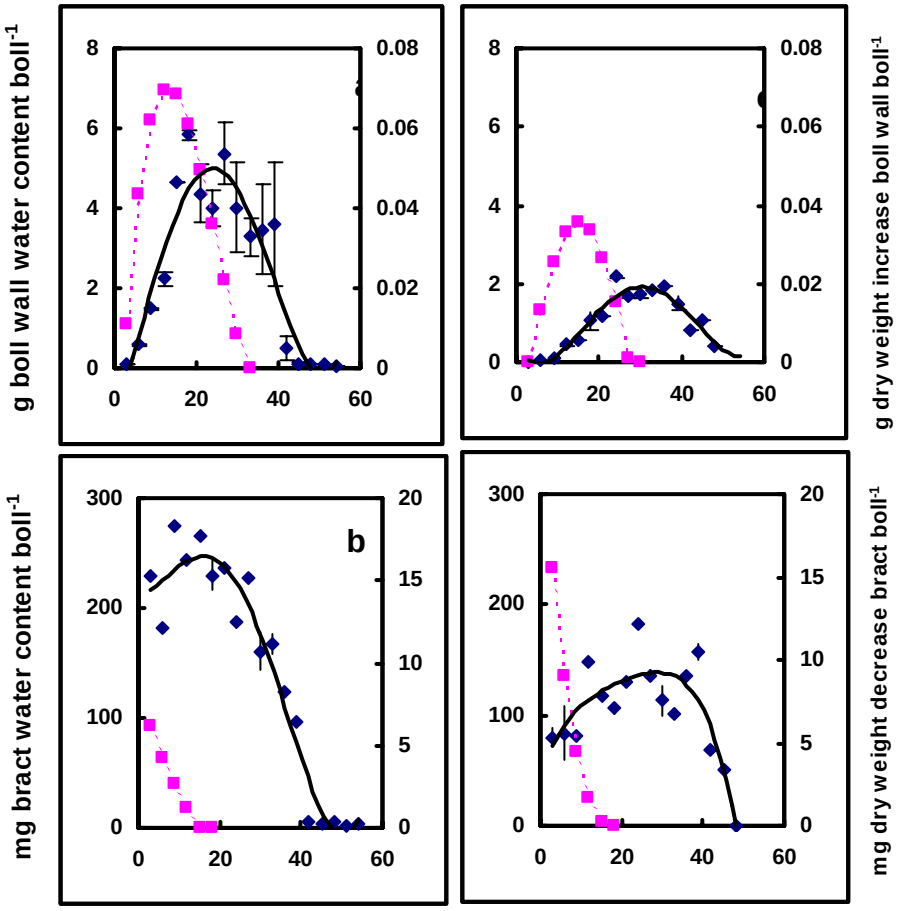


Fig-10: Rate of dry matter acumuation( ) and water content( ) against boll age in boll wall (a,c), bract (b,d) a in two different cultivar H- 6 (a, b) and G-cot (c,d). Vertical bars represent ±SD or

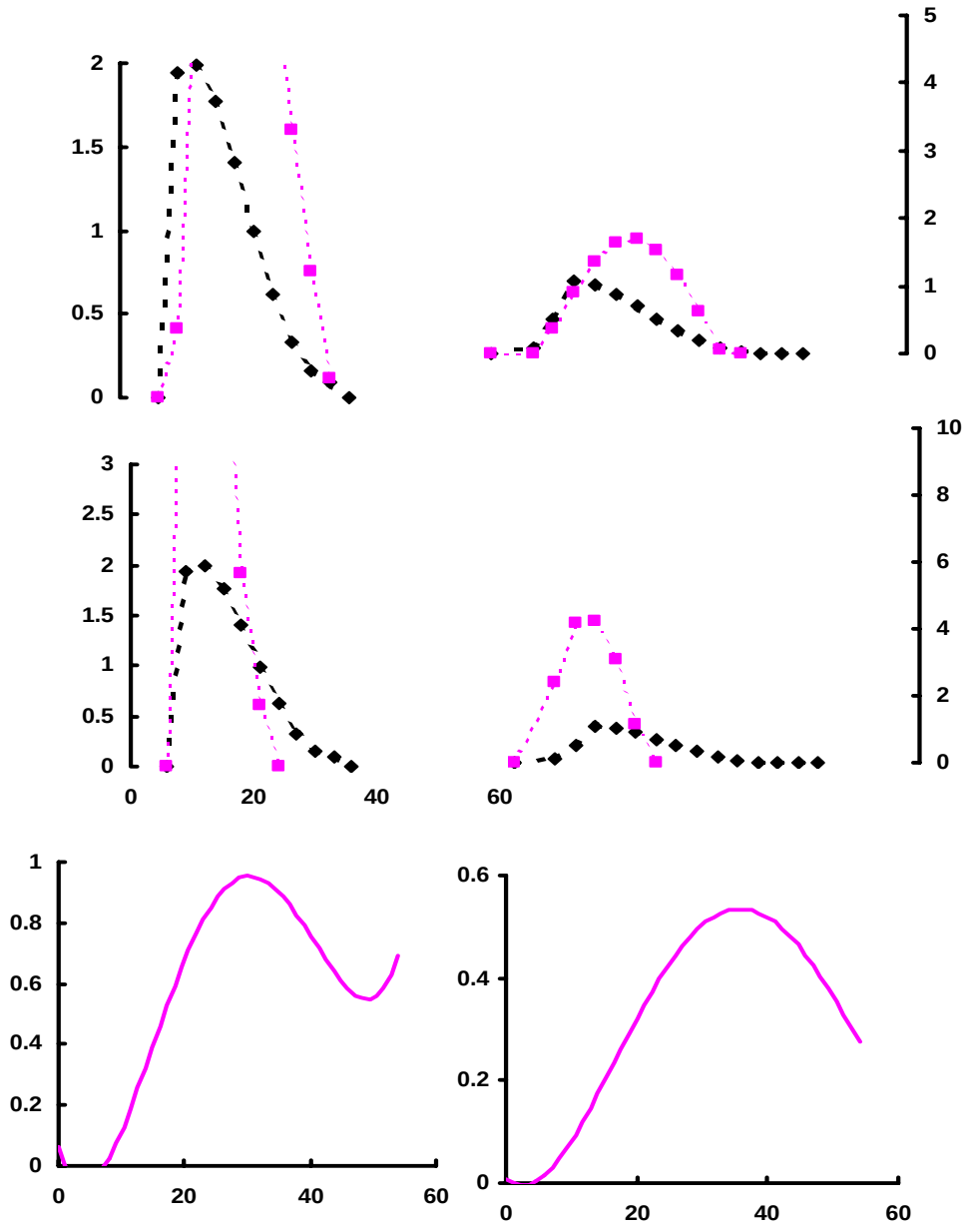
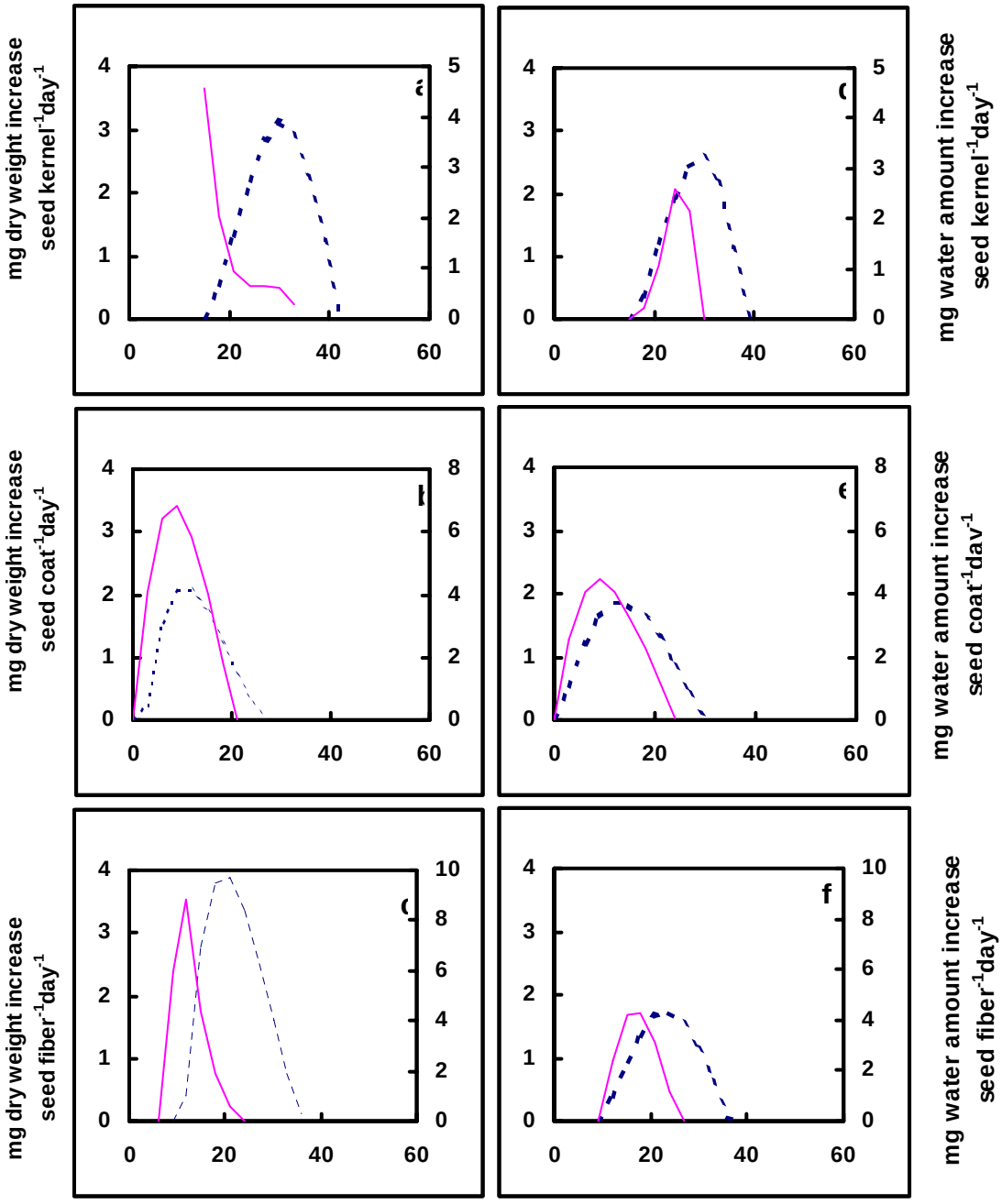


Fig-11: mm fiber length and dry weight (a, e), rate of elongation and rate of dry matter accumulation(b, f and rate of elongation and rate of water amount (c, g) against boll age, fiber: ouvle ratio (d, h) in two different genotype H- 6 (a, b, c,d) and G-cot (e, f, g, h)


mg dry weight increase seed kernel-1day-1
mg water amount increase seed kernel-1day-1
mg dry weight increase seed coat-1day-1
mg water amount increase seed coat-1day-1
mg dry weight increase seed fiber-1day-1
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a
c
b
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d
f
0 20 40 60
Days after anthesis

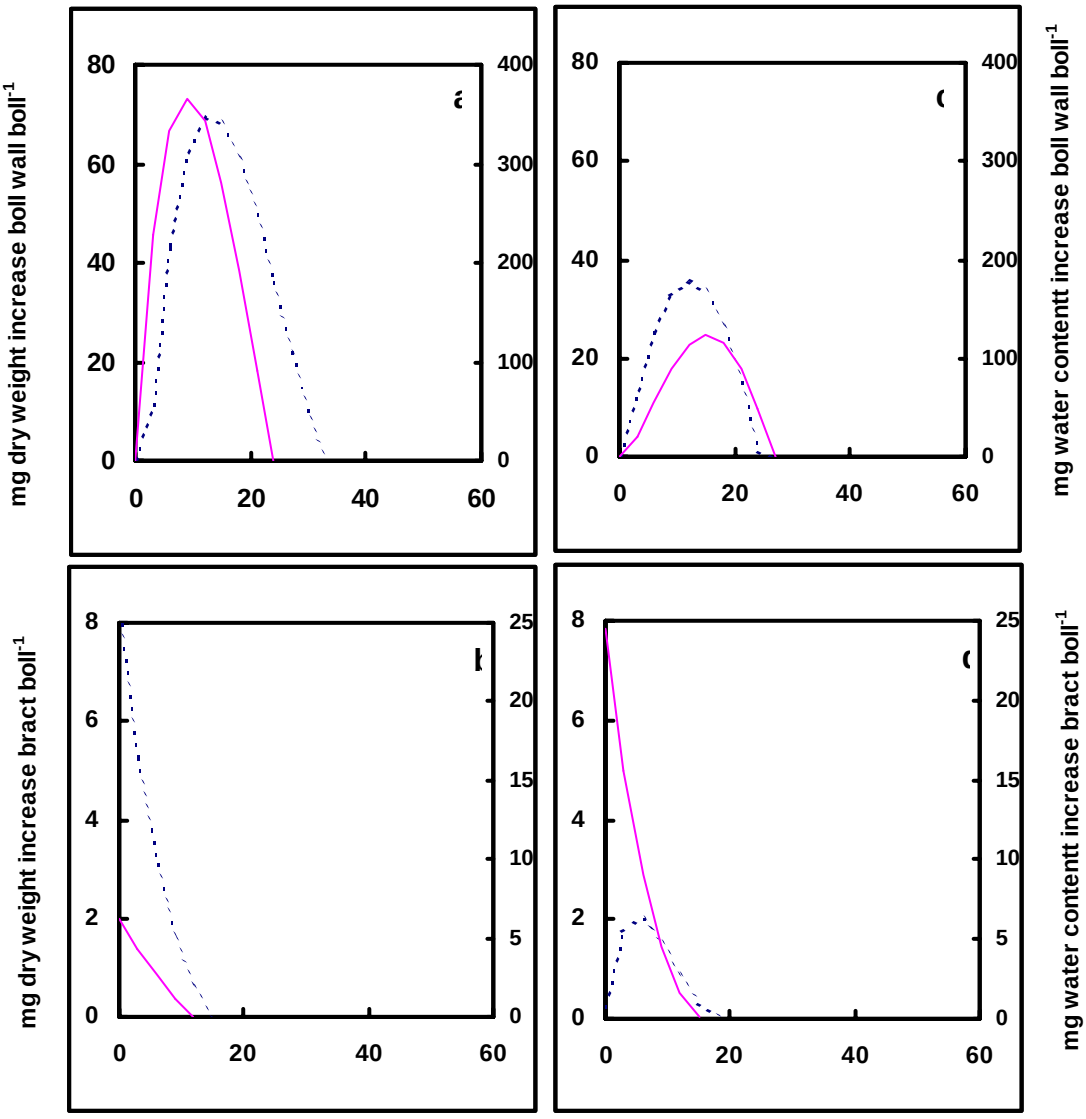


Fig- 13: Rate of dry matter acumuation(---) and rate of water content (—)against boll age in boll wall (a,b), bract (c,d) in two different cultivar H- 6 (a, b) and G-cot (d, e).

Table-1a. Comparison of growth parameters between two cotton genotypes H-6 and G. Cot

Parameters	H- 6	G. Cot
Number of seeds/boll	28	18
Number bolls/plant	33	26
Number of plants/ha	18,500	55,000
Total bolls/ha	6,10,500	14,30,000
Total yield/ha (g)	32,24,050	27,84,750

Table-1b. Comparison of dry weight and water amount between two cotton genotypes H-6 and G. Cot

Dry weight	H- 6	G. Cot
Ovule (kernel + seed coat) (mg)	90.95(48)	65(42)
Seed (ovule + fiber) (mg)	148.25(48)	94(39)
Total seed weight (mg)	4151(48)	1692(39)
Total boll weight (mg)	5281(48)	1946(39)
Water amounts	H-6	G. Cot
Ovule (kernel + seed coat) (mg)	102.55(15)	76(27)
Seed (ovule + fiber) (mg)	184.706(30)	126(27)
Total seed weight (mg)	5171(30)	2268(27)
Total boll weight (mg)	11179(18)	4460(21)

***Parentheses show ages at which maximum value obtained**

Table-2. Correlation coefficient for percent dry matter allocation amongst boll components of two cotton genotypes H-6 and G. Cot

H- 6	Bract	Boll wall	Fiber	Seed coat
Kernel	- 0.554	- 0.943	0.793	- 0.295
Seed coat	- 0.557	0.388	- 0.960	
Fiber	- 0.736	- 0.857		
Boll wall	0.468			
G. Cot	Bract	Boll wall	Fiber	Seed coat
Kernel	- 0.461	- 0.880	0.825	0.252
Seed coat	- 0.881	- 0.099	0.538	
Fiber	- 0.727	- 0.715		
Boll wall	0.153			

Table-3. Comparison of growth parameters between two cotton genotypes H-6 and G. Cot

Organ	Parameter	H- 6	G. Cot
Kernel	DW (mg)	57.35(48)	34.5(45)
	Rate of DMA	3.11(33)	2.55(36)
	Duration	18 - 45 DPA	24 - 42 DPA
	WA (mg)	29(36)	26(39)
	Rate of WA	4.56(15)	2.59(33)
	Duration	15 - 33 DPA	24 - 36 DPA
Seed coat	DW (mg)	33.6(48)	31(42)
	Rate of DMA	2.08(9)	1.850(15)
	Duration	3 - 24 DPA	3 - 30 DPA
	WA (mg)	84.4(18)	65(27)
	Rate of WA	6.80(9)	4.43(15)
	Duration	3 -18 DPA	9 - 30 DPA
Fiber	DW (mg)	60(36)	34(39)
	Rate of DMA	3.88(21)	1.70(27)
	Duration	9 - 36 DPA	15 - 39 DPA
	WA (mg)	101(30)	51(21)
	Rate of WA	8.79(12)	4.25(18)
	Duration	9 - 21 DPA	12 - 24 DPA
	Length	33.41 mm (36)	19mm(30)
	Rate of elongation	1.989	1.07
Boll wall	DW (mg)	1284 (36)	620(27)
	Rate of DMA	69(15)	35()
	Duration	3 - 30 DPA	6 - 27 DPA
	WA (mg)	5831(18)	2208(21)
	Rate of WA	366(9)	115(18)
	Duration	3 - 21 DPA	3 - 24 DPA
Bract	DW (mg)	85(15)	70(24)
	Rate of DMA	8.215(3)	1.99(12)
	Duration	3 - 18 DPA	6 - 27 DPA
	WA (mg)	274(9)	182.5(21)
	Rate of WA	6.18(3)	15.5(3)
	Duration	3 - 12 DPA	3 - 15 DPA

***Parentheses show age WA- water amount DW- dry weight**

Table-4a. Correlation coefficient between rate of dry matter accumulation and water amount in seed components in two cotton genotypes H-6 and G. Cot

Boll component	H- 6		G. Cot	
	r	df	r	df
Seed kernel	0.878***	13	0.816***	09
Seed coat	0.15 (NS)	11	0.527*	11
Seed fiber	0.705***	15	0.952***	11
Rate of elongation and Rate of water amount	0.888***	13	0.871***	11

Significance p level at 0.001 ≤ *, 0.01 ≤ **, 0. 1 ≤ ***
NS- Non significant

Table-4b. Correlation coefficient between rate of dry matter accumulation and rate of water amount in boll wall and bract in two cotton genotypes H-6 and G. Cot

Boll component	H- 6		G. Cot	
	r	df	r	df
Boll wall	0.529**	14	0.849***	8
Bract	0.994***	6	0.3 (NS)	7

Significance p level at 0.001 ≤ *, 0.01 ≤ **, 0.05 ≤ ***
NS- Non significant

Table- 5. ANOVA between two cotton genotypes for mean dry weight

Source	df	F- ratio
Kernel	23	3.58*
Seed coat	34	0.753
Fiber	29	11.29***
Boll wall	33	24.4***
Bract	33	12.331***

Significance p level at 0.001 ≤*, 0.001 ≤ **, 0.05 ≤ ***

Chapter III
Indole Acetic Acid And Phenyl Acetic Acid

SUMMARY

Two cotton genotypes H-6 (*Gossypium hirsutum*) and G. Cot (*Gossypium arboreum*) varying in final seed size and fiber length were selected for estimation of their endogenous free and conjugated IAA and PAA levels during entire growth period. Endogenous free and conjugated IAA and PAA level were higher during DMA phase in kernel and seed coat. Additionally they have close correlation with fiber elongation suggesting that auxin play an important role in DMA and cell elongation process. IAA and PAA levels were higher in H-6 kernel and fiber; whereas in G. Cot seed coat had more content of IAA and PAA. Thus, accumulation of IAA and PAA in various parts regulates the seed size and final yield. Long staple genotype H-6 had higher level of free IAA and PAA content than short staple genotype G. Cot which indicates that auxin is one of the important factors that determine fiber length. Both free IAA and PAA content were observed higher in H-6 boll wall. While PAA level was recorded high in G. Cot bract. In *in vitro* study, when different concentrations of IAA, NAA and PAA were supplemented to the media, percent promotion in dry weight, fiber length and seed size was remarkably higher in G. Cot than that of H-6. Both *in vivo* and *in vitro* findings suggest that auxin play an important role in cell division and cell elongation. From *in vitro* study it is clear that auxin had no clear effect on water uptake; this lead to the assumption that water uptake may be an independent parameter in the process of cell elongation.

Abbreviations: 4-Cl IAA - 4-Chloro indoleacetic acid, DMF- N, N-Dimethyl formamide, ELISA- Enzyme linked immunosorbent assay, IAA- Indole 3- acetic acid, NAA- Napthyl acetic acid, OPD - O-phenylene diamine, PAA- Phenyl acetic acid

INTRODUCTION

The phytohormone auxin is critical for plant growth and orchestrates many developmental processes. Auxins influence virtually every aspect of plant growth and development *i.e.* cell elongation (Rayle and Cleland 1992), cell division and apical dominance (Gazzarrini and Mccourt 2003), root elongation (Norcini et al. 1985), fertilization of ovules and fiber production in cotton (Beasley and Ting 1974). Numerous bioassays for auxin response have been described and these assays have proven useful in the isolation of endogenous auxins, identification of auxin precursors and the development of synthetic auxin-like compounds (Thimann 1977). One of the earliest noted auxin effects was in phototropism; the curvature of stem toward light source (Darwin 1880). Application of auxin to decapitated shoots can induce such bending in the absence of a light stimulus (Went 1926) and several non-phototrophic mutants are deficits in auxin signaling components (Harper et al. 2000, Tatemtsu et al. 2004).

Apart from occurring as a free acid, IAA also exists in conjugated forms (Cohen and Bandurski 1982). On average 95% of all IAA in a plant is conjugated into storage forms (Walz et al. 2002, Campanell et al. 2004). IAA conjugates have been described in which the IAA is linked through a peptide bond to amino acids or peptides (amid conjugates), or through an ester bond to various carbohydrates (ester conjugates). A variety of amide conjugates found in the plants studied are IAA-Ala, IAA-Asp, IAA-Leu, and IAA-Glu (Tam et al. 2000, Kowalczyk and Sandberg 2001). Common IAA-ester conjugates include IAA-myoinositol glycosides, IAA-myoinositol and IAA-Glc (Domagalski et al. 1987). Proposed functions of these conjugates include storage, transport compartmentalization, excess IAA detoxification and protection against peroxidative degradation (Cohen and Bandurski 1982). All the plants contained most of the IAA as derivatives, the cereal grains contained mainly ester IAA and the legume

seeds contained mainly peptidyl IAA (amide). Plant regulates IAA levels by modulation of IAA synthesis (Audus 1972) or through IAA oxidation (Good et al. 1956, Galston & Hillman 1961). Other than IAA many structurally similar compounds present in the plants have auxin like activity, which can also form the conjugated forms. These include IBA (Indole butyric acid) (Ludwig-Muller and Epstein 1993, Nordstrom et al. 2004), IPA (Indole propionic acid) (Schneider et al. 1985, Mohammad et al. 2003), and PAA (Phenyl acetic acid) (Hammad et al. 2003).

Most of present day knowledge about free and conjugated IAA originates from studies on seeds and seedlings. They are most abundant in mature seeds (Bandurski and Schulze 1977). This led to predict the hypothesis that seeds are the main sites of IAA storage, IAA conjugates and that conjugates are liberated as IAA, which can be used by the growing seedling (Cohen and Bandurski 1982). During initial period of seed development free and ester IAA level is high, drop in maturing seeds as sharp increase in amide IAA conjugates (about 80% of total pool of IAA) (Bialek and Cohen 1989). In orthodox seed the reduction in hormonal levels in the mature seed is a common phenomenon caused by the high degree of seed dehydration.

Although various quantitative analysis of IAA in mature seeds have been reported (Nowacki and Bandurski 1980, Bialek and Cohen 1986, Epstein et al. 1986), limited information is available on the dynamics of free and conjugated auxins during seed development (Gregorio et al. 1995) and it is limited to a few species mainly belonging to cereals and legumes (Sandberg et al. 1987, Bialek and Cohen 1989).

Hormone levels in seeds are commonly investigated by analyzing the whole seed without distinguishing tissues and cell types. However, this kind of information is critical for any hypothesis on the role of a hormone in embryogenesis and seed development as each seed component has a

different function in these processes (Hein et al. 1984). Cotton seed can be divided into kernel, seed coat and fiber that is economically important and hence, it is required to know the endogenous level to understand the growth and development of the individual part of cotton seed.

Seagull and Gialvalis (2004) showed that pre and post anthesis treatment with IAA result in significant increase in the number of fibers per ovule; additionally, the largest increase (58%) in fiber number was observed with post anthesis treatments. Previous observations of Jasdanwala et al. (1980) indicated that an increase in IAA levels is important for epidermal cells to differentiate into fibers. Although it is clear that ovules grown *in vitro* produce fibers differences in wall chemistry and protein profiles differ between fibers grown *in vitro* and *in vivo* (Davidonis 1999). Other differences between fibers grown *in vitro* and *in vivo* include differences in fiber length, secondary wall synthesis (Kim and Triplett 2001).

Further, earlier observation of Naithani et al. (1982) suggested that the levels of free auxin like substances in cotton fiber recorded maximum prior to the onset of fiber elongation and during elongation phase. Thaker and her coworkers (1986) indicated that the biosynthesis of IAA, as evidenced by the activity of IAA aldehyde dehydrogenase, increased significantly during elongation phase and showed close parallelism with rate of fiber elongation: and hence proved that auxin consumption is important for growth phenomena but not the leftover auxin.

Fruit development is a complex process that begins with the change from vegetative to a floral meristem and ends with mature fruit and viable seed. Although many tissues can synthesize auxin (Ljung et al. 2001), auxin transport is complex and highly regulated, involving many identified proteins. Chemical and genetic studies have revealed that transport of auxin to distant sites is clearly required for normal

development of plant; in later root development (Reed et al. 1998, Bhalerao et al. 2002) vascular development (Mattsson et al. 1999), phyllotaxis (Reinhardt et al. 2003), embryonic axis development (Friml et al. 2003). In the present study, apart from the reproductive structures (kernel, coat and fibers), endogenous IAA and PAA levels were checked in protective structures *i.e.* boll wall and bracts from both the genotypes. This may help to understand auxin role in organ development. Brenner and Cheikh (1995) stated that the concentration gradient of photoassimilates between source and sink tissue is likely the primary regulator for the current rate of transport and pattern of partitioning.

PAA (Phenyl acetic acid) is known to play an auxinomimetic role in plant growth regulation (Fries 1977, Hammad et al. 2003). It also participates in cell enlargement as IAA (Milborrow et al. 1975). The ability of PAA, a naturally occurring auxin, to initiate growth of callus and suspension cultures in several species is reported (Leuba and LeTourneau 1990). Elongation responses to combinations of IAA and PAA suggested that the compounds were acting additively and that they were affecting growth by the same mechanism (Small and Morris 1990). They have also suggested from petiole abscission in cotton that PAA was considerably less active than IAA in this response.

On the day of anthesis cotton ovules produce fiber when floated on the surface of a defined medium (Beasely and Ting 1973). Because of its economic importance and unique growth features, cotton fiber is the focus of much work involving plant cell wall development and elongation (Beasley et al.1974, Basra and Malik 1984, Delanghe 1986, Seagull 1990, Seagull and Gialvalis 2004). In earlier studies, Gokani and Thaker (2002) have estimated IAA and PAA level from developing fibers. However, no detailed study has been reported during the entire period of *in vitro* seed development and dry matter accumulation.

The aim of the present study was to compare the effect of auxin in both genotypes and to evaluate the effect of exogenous auxin on the fiber length, seed size, and seed dry weight. Indirect ELISA was performed using antibodies raised against IAA and PAA for quantification of their endogenous level. In the present study, endogenous free and conjugated IAA and PAA levels were estimated in all boll components of two cotton genotypes (H-6 and G. Cot) throughout the developmental period.

MATERIALS AND METHODS

(A) Raising of antibodies against IAA and PAA

(i) Preparation of PGR- BSA/Casein conjugate

Indole-3-acetic acid was conjugated with BSA and with casein by modified protocol of Weiler (1981). PAA-BSA/casein conjugation was carried out by following the methodology suggested by Gokani and Thaker (2002). For IAA or PAA conjugation, 52.3 mg of IAA or 100 mg of PAA were dissolved in 2 ml of DMF and 75 μl of tri-n- butylamine was added. After formation of the mixed anhydride (8 min reaction time at -15 ºC), the reaction mixture was added to 421 mg BSA or casein with constant stirring, dissolved in 22 ml of an equal mixture by volume of DMF and water. pH was maintained by addition of 420 μl 1M NaOH. After 1 h incubation at 0 ºC another 200 μl of 1M NaOH was added and stirring continued for 5 h in the dark. The reaction mixture was then dialyzed against 10% DMF for 16 h and then for 4 days against distilled water. Final volume was made with distilled water and stored at 0 ºC.

(ii) Immunization

Two rabbits were selected for each hormone. IAA/PAA-BSA conjugate was mixed with an equal amount of Freund's complete adjuvant and injected to the rabbits (Thaker 1995). Immunization was followed by intramuscular route. Booster injections were given periodically to raise the titer. Rabbits were bled (10 ml) from marginal ear vein and serum was separated. Antibodies raised against both conjugates (IAA-BSA and PAA-BSA) were tested for cross-reactivity with other naturally occurring and synthetic auxins. Antibodies against IAA or PAA did not react significantly with other naturally occurring auxins (Data not shown) and hence they were used for estimation of endogenous IAA and PAA content.

(iii) Purification of IgG

Purification was accomplished by ion-exchange chromatography. γ-Immunoglobulin (IgG) against IAA and PAA were collected by passing the serum through DEAE –cellulose column pre-equilibrated with 0.01M phosphate buffer (pH 8.0). The purified IgG was concentrated to the original volume of serum and preserved at 0 ºC.

(B) Extraction of IAA and PAA from the samples

Frozen samples of seed (kernel and seed coat), boll wall and bract (500 mg fresh weight) were powdered in pre-chilled mortar with liquid nitrogen and then mixed with 5 ml of 80% methanol (v/v). This mixture was stirred for 10 min and incubated for 48 h at 4 ºC in dark. The mixture was centrifuged at 10,000 g for 10 min and supernatant was collected. Pellets were washed twice with 80% methanol for complete extraction of hormone. The supernatant was directly used for IAA and PAA estimation. Conjugated IAA and PAA were extracted according to the method suggested by Bandurski and Schulze (1977). In brief, the hormone extract was allowed to hydrolyze with an equal amount of 2N KOH at room temperature for 2 h 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.

Calibration for IAA and PAA

A calibration curve was prepared for IAA and PAA showed linear relationship with concentration, in the range 50-600 ng/well and 100-600 ng/well, respectively. Dilution of samples and addition of internal standard was done in this calibration range.

(C) Indirect ELISA

To avoid cross-reactivity with IAA/PAA-BSA conjugated protein, IAA/PAA–casein conjugate was adsorbed on micro-titer plate (Tarson, INDIA). The conjugate was diluted (1:10) with coating buffer (10mM carbonate buffer, pH 9.7). The plate was incubated at 37 ºC for 3 h or overnight at 4 ºC. Then plate was coated with blocking buffer (PBS - T + 0.5% egg albumin) to block free protein binding sites of the well in order to check direct binding of antibodies on the well and incubated for 3 h at 37 ºC. After that antibodies against IAA and PAA were mixed with samples. Finally, plate was coated with anti-rabbit IgG, tagged with peroxidase and the color was developed using OPD. The reaction was terminated by addition of 6N sulfuric acid and plate was read at 492 nm using ELISA plate reader (μ quant, Biotek instruments, USA). After each step the plate was washed thrice with washing buffer (Phosphate buffer saline containing 0.05% Tween 20, pH 7.2). With each plate calibration curve was performed. All estimations were done in triplicate and reported as mean value with ± SD.

(D) Cotton ovule culture

Bolls were collected after one-day anthesis, from both the genotypes. They were kept under running tap water for 1-2 h and ovules were separated out under aseptic condition. Liquid basal media was prepared according to Beasley and Ting (1973) with or without IAA (mg L^{-1}), NAA (mg L^{-1}) or PAA (mg L^{-1}, 3 mg L^{-1}) and ovules acquired from ovary were floated on its surface. The cultured flasks were kept under dark condition at 35 °C. In preliminary experiments, different concentrations of IAA, NAA, and PAA were used and from that optimum exogenous levels were decided.

(i) *In vitro* growth analysis

In the present study, *in vitro* growth analysis was performed in terms of fresh weight, dry weight, water amount, fiber length and seed size. To

measure the effect of exogenous auxin levels (IAA, NAA and PAA) three flasks were selected for each treatments and fresh weight was taken and kept in oven at 70 °C. After 3 days, dry weight was measured and water amount was calculated as the mean difference of fresh weight and dry weight. In G. Cot, prolonged incubation with exogenous auxins producing callus so, experiment was terminated after 36 DPA.

(ii) *In vitro* fiber length and seed size measurement

During initial period, fiber length and seed size were measured with ocular micrometer and with a scale during the subsequent periods. The experiment was continued till the rate of fiber elongation declined. Similarly seed size was measured till it was stabilized. ANOVA was performed to determine the effect of exogenous auxins on the growth parameters like dry weight, water amount, fiber length and seed size of cultured ovules of each genotype.

RESULTS

Growth analysis

Growth analysis of two cotton genotypes is presented in chapter 2 (Figs. 2.1 to 2.13).

In vivo study

Endogenous IAA levels

(i) Estimation of IAA in ovule

In developing seed kernel of H-6, free IAA content increase slowly and peaked on 27 DPA (12.33 µg seed kernel^{-1}) and declined gradually from 39 DPA when boll opened (Fig. 3.2a). Conjugated IAA level remained steady initially up to 30 DPA, maximum IAA level was noted on 33 DPA (9.16 µg seed kernel^{-1}, Fig. 3.2d). In G. Cot kernel, free IAA remained low up to 30 DPA and peak was observed on 33 DPA (7.491 µg seed kernel^{-1}, Fig. 3.2a). Similarly, conjugated IAA level remained also low during initial period, up to 27 DPA and significant amount of conjugated IAA level was recorded during later stages from 30-45 DPA (Fig. 3.2d).

Changes in free and conjugated IAA levels were recorded in seed coat in both the genotypes throughout the developmental period. In H-6 seed coat, free IAA level remained very low up to 21 DPA. On 24 DPA (6.50 µg seed coat^{-1}) peak was observed and declined thereafter (Fig. 3.2b). While conjugated IAA level remained stable initially, sharp peak was obtained on 39 DPA (10.21 µg seed coat^{-1}, Fig. 3.2e) declined thereafter. In case of G. Cot seed coat, free IAA level was detected in negligible amount up to 9 DPA then gradual increase up to 27 DPA and achieved a maximum value 7.93 µg seed coat^{-1}; rapid decline was observed thereafter (Fig. 3.2b). Conjugated IAA content in G. Cot seed coat was significantly higher than free IAA. Conjugated IAA level remained low up to 21 DPA, significant amount of IAA level recorded from 24 DPA– 42 DPA and declined thereafter (Fig. 3.2e).

(ii) Estimation of IAA in fiber

Free IAA content in H-6 fiber, showed peak on 27 DPA (7.70 µg seed fiber^{-1}, Fig. 3.2c). Conjugated IAA level remained higher up to 36 DPA and rapidly declined after boll opened (Fig. 3.2f). In G. Cot free IAA level remained low initially, a sharp peak was observed on 24 DPA (6.02 µg seed fiber^{-1}) then stabilized in subsequent ages (Fig. 3.2c) while conjugated level remained almost steady throughout the developmental period except 21 and 33 DPA (5.14 and 4.68 µg seed fiber^{-1} respectively, Fig. 3.2f).

(iii) Estimation of IAA in boll wall and bract

As seed components, free and conjugated IAA content was also measured in boll wall and bract of both the genotypes (Fig. 3.3). In H-6 boll wall, during initial phase free IAA was detected in negligible amount up to 12 DPA, during 15-42 DPA marked IAA content noted and sharp peak obtained on 27 DPA (283 µg boll wall^{-1}, Fig. 3.3a) whereas rapid increase in conjugated IAA observed up to 18 DPA (983.17 µg boll wall^{-1}), remained higher up to 39 DPA and declined at maturity (Fig. 3.3c). In contrast to this result in H-6 bract, free IAA content was detected during initial phase ranging from 3-15 DPA and remained negligible thereafter (Fig. 3.3b). Moreover, conjugated IAA level was recorded higher till 39 DPA and declined thereafter.

In G. Cot boll wall, free IAA level gradually increased up to 21 DPA, peaked on 33 DPA (254 µg boll wall^{-1}) and gradually declined in subsequent stages (Fig. 3.3a). Whereas conjugated IAA level remained low up to 12 DPA increased rapidly and achieved a peak on 24 DPA (303.19 µg boll wall^{-1}) and gradual decrease in later stages was observed (Fig. 3.3c). In G. Cot bract (Fig. 3.3b, d), free IAA content was detectable during initial (3-12 DPA) and later ages (36-42 DPA), remained in negligible amount during 15-33 DPA whereas conjugated IAA content was higher up to 36 DPA and decline was observed thereafter.

In addition, total IAA content of H-6 boll was higher than the G. Cot. Maximum free IAA level in H-6 was noted on 27 DPA (305.05 µg $boll^{-1}$) whereas; in G. Cot maximum value was recorded on 33 DPA (269 µg $boll^{-1}$). In both the genotypes, IAA level drop after maturation (Fig. 3.1a).

Endogenous PAA level

(i) Estimation of PAA in ovule

Like that of IAA, changes in PAA level were measured in different boll components of two cotton genotypes throughout the developmental period. In H-6 seed kernel, PAA content increased from 18 DPA and remained high up to 39 DPA (22.40 µg) and then declined (Fig. 3.4a). In G. Cot kernel, PAA level remained absent during initial phase (up to 24 DPA), increased gradually and obtained maximum value on 36 DPA (6.02 µg) and declined thereafter (Fig. 3.4a). Thus, in H-6 kernel PAA content was many times higher than G. Cot.

Changes in endogenous level of PAA content in H-6 remained low in seed coat as compared to seed kernel. It showed gradual increase from 3-15 DPA, peaked on 15 DPA (8.54 µg), dropped during 18-21 DPA, again achieved higher level from 24-27 DPA and declined thereafter (Fig. 3.4b). In G. Cot seed coat, PAA content was absent during initial phase of 3-9 DPA, increased steadily thereafter, obtained sharp peak on 24 DPA (25 µg) and declined thereafter (Fig. 3.4b).

(ii) Estimation of PAA in fiber

In H-6 fiber (Fig. 3.4c), PAA content increased from 9 DPA and remained high up to 33 DPA and decreased gradually thereafter. However, in G. Cot fiber, amount of PAA was detected in negligible amount during 9-21 DPA, significant amount of PAA obtained during 24-48 DPA (Fig. 3.4c).

(iii) Estimation of PAA in boll wall and bract

In H-6 boll wall (Fig. 3.5a), after a short lag period of 9 DPA, increase in PAA level was detected during 12-27 DPA, slight fall was obtained after 27 DPA and reached to negligible level after boll opened. In G. Cot boll wall, PAA level was recorded from 30-36 DPA and then drastic decline was observed (Fig. 3.5a). In addition, in H-6 bract, PAA level gradually increased up to 15 DPA, decreased from 18-30 DPA and reached to negligible level thereafter. While, in G. Cot bract PAA level remained low up to 15 DPA, peaked on 18 DPA (72.97 µg boll bract^{-1}), declined and remained low till boll opened and then again declined (Fig. 3.5b).

Further, total PAA level per boll was recorded higher in H-6 than G. Cot (Fig. 3.1b). In H-6, PAA level increased rapidly up to 15 DPA, remained high up to 39 DPA and declined thereafter. In case of G. Cot, PAA level remained low up to 27 DPA, two peaks were observed on 30 DPA (629.12 µg PAA Boll^{-1}) and 39 DPA (730.16 µg PAA Boll^{-1}) but after boll opening (39 DPA) gradual decline was observed.

In vitro growth analysis

Exogenous application of different auxin concentrations on one day post anthesis ovules were checked in terms of dry weight, water amount, fiber length and seed size (Figs. 3.6 to 3.9). In control (without any hormones) treatment, maximum dry weight was restricted up to 3 mg in G. Cot but it was 21 mg in H-6 (Fig. 3.6a). When media was supplemented with 1 mg L^{-1} NAA (Fig. 3.6b), 1.5 times increase in dry weight was noted in H-6 while in G. Cot 6.33 times increase was obtained. Similarly, with IAA treatment rapid increase in dry matter accumulation was recorded in G. Cot as compared to H-6. In G. Cot, 18.33 times increase was noted while in H-6 only 1.19 times increase was observed (Fig. 3.6c). PAA (1 mg L^{-1}) treatment showed 3 times increases in H-6 and 10 times increase in G. Cot seed dry weight (Fig. 3.6d). Moreover, with increase in PAA concentration

(3 mg L^{-1}) 3 times higher dry weight in H-6 and 20 times higher dry weight in G. Cot seed was achieved (Fig. 3.6e). It is clear from the Fig. 3.7 that there was not much increment in water amount with auxin treatment in G. Cot or there was no significant difference between two genotypes with respect to auxin applications.

In the control treatment fiber initiation began at 3 DPA, after which fiber cell entered into elongation phase in both the genotypes. In control, H-6 fiber length was 2 mm followed by G. Cot with 0.4 mm (Fig. 3.8a). The application of NAA increased the length of short staple (12 times) and long staple genotype (3 times approximately) (Fig. 3.8b). Similarly, addition of IAA increased the G. Cot fiber length 11 times more where in H-6 fiber it was 3 times (Fig. 3.8c). However, application of PAA found to be more effective in G. Cot than H-6. PAA (1 mg L^{-1}) concentration increased the short fiber length 10 times more while in H-6 only 1.5 times increase was observed, though increasing the PAA concentration up to 3 mg L^{-1}, 14.37 times increase in G. Cot while 2.6 times increase in H-6 fiber length was observed (Fig. 3.8d, e).

Further, IAA and NAA found to be most effective auxin forms to increase the seed size. In control treatment, maximum size of H-6 seed was 19.0 mm^2 and it was 6.87 mm^2 in G. Cot. With NAA (mg L^{-1}) 4.485 times increase in G. Cot and 2.5 times increase in H-6 seed size was achieved (Fig. 3.9b) while with IAA treatment 8 times increase in G. Cot and only 2.08 times increase in H-6 was obtained. Moreover, in H-6 with IAA treatment callus induction was observed after 27 DPA (Fig. 3.9c). On the other hand PAA (mg L^{-1}) treatment showed approximately 2 times higher seed size in both the genotypes studied. With 3 mg L^{-1} PAA treatment, 4 times increase in G. Cot and 3 times increase in H-6 were obtained (Fig. 3.9d, e). Maximum increase in seed size was achieved with IAA though NAA was more stable auxin in media.

DISCUSSION

In vivo study

The principal findings of *in vivo* investigation of IAA and PAA accumulation in the developing cotton boll parts are (a) in general, free IAA levels were higher in H-6 whereas, conjugated were more in G. Cot (b) In H-6, IAA levels remained low in seed coat than that of kernel (c) In G. Cot as compared to kernel, seed coat has more free and conjugated IAA levels (d) In fiber, free and conjugated both IAA levels were higher in H-6 (e) PAA was higher in kernel and fiber of H-6 whereas in G. Cot seed coat has more PAA level than that of H-6 (f) In boll wall, IAA and PAA remained higher in H-6 whereas conjugated IAA was higher in G. Cot. In addition data suggest the significant differences between conjugated IAA levels amongst all the boll components of two genotypes studied (Table-1).

IAA in ovule

In the present study, maximum free IAA level in H-6 kernel was recorded during elongation and dry matter accumulation phases (21-45 DPA) (Fig. 3.2a) and declined during maturation phase. Although in G. Cot free IAA was higher during elongation phase but not extended up to completion of dry matter accumulation phase (Fig. 3.2a). Thus, differences in IAA level between two cotton genotypes with respect to growth phases may be responsible for their final yield. Further, in H-6 seed coat free IAA was higher up to 24 DPA when seed kernel showed low level of free IAA content that increased after 24 DPA. Contrary to this, in G. Cot higher degree of overlap was observed for kernel and seed coat for their IAA level (Fig. 3.2b, e). IAA estimation and dry matter accumulation phase showed similar pattern, which suggests that IAA play an important role in dry matter accumulation. Correlation between IAA and cell division in developing seed (Opik 1988) and changes in DMA (Bangerth et al. 1985) support the view that IAA might have increased cell division and thus DMA in H-6.

IAA in fiber

In developing fiber close correlation was obtained between length and free IAA level in H-6 ($p \leq 0.01$) and in G. Cot ($p \leq 0.001$) support the earlier work of Gokani and Thaker (2002) that endogenous IAA play an important role in fiber cell elongation (Table-3). Like that of free IAA, conjugated IAA was observed higher in long staple genotype than short staple genotype (Fig. 3.2c, f). Further, conjugated IAA and fiber length showed close correlation in both the genotypes, H-6 ($r= 0.501$, $p \leq 0.01$) and G. Cot ($r= 0.793$, $p \leq 0.001$) as it was observed for free IAA level that proves earlier record of our laboratory that conjugated IAA might have been protected from the enzymatic oxidation. Cohen and Bandurski (1982) indicated that conjugated forms of auxin also participate either in transport or remain as storage pool or function as auxin protectors from enzymatic attack. Other workers suggested that IAA is important for fiber initiation after fertilization (Joshi et al. 1967, Ramsey and Berlin 1976, Berlin 1986, Graves and Stewart 1988), differentiation (Jasdanwala et al. 1980, and Seagull and Giavalis 2004) and elongation (Thaker et al. 1986, Gokani and Thaker 2002).

IAA in boll wall and bract

IAA is an important plant hormone controlling a variety of development processes (Kowalczyk & Sandberg 2001). During the seed and fiber development boll wall and bract also play major role. In the present experiment, in boll wall free IAA with dry weight and with water amount (Table-2a) had close correlation. Like that of boll wall, IAA had also shown close correlation with dry weight and water amount (Table-2a) in bracts of both the genotypes. In boll wall, IAA and PAA remained higher in H-6 whereas conjugated IAA was higher in G. Cot; suggesting thereby poor mobilization of hormones to seeds and fiber.

PAA

PAA was higher in kernel and fiber of H-6 whereas in G. Cot seed coat has more PAA level than that of H-6. Thus, accumulation of IAA and PAA level in various parts regulates the seed size. Further, positive correlation between fiber length and PAA content in both the genotypes H-6 ($p \leq 0.001$) and G. Cot ($p \leq 0.001$, Table-3) suggest that PAA has a role in fiber elongation (Gokani and Thaker 2002).

Significant differences in PAA level ($p \leq 0.001$ for kernel, seed coat and fiber and $p \leq 0.01$ for boll wall and bract) was observed amongst all the boll components of both the genotypes studied (Table-1). In H-6 boll wall, PAA level was observed high as compared to G. Cot. Contrary to these results PAA level was noted higher in bract of G. Cot than H-6. From the presented data, it can be concluded that in high yielding genotype more PAA was present in kernel, fiber and boll wall whereas in G. Cot high PAA level was found in seed coat and bract and this may be responsible for final yield. As shown in Table- 4, there was positive correlation between water amount and PAA level in boll components in both genotypes. It is assumed that PAA may help in water uptake.

This *in vivo* investigation suggests that endogenous auxin play an important role in regulation of seed size and fiber length in cotton. Role of auxin in regulation of cell division and cell elongation is well known (Campanoni et al. 2003, Campanoni and Nick 2005). This lead to a speculation that if auxin is a cause of seed size and fiber length regulation then G. Cot seed should improve with exogenous application of auxin. To address this question, *in vitro* experiments were performed.

In vitro analysis

Looking to the results of *in vitro* analysis, IAA and PAA were found to be more promotive for seed dry weight in G. Cot than H-6 and dose dependent effect of PAA on dry weight of cultured ovules in G. Cot was obtained (Fig. 3.6d, e) while higher concentration of PAA was found to be inhibitory for H-6. Dry weight differences in both the genotypes were nullified with the help of IAA application (Table- 5). Extension of experiment for more than 36 days, induced callus in G. Cot seeds whereas in the same media H-6 seed grew normally, suggesting there by that endogenous status and requirement of hormones in media for optimum growth varies in both the genotypes studied.

Moreover both the genotypes showed promotion in fiber elongation with NAA and IAA (Fig. 3.8b, c) though earlier study suggested that IAA found to be better than NAA (Gokani and Thaker 2002). In the present study, it was observed that initial differences in the length of fibers amongst the genotypes were reduced with IAA and NAA, as shown in (Table-5). These results suggest that short staple genotype is deficient in endogenous levels of IAA and NAA and therefore, showed significant promotion when media was supplemented with either auxin. Additionally, remarkable increase in fiber length was obtained with PAA treatment in G. Cot. Initial difference in ovule size was nullified by supplementation of IAA (Table-5, Fig. 3.9). As shown in figures 3.6 and 3.9, with exogenous application of auxin, seed size and dry weight increased that leads to conclusion that auxin is involved in cell division and dry matter accumulation processes.

Contrary to *in vivo* experiment, *in vitro* study does not prove auxin effect in water uptake (Fig. 3.7). Many workers have correlated role of auxin in water uptake (Taiz 1984, Cosgrove 2001) and in seed development and/or in fruit development (Ozga and Reinecke 2003, Silveira et al. 2004). A concomitant action of auxins is to cause loosening of the cell wall (Cosgrove 2001) resulting in increased water uptake and cell enlargement.

Pless et al. (1984) explained that the function of 4-Cl IAA during seed development might also be the induction of cell enlargement to permit dry matter accumulation, which occurs subsequent to water uptake. However, in present in *vitro* study addition of auxin in media does not affect the water uptake in the seed, although, marked promotion was observed in seed size. This led to the assumption that water uptake may be an independent parameter in the process of cell elongation.

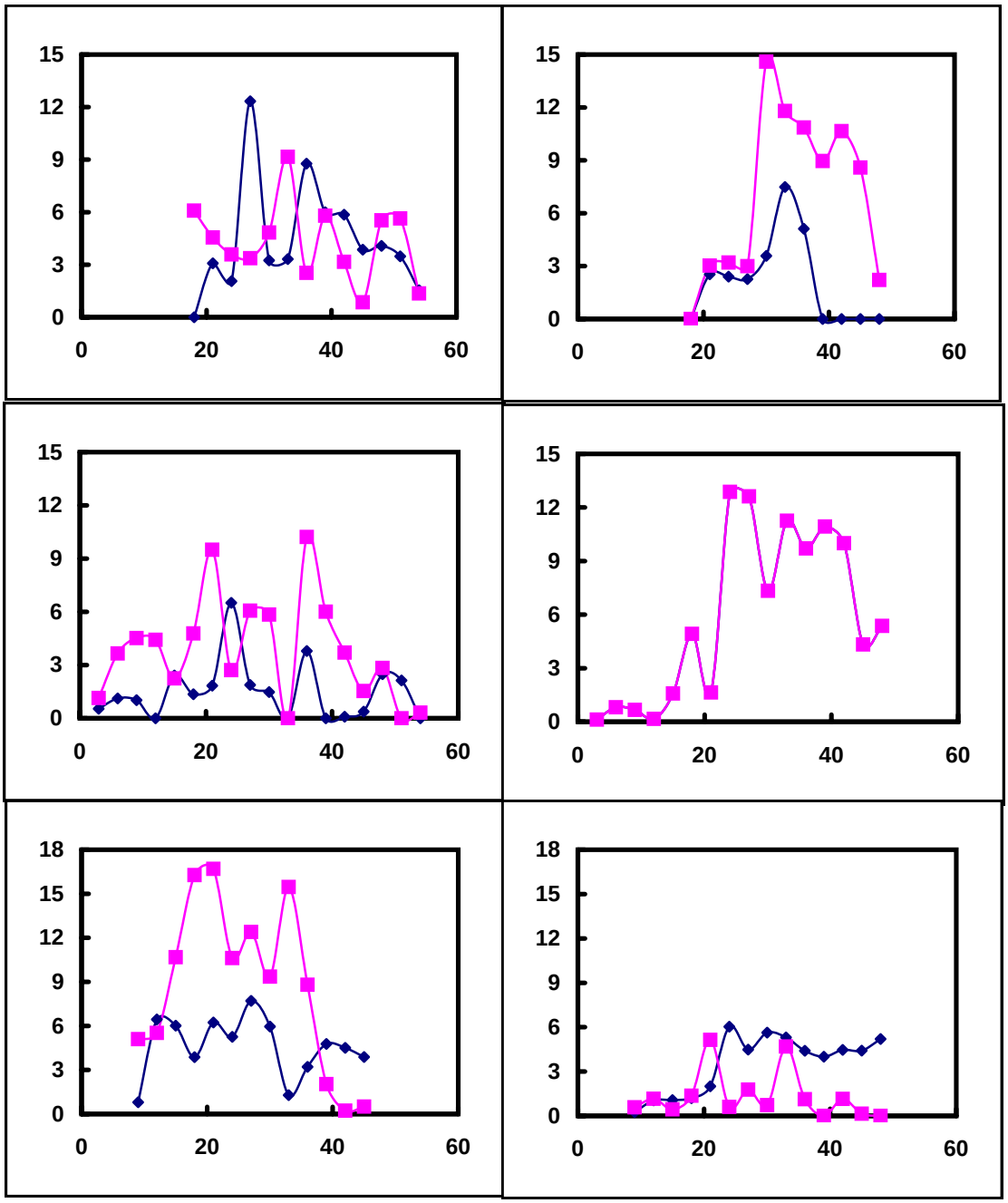


Fig-1 a,b,c- FreeIAA (blue) and conjugated IAAlevel (Pink)in H-6 and d,e,f: FreeIAA (blue)conjugat IAA levl (Pink) in G.cot

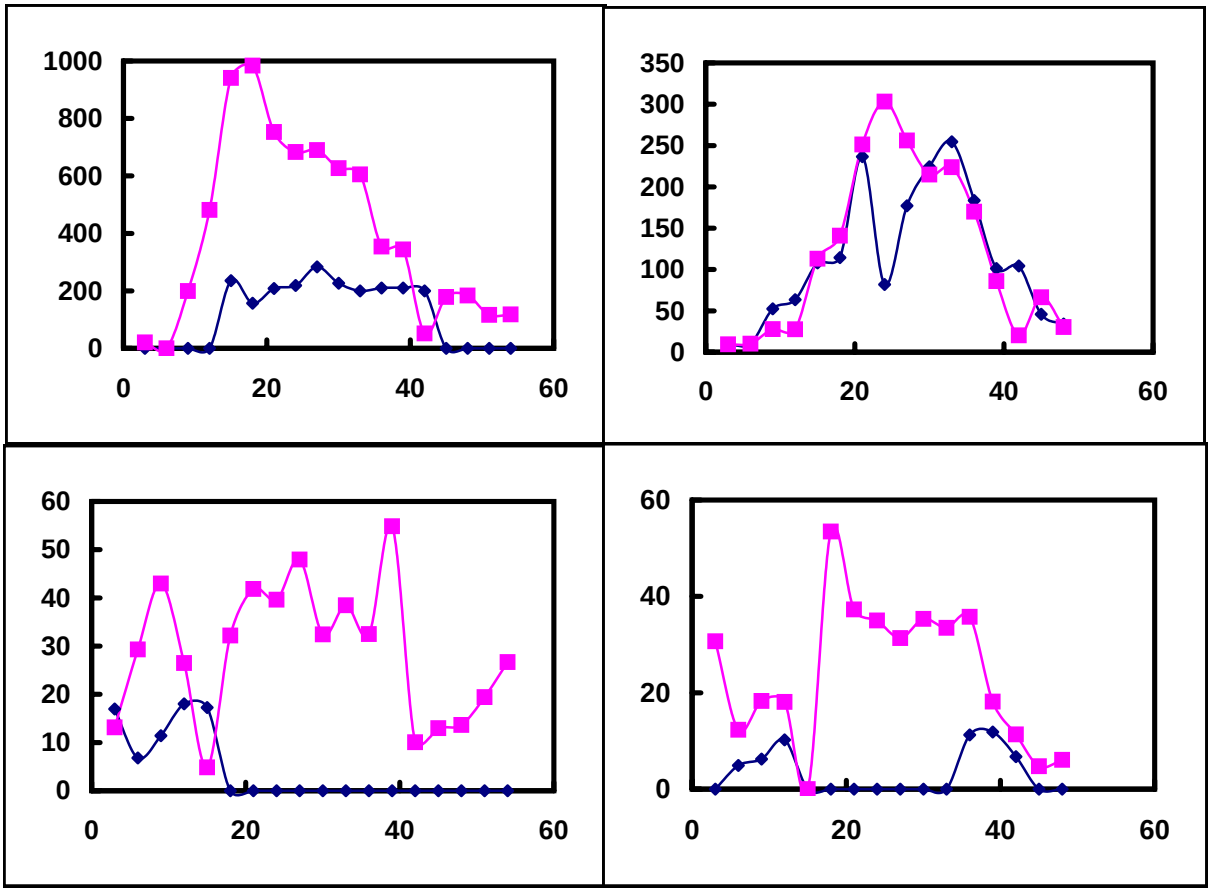


Fig-2a,b,- FreeIAA (blue) and conjugated IAAlevel (Pink)in H-6 and d,e: FreeIAA (blue)conjugat IAA levl (Pink) in G.cot

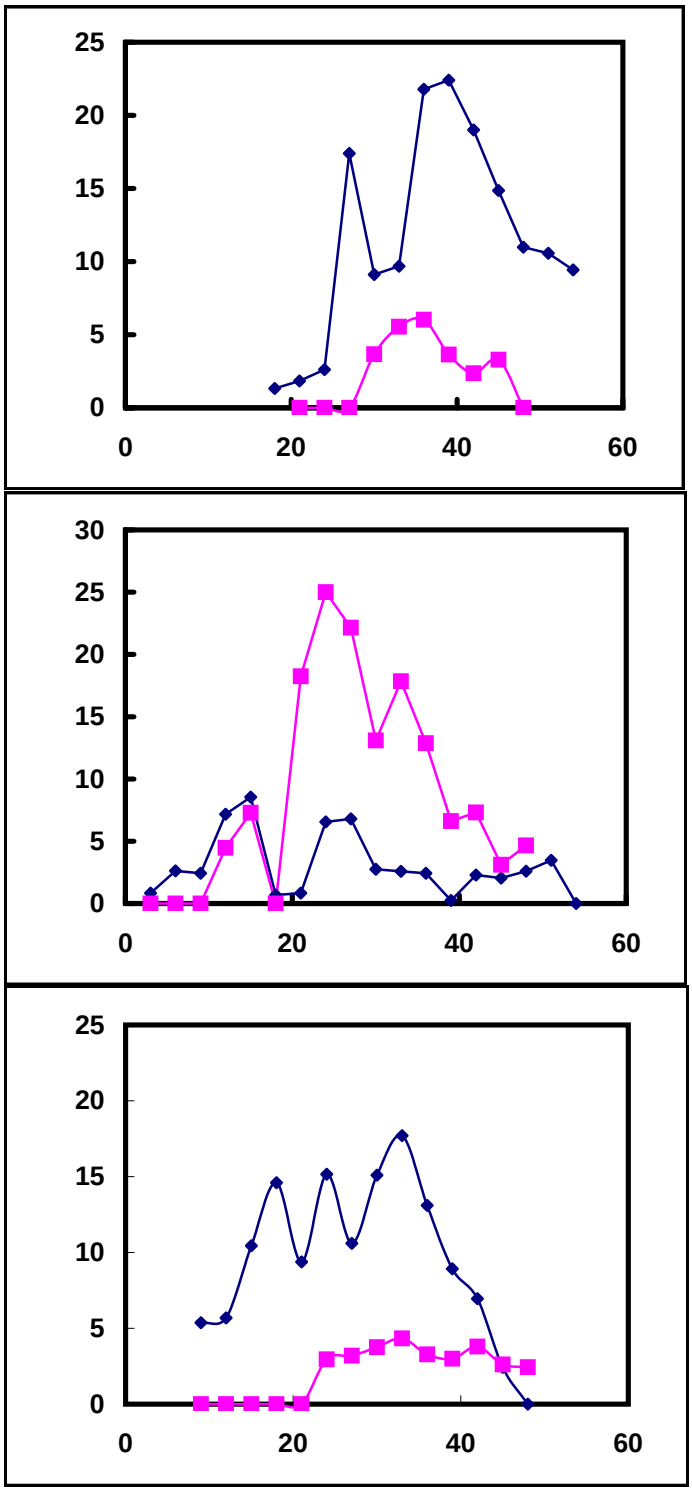


Fig-3 FreePAA (blue))in H-6 and FreePAA (Pink) in G.cot

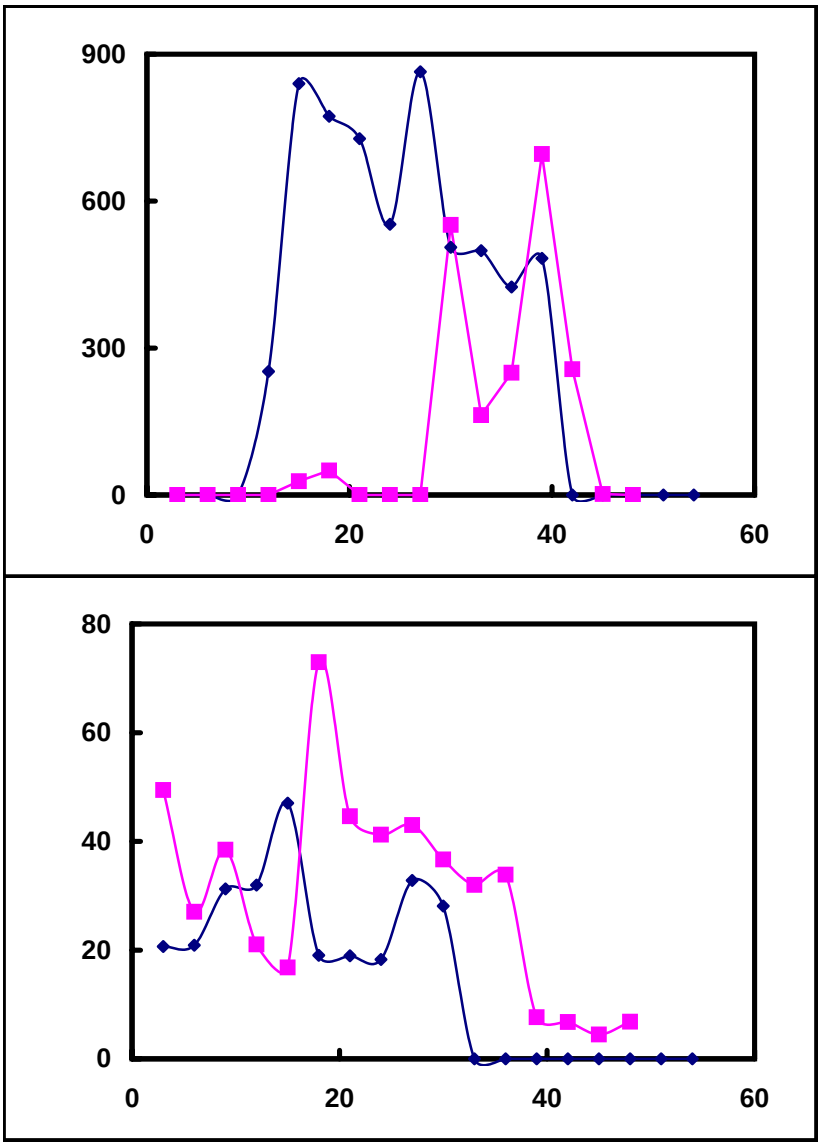


Fig-4 FreePAA (blue))in H-6 and FreePAA (Pink) in G.cot bw & br

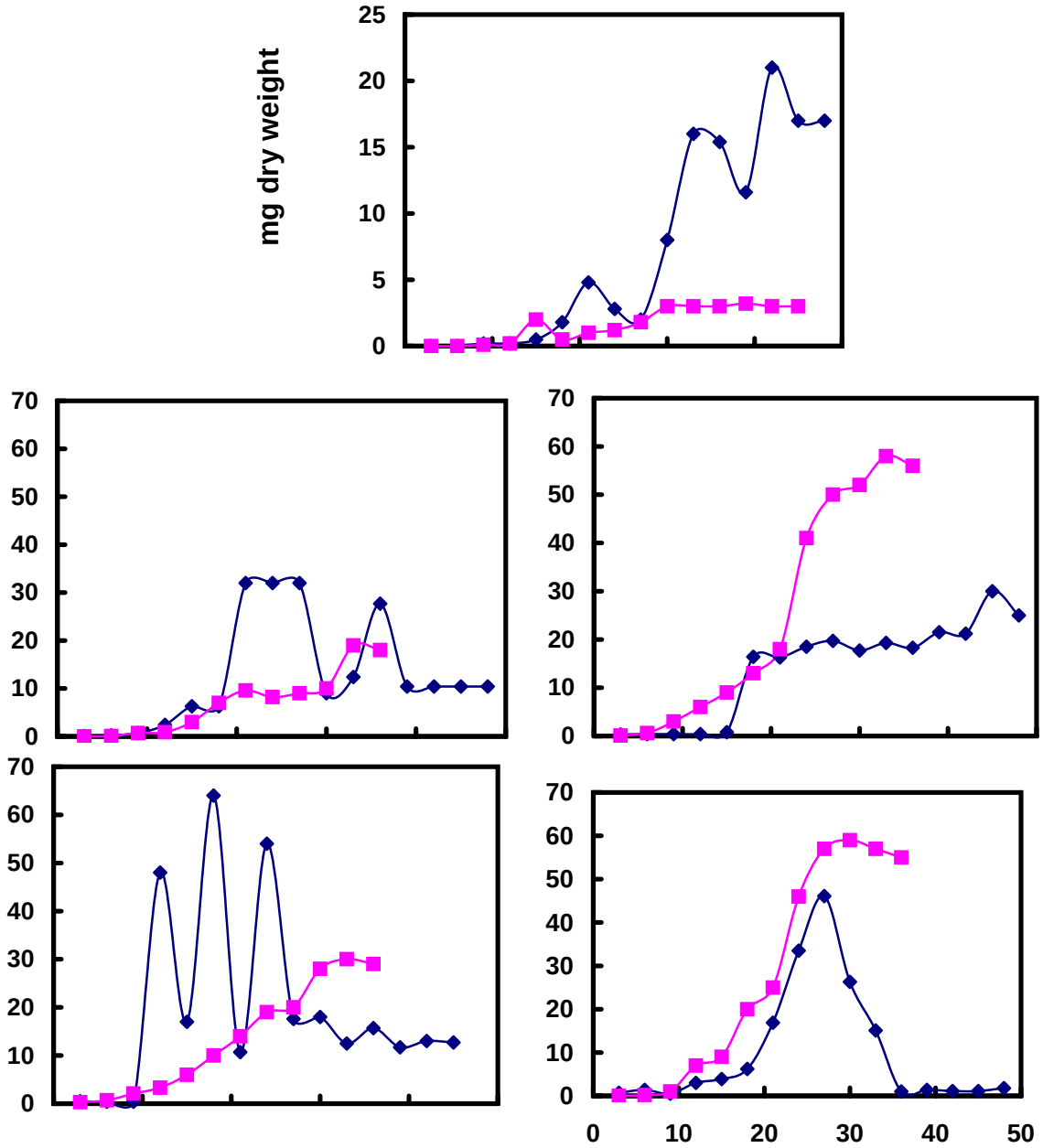


Fig-4: changes in in vitro dry weight, without hormone supplement (a), NAA(b), IAA(c), PAA mgL^{-1} (d) and PAA 3mgL^{-1} in H-6 () and G. Cot-13 ().

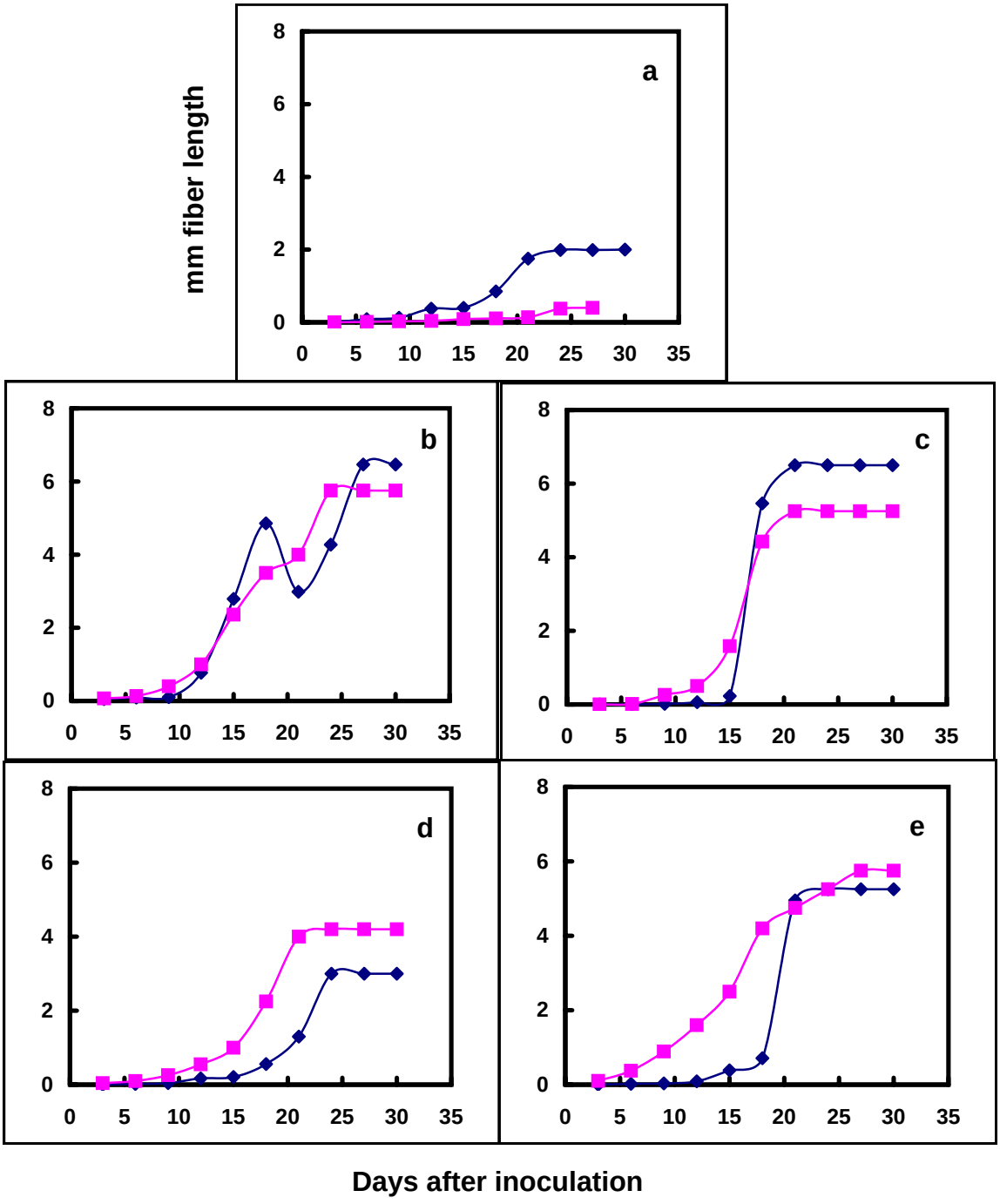


Fig-5: changes in in vitro fiber length, without hormone supplement (a), NAA(b), IAA(c), PAA mgL^{-1} (d) and PAA 3mgL^{-1} in H-6 () and G. Cot-13 ().

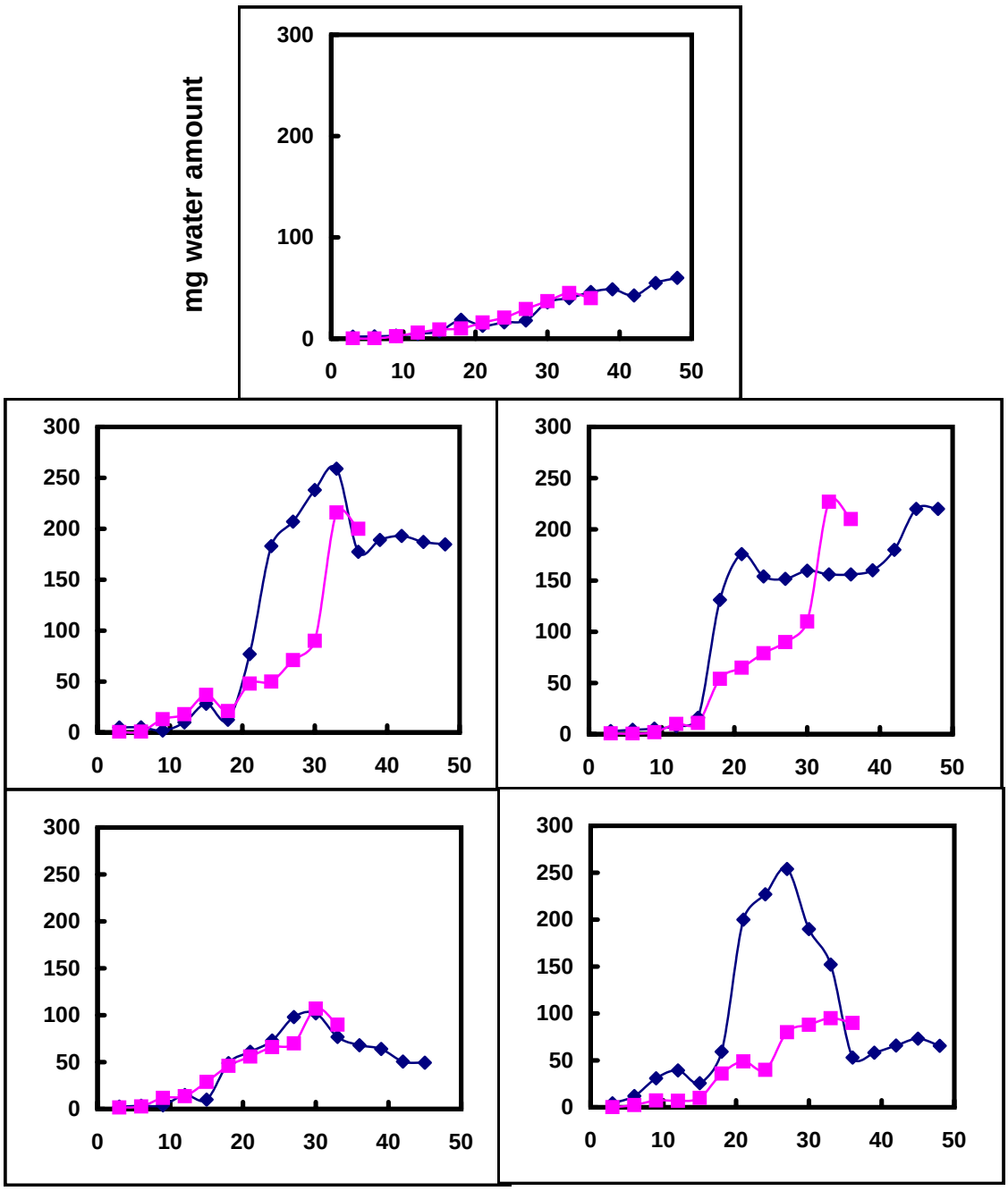


Fig-6: changes in in vitro water amount, without hormone supplement (a), NAA(b), IAA(c), PAA mgL^{-1} (d) and PAA 3mgL^{-1} (e) in H-6 () and G. Cot-13 ().

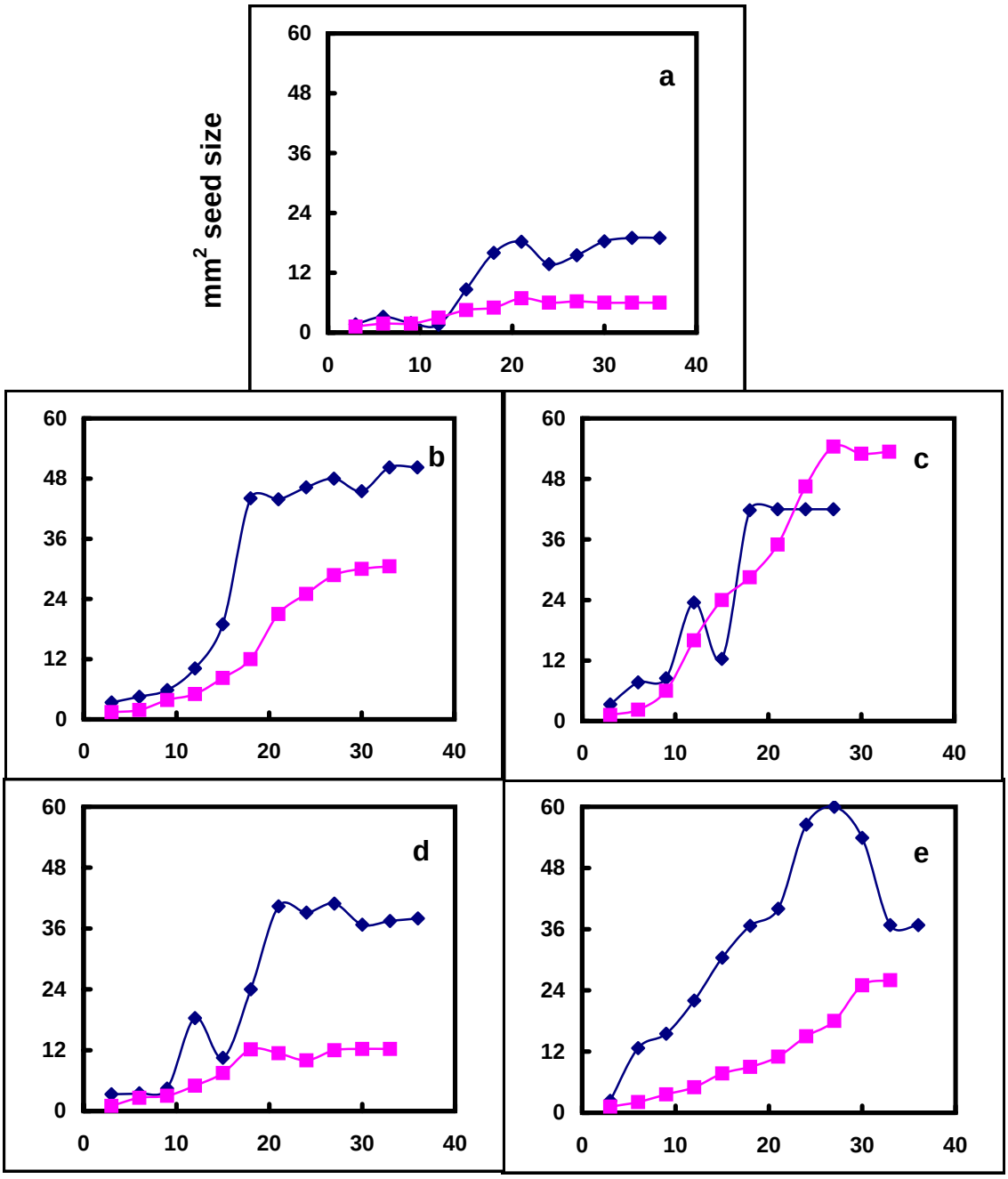


Fig-7: changes in in vitro seed size, without hormone supplement (a), NAA(b), IAA(c), PAA mgL^{-1} (d) and PAA 3mgL^{-1} in H-6 () and G. Cot-13 ().

Table-1. ANOVA between two cotton genotypes for their mean IAA and PAA levels in boll components

Source	Free IAA		Conjugate IAA		PAA	
	df	F- value	df	F- value	df	F- value
Kernel	23	2.732*	23	3.137**	22	14.394***
Seed coat	32	0.621	32	1.789*	31	7.567***
Fiber	25	1.957*	25	19.442***	27	26.995***
Boll wall	35	0.342	35	12.928***	32	4.777**
Bract	35	0.876	35	2.334*	31	6.05**

Significance level $p \leq 0.001$=*, $p \leq 0.01$=**, $p \leq 0.1$=***

Table-2a. Correlation coefficient between free IAA level with dry weight and water amount in boll wall and bract in two cotton genotypes: H- 6 and G. Cot.

Genotypes	Components	Free IAA and DW	Free IAA and WA
H-6	Boll wall	0.551**	0.82***
	Bract	0.306	0.547**
G. Cot	Boll wall	0.801***	0.706***
	Bract	0.725**	0.812**

Significance level p ≤ 0.001=*, p ≤ 0.01=**, p ≤ 0. 1=***

DW - dry weight

WA - water amount

Table- 2b. Correlation coefficient between conjugated IAA and water amount in boll components of two cotton genotypes: H-6 and G. Cot.

Source	H- 6	G. Cot
Kernel	0.332	0.751**
Seed coat	0.357*	0.716**
Fiber	0.81***	0.548*
Boll wall	0.924***	0.849***
Bract	0.836***	0.572**

Significance level p ≤ 0.001=*, p ≤ 0.01=**, p ≤ 0. 1=***

Table-3. Correlation coefficient between fiber length, endogenous free IAA and PAA levels in two cotton genotypes: H-6 and G. Cot.

Genotypes	Free IAA	PAA
	r	r
H-6	0.629**	0.892***
G. Cot	0.876***	0.843***

Significance level p ≤ 0.001=*, p ≤ 0.01=**, p ≤ 0. 1=***

Table-4. Correlation coefficient between endogenous PAA level and water amount in different boll components of two cotton genotypes: H-6 and G. Cot.

Source	H-6	G. Cot
	r	r
Kernel	0.115	0.735**
Seed coat	0.461*	0.799***
Fiber	0.775***	0.561*
Boll wall	0.92***	0.676**
Bract	0.693**	0.846***

Significance level p ≤ 0.001=*, p ≤ 0.01=**, p ≤ 0. 1=***

Table-5. ANOVA between two cotton genotypes for dry weight, fiber length, seed size and water amount with IAA/ NAA/ PAA treatments *in vitro*.

Dry weight	Control	IAA (1mg)	NAA (1 mg)	PAA (1mg)	PAA (3 mg)
F-value	5.795	0.676	9.769	8.15	25.883
p-value	0.02	0.425	0.004	0.01	3.75E-05
Fiber length	Control	IAA (1mg)	NAA (1mg)	PAA (1mg)	PAA (3 mg)
F-value	8.371	0.0958	0.355	3.6	2.214
p-value	0.009	0.342	0.558	0.07	0.0156
Seed size	Control	IAA (1mg)	NAA (1 mg)	PAA (1mg)	PAA (3 mg)
F-value	5.795	0.676	9.768	8.15	25.88
p-value	0.02	0.42	0.004	0.01	3.75E-05
Water amount	Control	IAA (1mg)	NAA (1 mg)	PAA (1mg)	PAA (3 mg)
F-value	1.139	2.776	3.448	0.065	4.238
p-value	0.295	0.107	0.075	0.8	0.049

Chapter IV
Gibberellic Acid

SUMMARY

Boll components of two cotton genotypes H-6 (bigger seed and long staple) and G. Cot (small seed and short staple) were analyzed to study the role of GA in seed development and fiber elongation, *in vivo* and *in vitro*. Different boll components *i.e.* Kernel, seed coat, fiber, boll wall along with septa and bracts were analyzed for endogenous GA content during entire period of their development. Endogenous GA level was estimated by indirect ELISA using antibodies raised against GA. Data presented in the present study showed that GA content per seed and per boll was many fold higher in H-6 than G. Cot throughout the developmental period. Moreover, H-6 kernel contained higher GA level than G. Cot, while GA content was found higher in G. Cot seed coat than H-6. Further, long staple genotype has shown distinctly higher GA content throughout the developmental period than short staple genotype suggesting thereby that GA is one of the important determinants in fiber length. Close correlation between endogenous GA content and water amount was recorded in all boll components. In *in vitro* studies when media was supplemented with GA_3 or GA_3+NAA, percent promotion in dry weight, water amount, seed size and fiber length was remarkably higher in low yielding genotype G. Cot, which indicates that this genotype may be deficient in endogenous GA_3 content. Further, GA_3+NAA were found to be more apposite, supporting the view that GA and auxin act synergistically. *In vivo* and *in vitro* findings reveal that GA is one of the important factors that determine final yield in cotton.

Abbreviations: BSA- Bovine serum albumin, ELISA-Enzyme linked immuno sorbent assay, GA- Gibberellic acid, IgG-γ-immunoglobulins, MS- Murashige and Skoog, PEG- Poly Ethylene glycol

INTRODUCTION

Gibberellins (GAs), a major class of plant hormones consists of more than 120 members (Murata et al. 2002). Gibberellins exist in angiosperms, gymnosperms, ferns, and probably also in mosses, algae and fungi (Salisbury and Ross 1992, Chaube and Singh 2000) and bacteria (Atzorn et al. 1988, Bottini et al. 1989). GAs play an important role in many aspects of plant growth (Richards et al. 2001) *i.e.* stem elongation (Zeevart 1983, Rood et al. 1989, Yang et al. 1996), seed germination (Leubner-Metzger et al. 1996), flower development (Nakayama et al. 2002), seed development (Davies 1995), fruit setting and anthocyanin biosynthesis (Weiss et al. 1992, Hooley 1994) to overcome seed and bud dormancy.

GAs are thought to be limiting factors controlling the fruit growth (Talon et al. 1990), as exogenous application of GA improves fruit set. Fruits also act as mobilization centers for nutrients, with hormones possibly modulating the process (Brenner and Cheikh 1995). Bioassay in tomato fruits showed that GA like activity follow bimodal pattern where two peaks coincide with cell division and cell expansion phases (Srivastva and Avtar 2005). Many other studies report internode elongation as a result of GA treatment (Kamijima 1981) and involvement of GA in cell division in cut cortex of tomato and cucumber (Asahina et al. 2002).

Seed as a rich source of many hormones (Crane 1969) are known to be essential for normal development of fruit; the size and shape of many fruits being determined by seed number and distribution. Many workers have observed that GAs level was high in immature seeds and decreased during seed maturation (McComb 1961, Rudrapal et al. 1992) which suggested that GAs play an important role in seed development. Gibberellin conjugates are preferentially formed during seed maturation and often are present in relatively high levels in maturing seeds (Hiraga et al. 1974a, b) and reversals of this trend upon imbibitions of the seed have been

observed in wheat and bean (Hashimoto and Rappaport 1966, Schneider 1983). It is observed that GAs involved in stimulating germination in tomato and *Arabidopsis* (Debeaujon and Koornneef 2000, Yamaguchi and Kamiya 2002, Ogawa et al. 2003). One of the GA effect in developing seed is to enhance cell elongation so that the radical can push through the endosperm, seed coat or fruit coat that restricts its growth (Salisbury and Ross 1992). Second, it also increases the growth potential of the embryo, as indicated by the reduced growth rate of GA-deficient embryos (Groot and Karssen 1987).

Despite of the many advances in recent years, many questions related to seed and fruit (fruit organ) development remained unanswered. A hypothesis for causal relationship with GAs suggests that the concentration and distribution of endogenous GAs should be positively correlated with the initiation of each of these physiological processes (Rood et al. 1989). The present day knowledge about gibberellins is derived from the exogenous applications on decapitate stem sections; however stem sections are composed of number of cell types which respond differently to exogenous gibberellins. Thus, with any experiment that can be responsive to exogenous supply of hormones a number of questions have to be addressed before it can be accepted that the observed hormone response is physiological (Trewavas 1991). Using cotton fiber as a model system, *in planta* and *in vitro* it was demonstrated that GA plays an important role in regulation of staple length (Gokani and Thaker 2001). However, no detailed study on cotton fruit and seed is reported. In the present study, two different genotypes of cotton, varying widely in growth of yield components were studied to evaluate role of endogenous GA. Endogenous GA was estimated using antibodies raised against GA-protein conjugates.

MATERIALS AND METHODS

(A) Raising of antibodies against GA

(i) Preparation of PGR- BSA/Casein conjugate

Plant growth regulators are haptens; therefore it is necessary to conjugate them with protein molecules *i.e*. BSA or casein to make them immunogenic. Gibberellic acid (GA_3) was conjugated with BSA and/or casein according to Gokani and Thaker (2002). GA_3 was coupled to free amino group of BSA via a mixed anhydride reaction. GA_3 (106 mg) and 75 µl tri- butylamine were dissolved in 2.5 ml DMF and cooled to 0 ºC. To this solution, 40 µl isobutylchlorocarbonate was added and the mixture was allowed to proceed for 20 min. Then, the solution was added, with thorough stirring to an ice-cold solution of 420 mg BSA in 22 ml DMF/ water (1:1 v/v). After 60 min, 0.2 ml 1M NaOH was added, and the mixture was stirred for 3 h and finally purified by dialysis against 10% aqueous DMF and 5 days against distilled water.

(ii) Immunization and (iii) Purification of IgG

As described in chapter- 3.

(B) Extraction of GA from the samples

It was performed as described in chapter- 3.

(C) ELISA and Calibration curve for GA

All the steps of ELISA were followed as described in Chapter -3. To test the sensitivity of the assay internal standards (150 ng GA_3) were mixed with samples. For preparation of calibration curve, standards (100 - 500 ng/well GA_3) were mixed with an optimum dilution of antibodies (1:10,000) instead of samples prior to coating on the plate. All

estimations were done in triplicates and reported as mean value with ±SD. In the present study, estimated endogenous value is expressed as μg GA per organ that is equivalent to GA_3 content.

(D) Cotton ovule culture

Bolls were collected after one-day anthesis and kept under running tap water for 1-2 h. Ovules were separated out under aseptic condition. Liquid basal media was prepared according to Beasley & Ting (1973) with or without GA_3 (mg L^{-1}), GA_3+NAA (mg L^{-1}, each) and ovules acquired from ovary were floated on its surface. The cultured flasks were kept under dark condition at 35 ºC. In preliminary experiments, different concentrations of GA_3 and GA_3+NAA were used and from that optimum exogenous levels were decided.

(i) *In vitro* growth analysis

In vitro growth analysis in terms of fresh weight, dry weight and water amount was performed for cultured ovules as described in Chapter - 3. Callus formation was observed in G. Cot after 36 DPA and hence experiment was terminated after the period.

(ii) *In vitro* fiber length and seed size measurement

In vitro fiber length and seed size measurement was carried out as described in Chapter 3. Results obtained in control treatment were considered as 100% and respective increment in growth parameters was calculated for both the genotypes (Table-4).

Data analysis

Correlation coefficient was worked out between endogenous GA level and water amount during boll development. ANOVA was performed between both the genotypes to check the significant difference on their mean dry weight, fiber length, seed size and water amount with or without exogenous GA_3 application.

RESULTS

In vivo study

Endogenous GA levels

(i) Estimation of GA in ovule

In developing kernel of H-6, GA content remained low from 18-24 DPA, significant GA level was recorded during 27-39 DPA and gradually decreased in later stages (Fig. 4.1a). Maximum GA content was recorded on 33 DPA (6.28 μg seed kernel^{-1}). In G. Cot kernel lag phase was observed from 21-24 DPA, increased gradually and peaked on 33 DPA (8.22 μg seed kernel^{-1}) then declined gradually (Fig. 4.1a).

In H-6 seed coat (Fig. 4.1b), increase in GA level was recorded up to 24 DPA and decreased gradually thereafter. Maximum value (5.11 μg seed coat^{-1}) was obtained on 24 DPA. Changes in GA level in seed coat of G. Cot showed lag phase during initial period up to 9 DPA, increased and obtained a peak on 24 DPA (6.57 μg seed coat^{-1}) then decreased and stabilized in subsequent stages (Fig. 4.1b).

(ii) Estimation of GA in fiber

As shown in figure 4.1c, in H-6 fiber significant GA level was recorded from 15-36 DPA and decreased gradually after that. Maximum GA content was observed on 27 DPA (9.1 μg seed fiber^{-1}), whereas in developing fiber of G. Cot, initially GA content remained low then increased, remained higher during 21-30 DPA and declined thereafter. Peak value was recorded on 24 DPA (4.59 μg seed fiber^{-1}, Fig. 4.1c).

(**iii) Estimation of GA in boll wall and bract**

Throughout the boll developmental period, fluctuation in GA level was recorded in boll wall and bract of both the genotypes; H-6 and G. Cot (Fig. 4.2a, b). In H-6 boll wall, GA content was detected in negligible amount from 3-21 DPA and significant amount recorded from 24-39 DPA, remained absent in later stages (Fig. 4.2a). In boll wall of G. Cot, GA content increased gradually; from 3-21 DPA then fall was observed. After that GA level increased, obtained maximum value on 36 DPA (104.40 μg $boll^{-1}$) and finally declined at maturity (Fig. 4.2a).

In bract of H-6, remarkable value of GA was recorded during initial period ranging from 3-18 DPA, but decreased thereafter. After 30 DPA GA content remained absent. Maximum amount of GA recorded on 15 DPA (48.44 μg boll $bract^{-1}$, Fig. 4.2b). Whereas, GA content in G. Cot bract increased initially from 3-15 DPA, and declined thereafter. Peak value was obtained on 15 DPA (43.15 μg boll $bract^{-1}$, Fig. 4.2b).

As shown in figure 4.3a, in H-6 and in G. Cot seed GA content remained higher during 15-39 DPA and declined after that when boll opened. Moreover, in H-6, GA content remained higher throughout the seed developmental period as compared to G. Cot. Maximum amount was recorded on 27 DPA (16.65 μg seed $boll^{-1}$) in H-6 and on 33 DPA (13.07 μg seed $boll^{-1}$) in G. Cot respectively. GA level per boll was remarkably higher in H-6 than G. Cot (Fig. 4.3b). In addition, in H-6 maximum GA content recorded was 866.03 μg $boll^{-1}$ (27 DPA) while in G. Cot it was half the value of H-6; 471.36 μg $boll^{-1}$ (33 DPA, Fig. 4.3b).

In vitro growth analysis

In the present study, one-day-old ovules were cultured in liquid media (MS media) to evaluate the effect of GA_3 and its combination with auxin on dry weight, water amount, fiber length and seed size with respect to control treatment (without hormones).

In the control treatment, final seed dry weight was 21 mg seed^{-1} in H-6 and 3 mg seed^{-1} in G. Cot, while GA_3 (1 mg L^{-1}) treatment showed approximately 2 times increase in H-6 and 3 times increase in dry weight of G. Cot seed (Fig. 4.4a, b). Moreover, when media was supplemented with GA_3+NAA (1 mg L^{-1} each), 7.16 times increase in H-6 and 30 times increase in G. Cot seed dry weight was obtained (Fig. 4.4c).

Similarly, when water amount in cultured ovule was calculated, in control treatment maximum water amount was recorded as 64 mg seed^{-1} in H-6 and 20.8 mg seed^{-1} in G. Cot. Whereas with GA_3 application (1 mg L^{-1}) 7.18 and 9.37 times increase in water amount of H-6 and G. Cot seed was achieved, respectively. And with GA_3+NAA treatment (1 mg L^{-1}), 6.89 times in H-6 and 12.69 times higher water amount in G. Cot seed was obtained (Fig. 4.5).

When one-day post anthesis ovules were cultured without hormone, maximum fiber length recorded in H-6 and in G. Cot was 1.99 mm and 0.4 mm, respectively (Fig. 4.a). When ovules were cultured with GA_3 (1 mg L^{-1}) 5.7 mm length in H-6 and 5 mm length in G. Cot was obtained (Fig. 4.6b). However, with GA_3+NAA (1 mg L^{-1}) treatment 15.5 mm and 9.0 mm length were recorded in H-6 and G. Cot fiber, respectively. This result matched as 8.15 times and 22.5 times increase in fiber length in H-6 and G. Cot, respectively (Fig. 4.6c).

Changes in seed size of cultured ovule was recorded till it was stabilized. In G. Cot, seed size was measured up to 36 DPA because prolong incubation induced callus formation in the media. In control treatment, maximum size of H-6 seed was 19.0 mm^2 and it was 6.87 mm^2 in G. Cot (Fig. 4.7a). As compared to control treatment, when 1 mg L^{-1} of GA_3 was supplied to the media 5.36 times increase in H-6 and 10.29 times increase in G. Cot seed size was obtained, whereas with GA_3+NAA (1 mg L^{-1} both) application 6.42 times in H-6 and 12.94 times increase in G. Cot seed size was recorded (Fig. 4.7b, c).

In the present study our aim was to study the relationship between endogenous GA content and growth phases of seed components as well as its endogenous level in protective structures along with effects of exogenous GA_3 application on seed development. Major findings of *in vivo* and *in vitro* studies are (i) H-6 seed and boll had higher level of GA than G. Cot (ii) GA content in kernel was higher throughout the developmental period in H-6 than G. Cot, whereas the average value of GA remained higher in G. Cot seed coat till maturation period (iii) Further, long staple genotype (H-6) showed distinctly higher GA content throughout the developmental period than short staple genotype (G. Cot). (iv) *In vitro* study showed that percent promotion in all parameters studied (dry weight, water amount, seed size and fiber length) was remarkably higher in small seed than bigger seed. (v) Close correlation between water amount and GA content was obtained for all the boll components of both the genotypes.

DISCUSSION

In vivo study

In the present research work, GA contents in different boll components of two different cotton genotypes showed marked variation amongst the boll components and between the two genotypes studied (Table-1a, 1b).

As shown in figure 4.3a, when GA level was tested in seed, high yielding genotype H-6 showed distinct high level of GA than G. Cot. In addition, GA level was remarkably higher during cell elongation and DMA phases and declined at maturity. The high level of GAs found in young seeds support their role in seed development (Garcia-Martinez et al. 1997), on the other hand Groot et al. (1987) have reported that exogenous GA increased the seed weight and delayed seed dehydration of the GA deficient ga-1 mutant of tomato seed.

Comparison of GA contents between H-6 and G. Cot kernel revealed that it remained higher throughout the developmental period in H-6 than G. Cot. Decline in the bioactive GA at later phases, may be its conversion to conjugated form. From the earlier work, it is clear that preferentially during seed maturation gibberellin conjugates are often formed and relatively high levels present in maturing seeds (Hiraga et al. 1974a, b). White et al. (2000) explained in maize that GAs level declines during embryogenesis as ABA levels peak. Moreover, the distribution of hormones in different parts of seed is not uniform (Thomas et al. 1978). Average value of GA remained higher in G. Cot seed coat till maturation phase. It is interesting to note that in G. Cot seed coat GA remained higher in later stages also. There are many reports, which suggest that the seed coat play an important role in seed development (Kang et al. 1999). It has been suggested that GAs are involved in the early and late

stages of seed development (Lange 1997, MacMilan 1997, Swain et al. 1997). Data presented in this thesis suggesting that it involved in total seed development period.

The formation of seeds is intimately linked with fruit growth and development (Koltunow et al. 2002, Ozga et al. 2002). In the present study, many times higher endogenous GA content in H-6 fruit (boll) as compared to G. Cot (Fig. 4.3b) coincides with the higher weight and larger size boll. These observations are supported by the results in okra fruit where GA level in a green fruit tissue are intimately associated with cell expansion and increase in fresh weight (Koshioka and Nishijima 1996).

As shown in Table-2, close correlation between water amount and GA content was obtained for all the boll components of both the genotypes; this showed that GA may play important role in water uptake and that may be the reason for cell expansion. Further, water uptake due to GA treatment that results in cell expansion is well known (Katsumi and Kazama 1978, Miyamoto and Kamisaka 1988). Mahouachi et al. (2005) showed that water stress in citrus fruit decreased the level of GA content and increased the ABA level. It is suggested that the balance between ABA and GA content may provide the temporal control over the germination (White and Rivin 2000, Yang et al. 2001).

Further, long staple genotype (H-6) showed distinctly higher GA content throughout the developmental period studied than short staple genotype (G. Cot.) (Fig. 4.1c), this result suggests that short staple genotype may be deficient in bioactive GA which supports the earlier work of our laboratory that GA has an important role in regulation of fiber elongation (Gokani and Thaker 2002).

The involvement of gibberellins in the reproductive development of a wide variety of higher plants (Pharis and King 1985) led to the practical use of GA applications in fruit production. Aim of the study was to evaluate the role of GA in total boll development, so endogenous GA levels were also measured in boll wall and bract. In H-6 boll wall, GA level was higher than G. Cot while in bract GA level remained almost equal or higher in G. Cot. Why and how GA is more translocated/ stored in seed coat and bract in G. Cot and in kernel, fiber and boll wall in H-6 is not clear.

These observations lead to two probabilities (i) higher GA level is responsible for the long fiber length and (ii) higher GA level participates in controlling seed size and finally high yield. If these probabilities are true than exogenous application should promote fiber length in short staple genotype as well as seed size in small seeded genotype.

Early studies involving exogenous application of various hormones to developing fruit indicate their roles at different stages of fruit development (Crane 1964). However, the fact that the effects of exogenous application may not be a true representation of their cellular role has led to an examination of the endogenous levels of hormones during fruit development and ripening. Thus, Trewavas (1991) suggested that an isolated system is required to compare and study the response of endogenous and exogenous hormonal concentrations and physiological response. To address this, *in vitro* experiment was conducted.

In vitro study

In the present study, initial difference in water amount observed in control treatment was nullified with application of GA_3 and GA_3+NAA (Fig. 4.5 Table-3a). Thus, GA_3 level can control water amount. When GA_3+NAA (1 mg L^{-1}) was added to the media remarkable increase in dry weight was observed in both the genotypes with respect to controls (Fig. 4.4), though GA_3 alone (1 mg L^{-1}) was not effective. There was no remarkable increase in dry weight but clear increase in seed size could be correlated with the simultaneous increment in water amount (Table- 3b) that proves GA play an important role in water uptake. Further, percent promotion in all parameters studied was remarkably higher in small seed than bigger seed (Table- 4). This result suggests that small seed may be deficient in their GA level and therefore, showed significant promotion when media was supplemented with GA_3.

Gialvalis and Seagull (2001) and many other workers suggest that GA and auxin treatments induce more epidermal cells that differentiate into fibers. In auxin dependent and independent suspension culture of cotton ovules, gibberellin induced increase in cell elongation has been reported (Beasley et al. 1974, Davidonis 1990). With GA_3 treatment significant percent promotion was observed in G. Cot fiber length than H-6 (Table-4). When the ovules were grown on the media containing both GA_3+NAA additive effect in an increase in fiber length was observed in both the genotypes. These results support the previous conclusion that auxin plays a very important role in fiber elongation (Naithani et al. 1982). More promotion was observed in short staple (G. Cot) than long staple (H-6) when GA_3 or GA_3+NAA was supplemented to the media (Fig. 4.6, Tables- 3a, 4). Earlier work of Gokani and Thaker (2002) indicated from culture experiment that GA_3 has an important role in regulation of fiber elongation. These *in vivo* and *in vitro* results indicated that long staple has enough endogenous level of this hormone.

In addition, statistical analysis for control treatment showed that there was significant difference between the genotypes for water amount ($p \leq 0.001$), dry weight ($p \leq 0.01$), fiber length ($p \leq 0.001$) and seed size ($p \leq 0.001$) (Table- 3a). The differences in water amount, fiber length and seed size became insignificant with GA_3 application (Table- 3a) and thus support the hypothesis proposed in this work. Moreover, data presented from endogenous GA estimation and exogenous application on two cotton genotypes varying in their staple length and seed size suggest that GA is involved in regulation of fiber elongation and seed size determination. The present study indicates that GA and auxin act synergistically or additively in stimulating cell elongation (Ross et al. 2003).

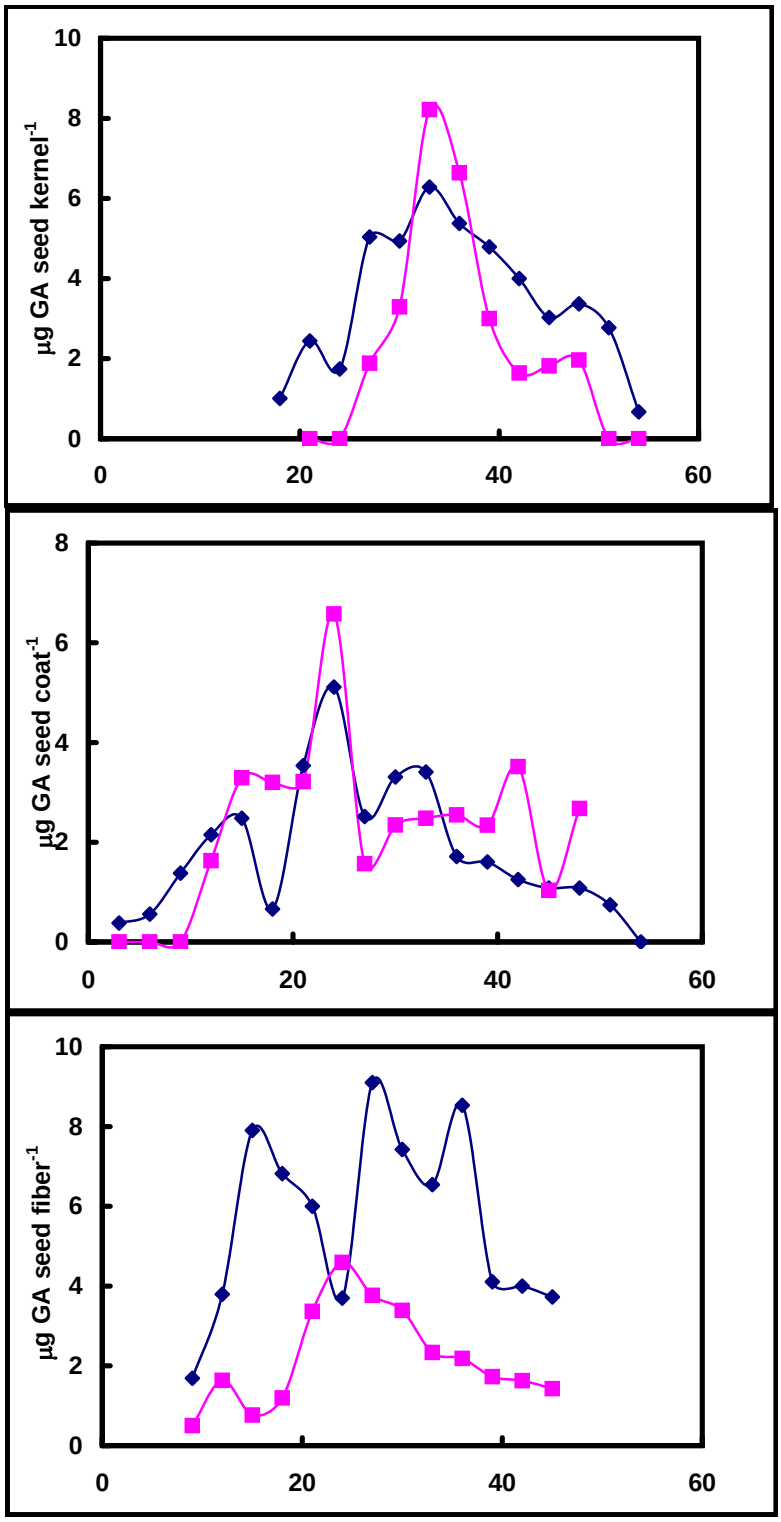


Fig-1:Changes in free GA content in seed kernel (a), seed coat (b) and seed fiber (c) in two cotton genotypes H-6 (♦) and G. Cot -13 (■)

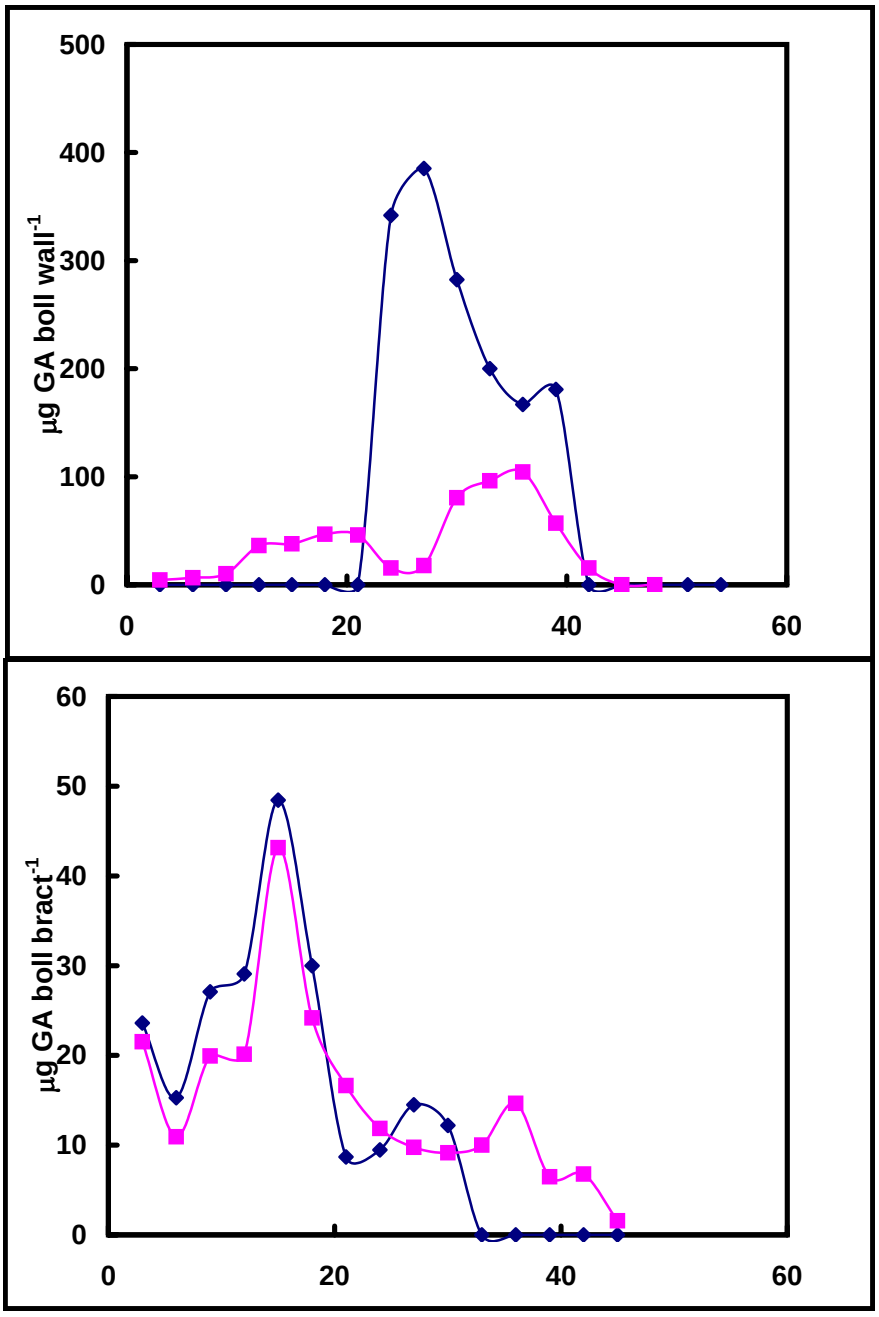


Fig-2: Changes in free GA content in boll wall (a) and bract (b)in two cotton genotypes H-6 (◆) and G. Cot -13 (■)

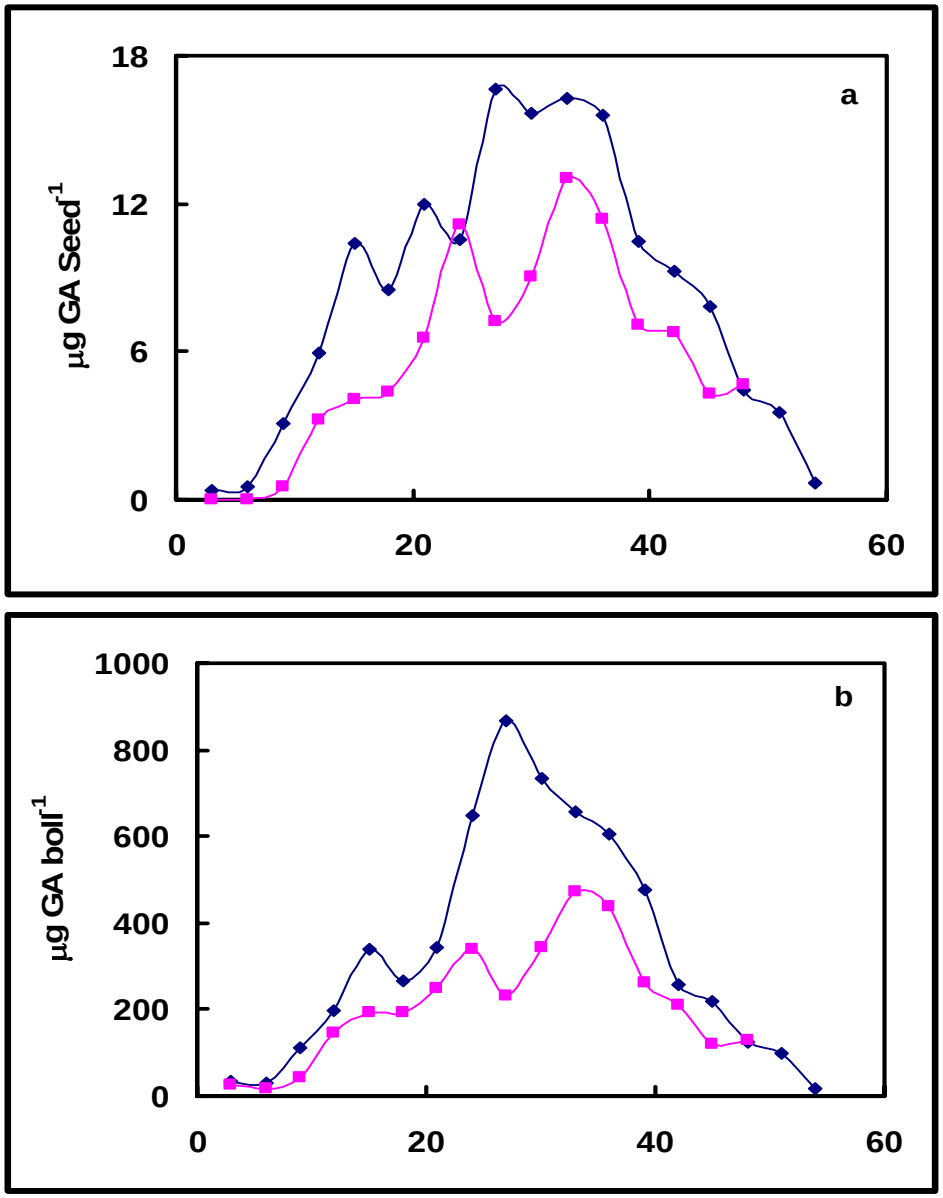


Fig-3: Changes in free GA content in seed (a) and boll (b) of two cotton genotypes H-6 (♦)and G. Cot -13 (■)

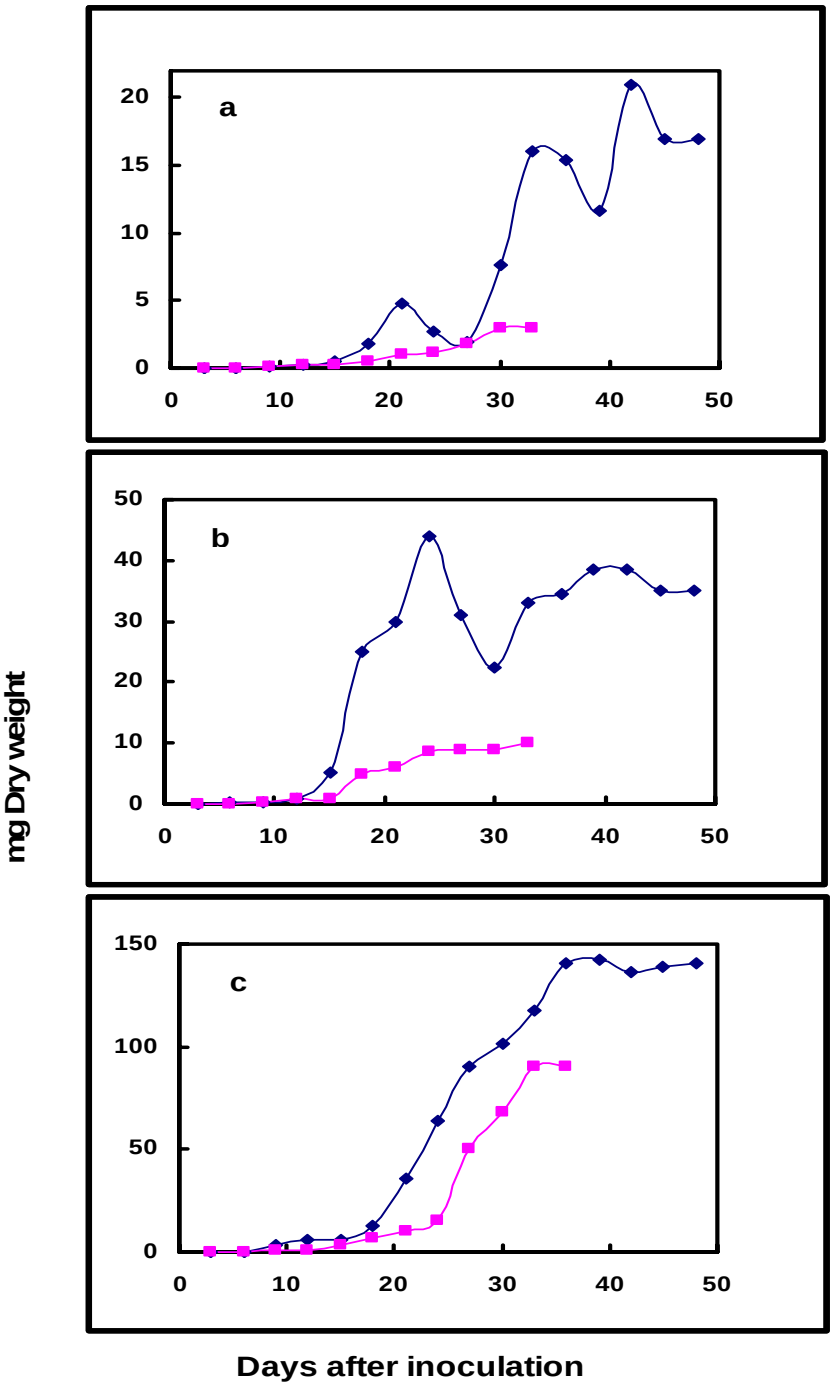


Fig-4: Changes in dry weight without hormone (a), with GA (1mg L^{-1}) (B) , GA+NAA (1mg L^{-1}) (c)

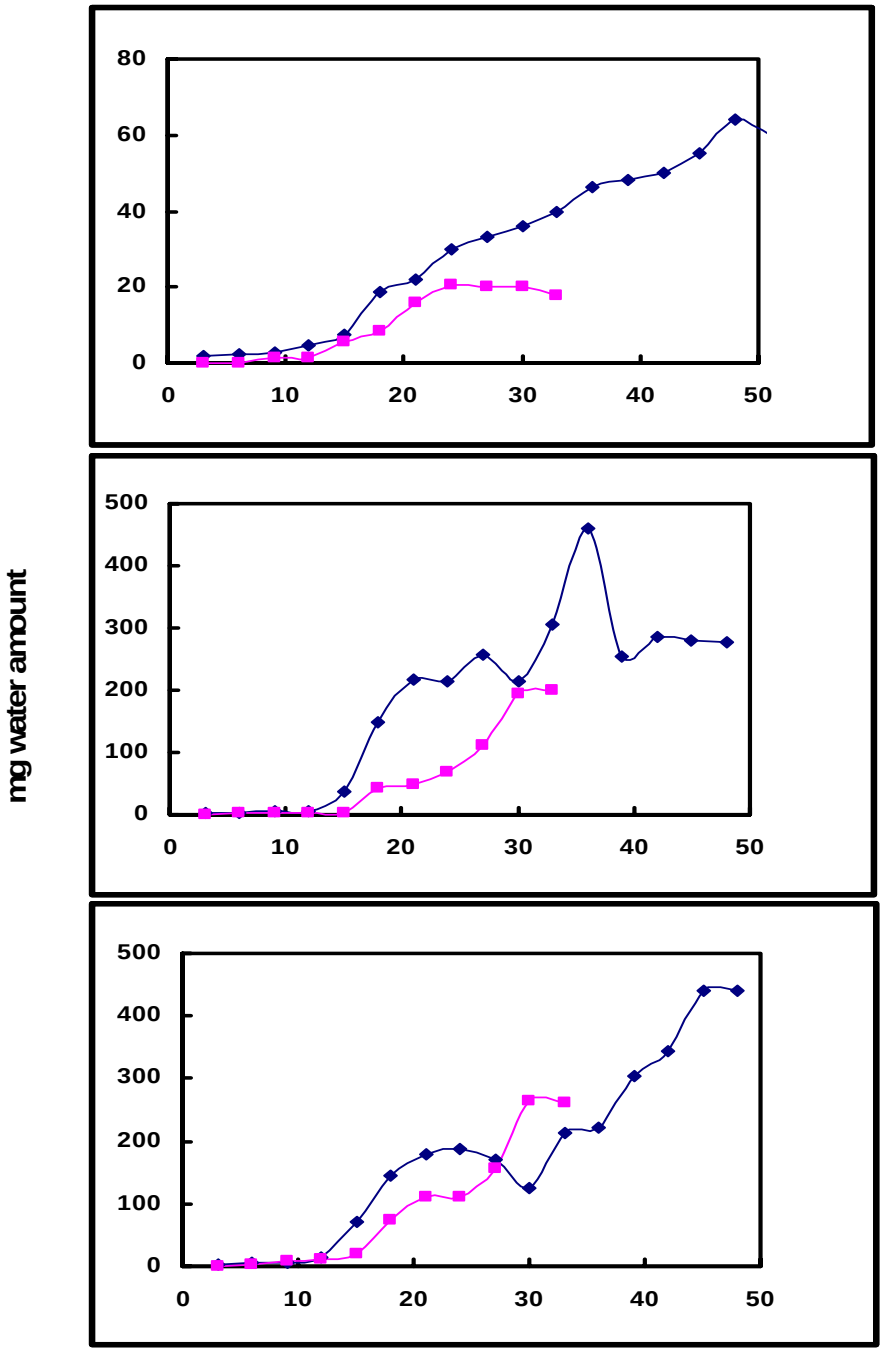


Fig- 5 Changes in water amount without hormone (a), with GA (1mg L^{-1}) (B) , GA+NAA (1mg L^{-1}) (c)

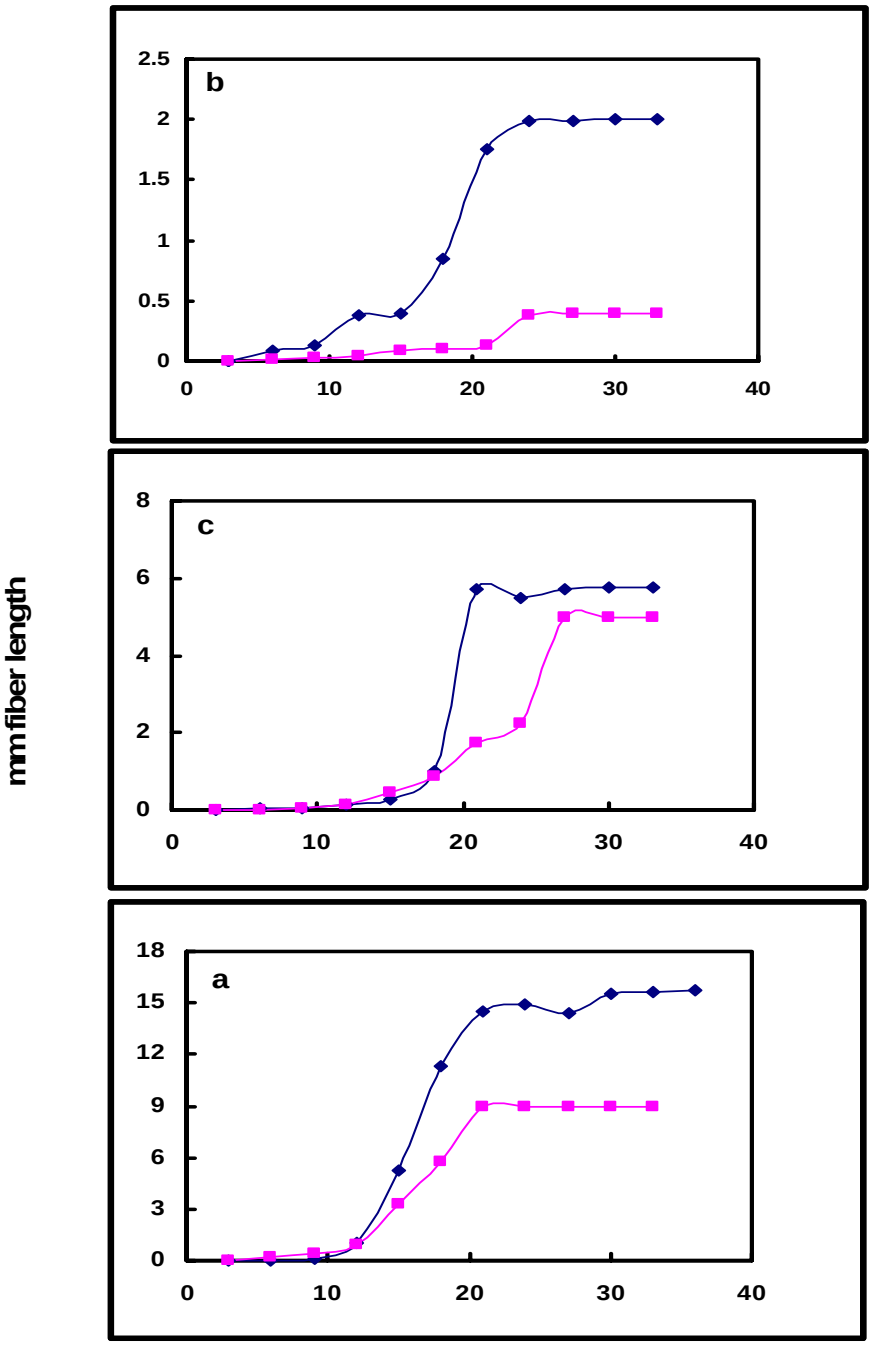


Fig-6: Changes in fiber length without hormone (a), with GA (1mg L^{-1}) (B) , GA+NAA (1mg L^{-1}) (c)

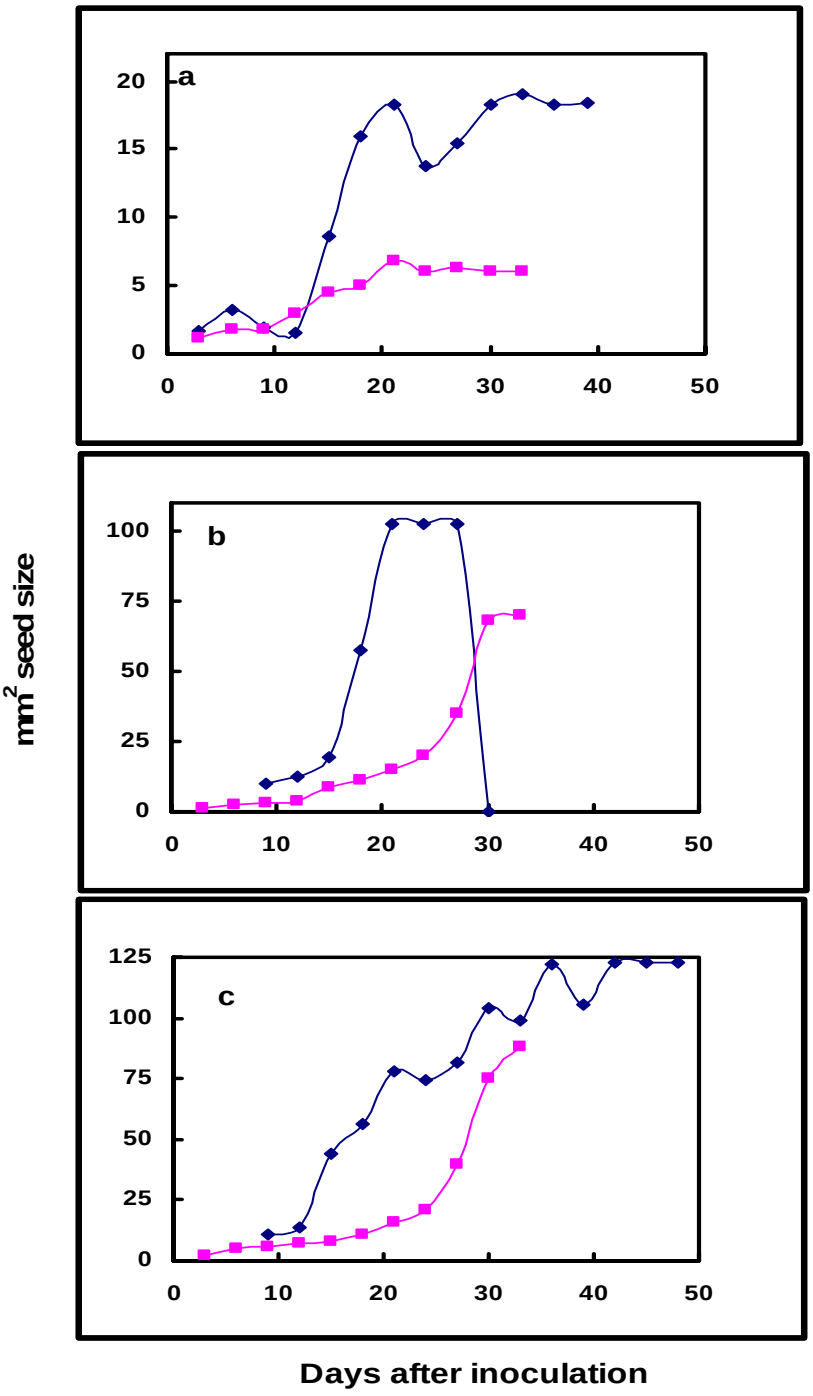


Fig- 7: Changes in seed size without hormone (a), with GA (1mg L^{-1}) (B) , GA+NAA (1mg L^{-1}) (c)

Table-1a. ANOVA amongst all boll components of two cotton genotypes for endogenous GA content

Genotypes	GA	
	df	F- value
H-6	4,72	2.498***
G. Cot	4,69	2.513***

Significance level p ≤ 0.001=*, p ≤ 0.01=**, p ≤ 0.1 =***

Table-1b. ANOVA between two cotton genotypes for their endogenous GA content in boll components

Source	GA	
	df	F- Value
Kernel	21	1.675
Seed coat	32	0.529
Fiber	24	20.756***
Boll wall	27	3.426**
Bract	29	2.240*

Significance level p ≤ 0.001=*, p ≤ 0.05=**, p ≤ 0.1 =***

Table- 2: Correlation between water amount and endogenous GA content in all boll components in two cotton genotypes: H-6 and G. Cot

Source	H-6	G. Cot
	r	r
Kernel	0.625**	0.632**
Seed coat	0.668***	0.66**
Fiber	0.803***	0.883***
Length	0.439*	0.504*
Boll wall	0.561**	0.608**
Bract	0.763***	NS

Significance level p ≤ 0.001=*, p ≤ 0.01=**, p ≤ 0. 1=*,**

NS= non significant

Table-3a. ANOVA between two cotton genotypes for water amount, dry weight, fiber length and ovule size of cultured ovules supplemented with GA_3/ GA_3+NAA/ without hormone

Water amount	Control	GA_3	GA_3+NAA
F-value	4.225	4.241	4.241
p-value	0.006	0.01	0.1
df	27	26	26
Dry weight	Control	GA_3	GA_3+NAA
F-value	7.417	14.129	5.925
p-value	0.01	0.001	0.02
df	26	26	26
Fiber length	Control	GA_3	GA_3+NAA
F-value	10.237	0.627	1.862
p-value	0.01	0.4 (NS)	0.1
df	21	21	21
Seed size	Control	GA_3	GA_3+NAA
F-value	10.640	2.364	9.280
p-value	0.003	0.1	0.005
df	23	19	26

Table-3b. Correlation between *in vitro* water amount and seed size with or without hormone treatments in two cotton genotypes: H-6 and G. Cot

Treatment	H-6	G. Cot
	r	r
Control	0.893***	0.922***
GA_3	0.898***	0.990***
GA_3+NAA	0.765**	0.978***

Significance level p ≤ 0.001=*, p ≤ 0.01=**, p ≤ 0. 1=***

Table-4. **Percent promotion in fiber length, dry weight, water amount and seed size of cultured ovules with GA_3 and GA_3 +NAA treatments in two cotton genotypes: H-6 and G. Cot**

Treatment	Fiber length		Dry weight		Water amount		Seed size	
	H-6	G. Cot	H-6	G. Cot	H-6	G. Cot	H-6	G. Cot
GA_3	286.43	1250.0	209.52	333.33	718.75	1000.0	539.47	1029.4
GA_3+NAA	778.89	2250.0	676.19	3000.0	689.06	1320.0	642.11	1294.1

Chapter V

Zeatin

SUMMARY

Involvement of zeatin in boll development was studied. From the growth analysis variation in dry weight, fiber length and final yield in boll components was observed between two cotton genotypes (H-6 and G. Cot). At different physiological developmental stages from the day of anthesis till boll opened, endogenous free and conjugated zeatin levels were measured. Zeatin content remained higher during cell division and expansion phases in kernel, seed coat in H-6 whereas in G. Cot its level was low and also for short period. In H-6 boll wall free zeatin content was higher than that of G. Cot while in case of bract, its level was noted for longer period in G. Cot. While in fiber it remained stable throughout the developmental period in both the genotypes. Endogenous zeatin level indicated its role in cell division, cell expansion and dry matter accumulation but may not have role in fiber elongation**.** *In vitro* study suggested that initial difference specifically in dry weight and seed size was nullified by KiN treatment. Potential role of zeatin in cotton boll development is discussed.

Abbreviations: CKs- Cytokinins, DHZ- Dihydrozeatin, KiN – Kinetin, BAP – 6, Benzyl amino purine, 2ip- 2, iso pentyl

INTRODUCTION

Cytokinins (Cks) are well recognized as one of the major group of plant hormones (Momtaz 1998), regulate various developmental process including cell division, root and shoot growth and branching, chloroplast development, leaf senescence, stress response, pathogen resistance and sink strength (Mok and Mok 2001, Schmulling 2002, Kakimoto 2003). All native cytokinins are derivatives of adenine bearing an isoprenoid, isoprenoid-derived or aromatic substituent at the N^6- position of the purine ring (Gaudinova et al. 2005). 2ip and zeatin are isoprenoid type of cytokinins whereas KiN and BA are of aromatic type (Nikolic et al. 2006).

Zeatin, the first naturally occurring cytokinin (Letham 1964, Letham and Palni 1983) promotes various developmental processes such as cell expansion, breakdown of storage material, water uptake, increase in fresh weight (Chen and Leisner 1985, Pospisilova et al. 2000) and flower and fruit set (Weaver et al. 1965, Lilove and Andonova 1976). Van Staden and Dimalla (1978) showed that high endogenous zeatin levels break dormancy in potato tubers. In general, actively dividing tissues *i.e.* roots or shoot apices have higher zeatin activity than the mature tissues (Letham and Williams 1969, Nagata 1972). Extensive study of zeatin and cell division suggested its role in cell division and cell expansion in *in vivo* (Short and Torrey 1972a, b) and *in vitro* systems (Miller 1965, Fosket and Torrey 1969).

Numerous effects of phytohormone on flower and fruit development have been reported (Wafler and Meier 1994, John 1995). It has become clear that zeatin plays an important role in inducing fruit set in several plants (Letham 1967, Crane 1969). The high cytozeatin activity of fertilized fruits may be considered as a result of strong sink activity. In tomato plants, it was

observed that less zeatin was metabolized by parthenocarpic fruits than seeded fruits and fruit flesh than by isolated seed (Palmer et al. 1981). From the exogenous application of plant growth regulators to the mango fruits it is assumed that auxin, cytokinin and GA were involved in fruit development. Lack of any of the regulators may lead to reduction in fruit set (Chen 1983).

Cytozeatin are known to occur in seeds and seed components (Lorenzi et al. 1978). Zeatin, zeatin riboside and zeatin ribotide have been reported in developing seeds and receptacles of apple fruitlets (Letham and Williams 1969), watermelon seeds (Prakash and Maheshwari 1970), *Curcubita pepo* seeds (Gupta and Maheshwari 1970), lupin seeds (Koshimizu et al. 1967) and coconut endosperm (Letham 1968). Further, it has been recorded that zeatin riboside or zeatin content became low at the end of maturation while at the beginning of seed development its level was high (Musatenko et al. 2004) suggesting their involvement in the physiology of seed development. Cytokinin releases seed dormancy in contrasts with ABA inhibition of germination (Pospisilova et al. 2000).

Zeatin acting both in synergy and antagonism with other plant hormones, influence a wide range of events during plant growth. The balance between auxin and cytozeatin controls a wide range of processes in plant development including the formation of roots, shoots and callus tissue *in vitro* (Skoog and Miller 1957, Coenen et al. 2003), the out growth of shoot axillary buds (Sachs and Thimann 1967) and the formation of lateral roots (Whightman et al. 1980, Hinchee and Rost 1986). Tobacco and soybean callus require exogenous zeatin for cell proliferation *in vitro* in the presence of auxin.

Plant responses to CKs are often judged from their response to exogenously applied CKs. However, when CKs are exogenously applied to plants, it is necessary to take into consideration that they can increase the content of endogenous CKs by their uptake and promotion of CK biosynthesis. On the other hand; they can increase cytokinin oxidase activity that results in CK degradation (Hare et al. 1997) or converted in to inactive form. Glycosyl conjugates of zeatin are found important for storage and provide protection against degradative enzymes (Martin et al. 1999). Yang et al. (2001) showed that KiN has no important role in fiber development. However, little is known about its effect on cotton seed development. In the present study, throughout the developmental period endogenous free as well as conjugated zeatin content was measured from all the boll components. To check the effect of exogenous kinetin on seed development in terms of dry weight, water amount, seed size and fiber length *in vitro* study was performed.

MATERIALS AND METHODS

(A) Raising of antibodies against zeatin

(i) Preparation of zeatin- BSA/Casein conjugate

Zeatin-BSA conjugation was prepared by following the method described by Weiler (1980). For preparation of zeatin-BSA conjugate, 10.3 mg zeatin was suspended in 1 ml of methanol and 5 ml of 0.01M Na_2SO_4 was added over a period of 7 min. The solution was stirred for another 13 min. And 0.3 ml of 0.1M ethylene glycol (30 μmol) was added. After 5 min, the reaction mixture was added drop wise to a stirred solution of 110 mg BSA in 5 ml distilled water. During the addition of the oxidized riboside the pH was kept constant between 9.2 to 9.4 by addition of 5% K_2CO_3. After 60 min solid $NaBH_4$ (5 mg) was added and its repeated addition was done after 40 min. Then, pH was adjusted to 6.5 by the addition of 1M acetic acid and the solution was stirred for another 2 h. To avoid the reaction with injected protein zeatin-casein conjugate was prepared (same as zeatin-BSA conjugate). The conjugate was purified by dialysis against water for 3 days at 4 ºC and stored at 0 ºC.

(ii) Immunization and (iii) Purification of IgG

As described in chapter -3.

(B) Extraction of zeatin from the samples

It was performed as described in chapter- 3.

(C) Indirect ELISA and calibration curve for zeatin

A calibration curve was prepared for zeatin showed linear relationship with concentration in the range of 25-250 ng/well. Dilution of samples and addition of internal standard was done in this calibration range. All the estimations were done in triplicates and reported as mean value with ±SD.

(D) Cotton ovule culture

One-day post anthesis bolls were collected from both the genotypes. They were kept under running tap water for 1-2 h and ovules were separated out under aseptic condition. Liquid basal media was prepared according to Beasley and Ting (1973). Media was supplemented with or without KiN (1mg L^{-1}), GA+NAA+KiN (1mg L^{-1} all) and GA+NAA (1mg L^{-1} both) and ovules were floated on its surface. The cultured flasks were kept under dark condition at 35 ^{o}C. In the preliminary experiments, different concentrations of KiN, GA and NAA were used and from that optimum exogenous level were decided.

(i) *In vitro* growth analysis

In the present study, *in vitro* growth analysis was performed for cultured ovules in terms of fresh weight, dry weight and water amount as described in Chapter -3. Callus formation was observed in G. Cot after 36 days and hence experiment was terminated after the period.

(ii) *In vitro* fiber length and seed size measurement

In vitro fiber length and seed size measurement was carried out as described in Chapter 3.

RESULTS

In vivo study

Endogenous zeatin level

(i) Estimation of zeatin in ovule

Endogenous zeatin level in developing seed kernel of both H-6 and G. Cot showed a gradual increase from 20-36 DPA. In H-6, after a decline up to 42 DPA the zeatin level was stabilized till maturity (Fig. 5.1a) whereas it declined gradually in G. Cot. Maximum value was obtained on 36 DPA in both the genotypes, H-6 (3.42 μg zeatin seed kernel^{-1}) and G. Cot (3.03 μg zeatin seed kernel^{-1}). The conjugated zeatin level in H-6 (2.38 μg zeatin seed kernel^{-1}) and G. Cot (1.96 μg zeatin seed kernel^{-1}) kernel increased up to 33 DPA and declined thereafter (Fig. 5.1d) but in G. Cot, the level remained lower than that of H-6.

In H-6 seed coat, free zeatin content increased rapidly up to 15 DPA (2.1 μg zeatin seed coat^{-1}) then remained negligible in subsequent periods (Fig. 5.1b). While in case of G. Cot seed coat, free zeatin level was recorded only up to 6 DPA (Fig. 5.1b). Conjugate zeatin level increased up to 18 DPA in H-6 and 24 DPA in G. Cot seed coat and declined gradually thereafter. Initially, H-6 seed coat showed higher conjugated zeatin content than that of G. Cot (Fig. 5.1).

(ii) Estimation of zeatin in fiber

In developing H-6 fiber, free zeatin amount increased gradually during initial phase (up to 15 DPA). However after 15 DPA, zeatin level remained stable for subsequent periods. As shown in figure 5.1c, free zeatin level in G. Cot fiber remained much higher than H-6 throughout the developmental period. Maximum value was recorded on 15 DPA in H-6 (2.81 μg zeatin seed fiber^{-1})

and on 27 DPA (4.77 μg zeatin seed fiber^{-1}) in G. Cot. Contrary to free zeatin level, conjugated zeatin level in H-6 fiber remained low up to 15 DPA then increased up to 36 DPA and declined thereafter while in case of G. Cot fiber, it was higher up to 18 DPA and declined thereafter (Fig. 5.1f).

(iii) Estimation of zeatin in boll wall and bract

Free and conjugated zeatin level was also measured in boll wall of H-6 and G. Cot throughout the boll development period (Fig. 5.2a, c). Initially, in H-6 boll wall lag phase was observed from 3-9 DPA then gradually increased up to 27 DPA (358.64 μg zeatin boll wall^{-1}). After 33 DPA, free zeatin content was at negligible level. In G. Cot boll wall, free zeatin level was noted during 30-39 DPA. In subsequent period it was at negligible level (Fig. 5.2a).

In H-6 boll wall, conjugated level was higher than G. Cot throughout the development period. In H-6 boll wall, conjugated zeatin level increased rapidly up to 18 DPA then decreased and stabilized up to 39 DPA. Maximum value was obtained on 39 DPA (74.22 μg zeatin boll wall^{-1}) (Fig. 5.2c). While in G. Cot, conjugate zeatin level increased up to 21 DPA (335.88 μg zeatin boll wall^{-1}) and declined gradually in subsequent periods (Fig. 5.2c).

In H-6 bract, free zeatin level increased initially and achieved maximum value on 9 DPA (28.15 μg zeatin boll bract^{-1}); subsequently decreased to 1.50 μg on 15 DPA and negligible level was recorded thereafter. Estimation of endogenous zeatin level in G. Cot bract showed that free zeatin level increased up to 24 DPA (31.53 μg zeatin boll bract^{-1}) and declined thereafter (Fig. 5.2b). As shown in figure 5.2d, in H-6 bract conjugate zeatin content increased up to 15 DPA then declined gradually. While in G. Cot bract, conjugate zeatin content remained almost constant throughout the boll developmental period (Fig. 5.2d).

Free zeatin content per boll calculated for both the genotypes showed that during initial period (up to 27 DPA), it was many fold higher in H-6 boll as compared to G. Cot. After 30 DPA, free zeatin content declined gradually in H-6 (Fig. 5.3b). While in case of G. Cot boll, free zeatin level remained low up to 9 DPA, increased up to 39 DPA and declined in subsequent periods (Fig. 5.3b). Maximum free zeatin level was recorded on 27 DPA (461.33 μg) and 39 DPA (199.52 μg) in H-6 and G. Cot, respectively. Zeatin content at seed level (kernel + seed coat + fiber) from the two genotypes tested showed that during initial period it was higher in H-6; after 21 DPA it was noted distinctly higher in G. Cot seed (Fig. 5.3a).

In vitro growth analysis

Effect of exogenous hormonal level on one-day post anthesis ovules were checked in terms of dry weight, water amount, fiber length and seed size. Results obtained in control treatment were considered as 100% and respective increment in growth parameters was calculated for both the genotypes. In control treatment (without hormones) dry weight was restricted up to 21 mg in H-6 and 3 mg in G. Cot, respectively (Fig. 5.4a). When media was supplemented with 1 mg L^{-1} KiN, final dry weight achieved was 32 mg in G. Cot seed (1067% increment) while in H-6 it was obtained only 25 mg (147% increment) (Fig. 5.4b). With GA_3+NAA+KiN (1 mg L^{-1} all) application, both genotypes showed remarkable promotion in dry weight. In H-6, final dry weight was 94.3 mg whereas in case of G. Cot it was 62.2 mg (Fig. 5.4c). Moreover, GA_3+NAA treatment was found to be more promotive for dry weight increment (136.6 mg in H-6 and 90 mg in G. Cot, Fig. 5.4d). Additionally, more promotion was obtained in G. Cot as compared to H-6 (Table-2).

When KiN was added with auxin and GA_3 or separately, variation in water uptake was observed. In control treatment, final water amount recorded was 64 mg in H-6 and 20.8 mg in G. Cot, respectively (Fig. 5.5a). When media was supplemented with 1 mg L^{-1} KiN, maximum water amount was 210 mg in H-6 and 90.0 mg in G. Cot respectively. These results can be expressed as 350% increase in H-6 and 454.5% increase in G. Cot as compared to control treatment (Fig. 5.5b). While, when GA_3+NAA+KiN was supplemented, maximum water amount recorded was 473.33 mg in H-6 and 120 mg in G. Cot, respectively (Fig. 5.5c). With GA_3+NAA, seed showed 441.3 mg water amount in H-6 and 264 mg in G. Cot. Thus, GA_3+NAA treatment was found to be more promotive for G. Cot (Fig. 5.5d).

In control treatment, fiber initiation began at 3 DPA, after which fiber cell entered into elongation phase. In H-6 control, final fiber length was nearly 2 mm followed by 0.4 mm in G. Cot (Fig. 5.6a). With KiN treatment, final fiber length in H-6 was 5.25 mm and 2.2 mm in G. Cot, respectively (Fig. 5.6b). Addition of GA_3 and auxin showed 787.5% promotion in H-6 and 2250% in case of G. Cot. With GA_3+NAA treatment, maximum length was 15.75 mm in H-6 and 9 mm in G. Cot (Fig. 5.6d). Thus, with exogenous application, more promotive results were observed for G. Cot (Table-2). Fiber length was decreased when KiN was supplied along with GA_3 and auxin. The application of GA_3+NAA+KiN (1 mg L^{-1} all) showed 550% promotion in H-6 and 1938% in G. Cot fiber. At the time, the corresponding length was 11 mm in H-6 and 7.75 mm in G. Cot, respectively (Fig. 5.6c).

In control treatment, final seed size remained 19 mm^2 and 6.87 mm^2 in H-6 and G. Cot, respectively (Fig. 5.7a). When media was supplemented with 1 mg L^{-1} KiN, final seed size was 27 mm^2 in H-6 and 36 mm^2 in G. Cot (Fig.

5.7b). Whereas GA_3+NAA showed final seed size 123.17 mm^2 in H-6 and 88 mm^2 in G. Cot (Fig. 5.7d). As shown in figure 5.7c, GA_3+NAA+KiN treatment was found to be most effective for seed size with respect to other hormonal combinations. When gibberellin (GA_3), auxin (NAA) and kinetin were applied together, final seed size was 200 mm^2 in H-6 and 130 mm^2 in G. Cot that was 1089% promotion in H-6 and 2167% promotion in G. Cot. Looking to the results, it is clear that more promotion was achieved in G. Cot (small seed) than H-6 (big seed).

DISCUSSION

In vivo analysis

In H-6 kernel, steep increase in free zeatin content during cell division and cell elongation phases (18-36 DPA) was observed. After a slight decline, it was stabilized during dry matter accumulation (36-45 DPA) and maturation phases (45-54 DPA). Comparison of endogenous zeatin level in seed kernel revealed that the H-6 kernel has higher free zeatin content in later stages also. While in case of G. Cot kernel, after cell elongation phase (36 DPA) a gradual decline in free zeatin level was observed (Fig. 5.1a). Higher zeatin level for longer period of development may facilitate superior growth of kernel in H-6 than G. Cot. Many workers have suggested the involvement of cytokinin in cell division and cell expansion (Ravishanker et al. 1995, Taylor and Cowan 2001, Iqbal et al. 2006). Daskalova and his co-workers have proposed (2007) that increase in CK concentration at the beginning of seed development would stimulate cell division, enhance seed size, grain filling and hence seed yield. Additionally, Gaudinova et al. (2005) proved in pea leaves that zeatin reductase enzyme convert the active zeatin into DHZ, which is resistant to cytokinin oxidase (CKX) and so preserve the high activity of zeatin. However, there was no remarkable difference in conjugated zeatin content in kernel of both the genotypes (Fig. 5.1d).

Though much research is carried out on the seed development but very little is known about the endogenous control of seed coat development. Looking to the results of zeatin estimation in seed coat, H-6 seed coat has rapid increment in free zeatin level during initial phase (cell division and cell expansion phases, 3-15 DPA), at this time rate of water uptake was also found to be higher (Chapter-2: Fig. 2.9b). Cytokinins promote water uptake and

increase fresh weight is well known (Chen and Liesner 1985). Nooden et al. (1985) showed in soybean that increasing zeatin content in seed coat increased the water imbibition. Contrary to H-6, G. Cot seed coat showed almost negligible level of free zeatin. Additionally, in H-6 seed coat conjugated zeatin level increased up to 18 DPA (during cell division phase); and declined thereafter. At the same time free zeatin content was increased rapidly in H-6 kernel tissue suggesting transport of zeatin from seed coat to kernel tissue. Though the grain-sink potential is genetically determined factor but is also a matter of competition for assimilates and space created by number of seed coat cells. The negligible level of free zeatin during cell division and expansion phase in G. Cot seed coat may restrict the seed size. Gupta et al. (2003) indicated that in wheat, genotype with larger grains possess higher cytokinin like substances.

Thomas et al. (1978) suggested that the distribution of hormones in different parts of seed is not uniform. Cotton fibers are single celled outgrowth from individual epidermal cells on the outer integument of the ovule. In the present study, G. Cot fiber showed higher zeatin content (free) than H-6 throughout the developmental phases (Fig. 5.1c). As longer staple genotype H-6 has less free zeatin content and short staple genotype G. Cot has higher level of free zeatin suggesting that endogenous zeatin has no role in fiber length determination. Yang et al. (2001) has reported that kinetin has no effect on dry matter or cellulose accumulation in fibers. Beasley (1973) explained that exogenous kinetin promotes an increase in size of unfertilized ovules yet does not induce fiber growth.

The present work suggests that distribution of hormones at different physiological stages is a limiting factor in cotton seed development. The *in vivo* study lead to conclude that compared to G. Cot; higher zeatin content in H-6

seed coat and kernel may permit bigger seed size and higher dry weight per seed. According to the “competing sinks” hypothesis fruit growth can be limited with concentration of nutrients as they are needed for the building of new tissues and that the higher concentration of hormones in seed are necessary in order to create a strong sink. Cytokinins have been implicated in such a hypothesis and it has been demonstrated that sugars and amino acids can be transported to regions of high cytokinin levels (Mothes 1961, Luckwill 1977). H-6 boll wall has higher level of free zeatin content than G. Cot during initial period (up to 27 DPA) lead to conclude that higher levels of zeatin in H-6 boll wall allow more spatial growth in H-6 and thus affect the final yield.

In addition, in H-6 bract free zeatin level was higher during initial period of boll development (up to 12 DPA); it is interested to note that bract zeatin level started to decline when boll wall started to accumulate higher amount of zeatin (Fig. 5.2b). While in case of G. Cot, bract remained green for longer time till boll opening. This observation matched with endogenous free zeatin level, which remained higher till 39 DPA (when boll opened). Zaffari et al. (1998) observed in banana leaves that the endogenous cytokinin has direct correlation with chlorophyll synthesis.

In the present study, all developing boll components of two cotton genotypes (H-6 and G. Cot) varying in their seed index and final fiber length showed marked variation in their endogenous zeatin content at different growth phases (Figs. 5.1, 5.2). As shown in figure 5.3, H-6 boll has many folds higher amount of free zeatin in early phase of boll development than G. Cot that may be responsible for final boll growth in these genotypes. Endogenous estimation of free and conjugated zeatin level in seed and protective components match with the earlier observation of endogenous auxin and GA estimation (chapter-3 and 4) in different boll components; hereby indicating the role of promotory hormones in fruit development.

In vivo study suggesting hereby that endogenous zeatin content has correlation with cell division, expansion and dry matter accumulation. However, endogenous zeatin content has no influence on the fiber length. To exploit the relationships between kinetin (synthetic CK) and seed dry weight, water amount, seed size and fiber development (elongation); *in vitro* experiments were performed.

In vitro analysis

The *in vitro* culture of fertilized cotton ovule enables one to assess the role(s) of different plant growth regulators. Control results were considered as 100% and respective increment with treatments was calculated for all studied parameters (Table-2). In the present study, initial difference in dry weight observed in control treatment was nullified with exogenous application of (1 mg L^{-1}) kinetin (Fig. 5.4, Table-1). Additionally, GA_3+NAA+KiN (1 mg L^{-1} each) showed further promotion in dry weight of seed in both the genotypes though G. Cot showed higher promotion (2000%) than H-6 (502%) (Fig. 5.4c, Table-2). However, maximum dry weight was obtained with GA_3+NAA (1 mg L^{-1} each). This, support the earlier results of chapter 3 and 4 that GA_3 and NAA play an important role in dry matter accumulation. Moreover, many times higher percent promotion in G. Cot suggests that G. Cot seed is deficient in endogenous cytokinin level and thus support the results of *in vivo* experiment.

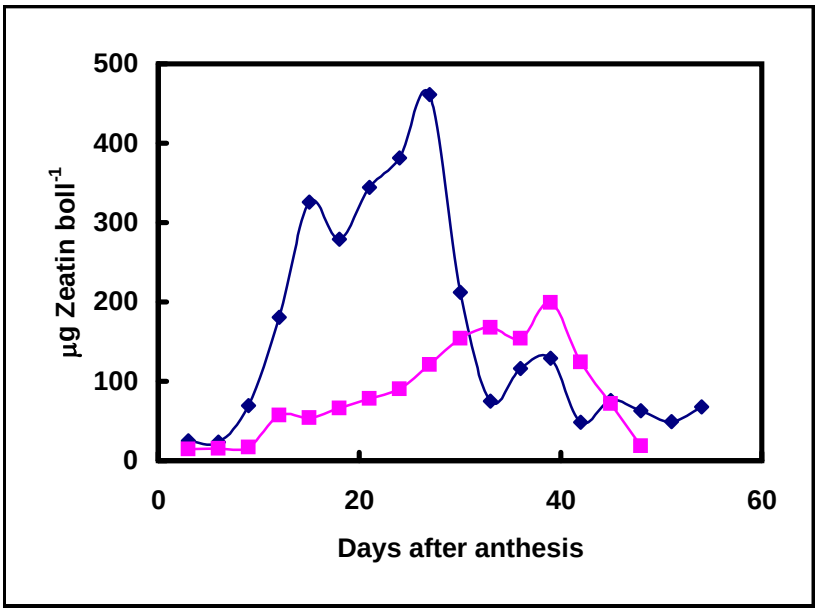
Similar to dry weight when water amount was calculated; it was clear that GA_3+NAA+KiN was more effective than KiN alone. However, GA_3+NAA treatment showed maximum water accumulation in both the genotypes. There was close correlation between dry weight and water amount with

exogenous treatments where kinetin applied alone (r= 0.961 in H-6 and r= 0.976 in G. Cot) or with combination of GA_3 and NAA (r= 0.806 in H-6 and r= 0.895 in G. Cot). As shown in Table-3, similar kind of correlation was observed between water amount and seed size in both the genotypes. Thus, there is synergistic relationship between auxin, gibberellin and cytokinin. Many workers have accepted the concept that plant growth is mediated through the balance of many phytohormones (Momtaz et al. 1998, Patel and Thaker 2007). Skoog and Miller (1957) have demonstrated a clear interaction between auxin and cytokinin with regard to organogenesis in plant tissue cultures. Nishinari and Syono (1980) had reported similar results in soybean cell culture.

With KiN treatment, nearly about 5 times increment in G. Cot fiber length was observed as compared to control while with GA_3+NAA+KiN application G. Cot fiber length was nearly about 8 mm. However, when KiN was removed and only GA_3+NAA was supplied to the media; many fold increase in fiber length was recorded in both the genotypes. Further this, promotion was much higher in G. Cot than H-6, which justifies the *in vivo* results that zeatin (cytokinin) has no role in cotton fiber development rather it proves inhibitory. Momtaz et al. (1998) showed that higher concentration of kinetin narrowed the stimulatory effect of both GA_3 and IAA and thus non promotive for fiber development.

Similarly, statistical analysis showed that there was a significant difference between the two genotypes for their seed size, which became less significant with GA_3+NAA+KiN and insignificant with KiN treatment (Table-1). Therefore, support the hypothesis that KiN increases seed size and hence regulates cell division. Compared to G. Cot, H-6 exhibited less promotory effect of exogenously applied KiN on all the studied parameters; lead to assume that

it has optimum endogenous content. Wobus and Weber (1999) have proposed that plant hormones including IAA, ABA, ZR and GA are closely associated with grain development. In conclusion, the results presented here showed that complete plant growth is a consequence of synergistic effects of promotory phytohormones (gibberellins, auxins, cytokinin).


500
400
300
200
100
0
µg Zeatin boll-1
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60
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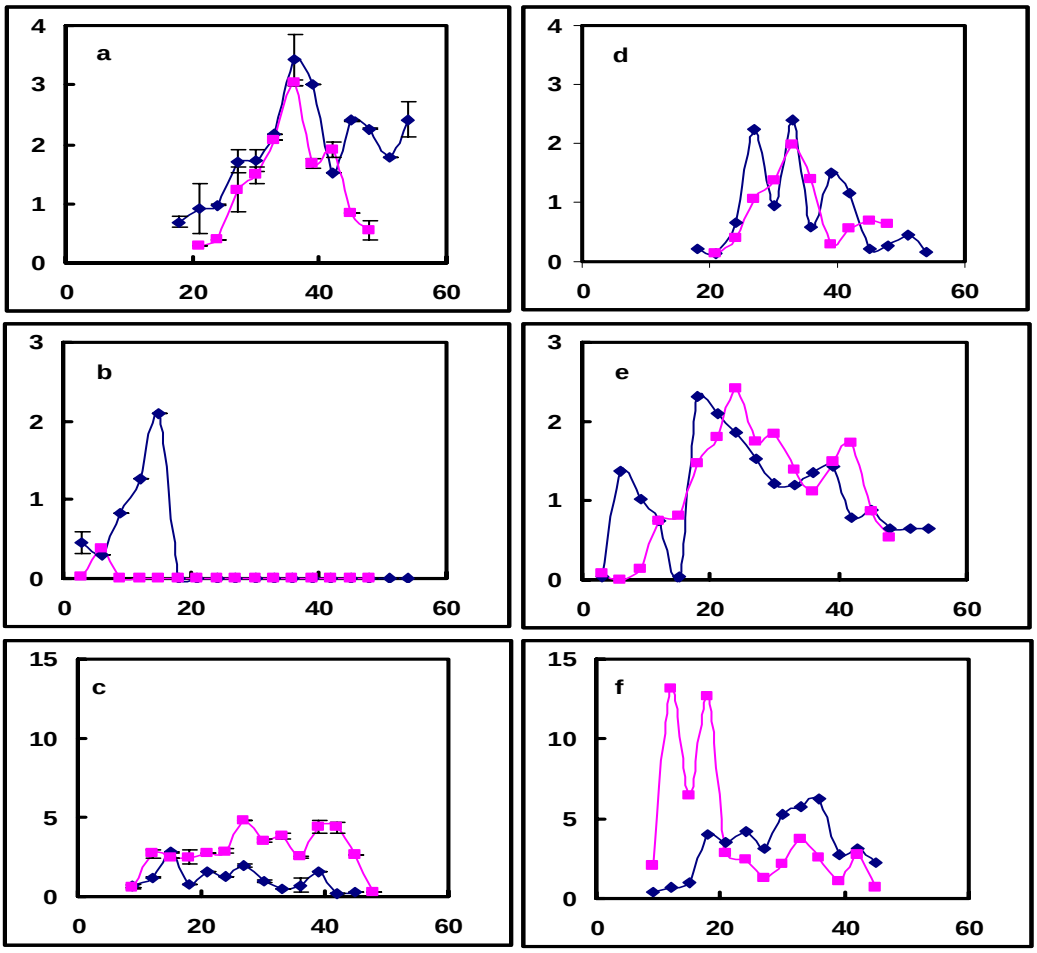

Fig-2: Changes in free and conjugate zeatin content in kernel (a,d), seed coat (b, e) and fiber (c, f) against boll age in two cotton genotypes H-6 ( ) and G. Cot ( ). Vertical bars represent ±SD or are within the symbol

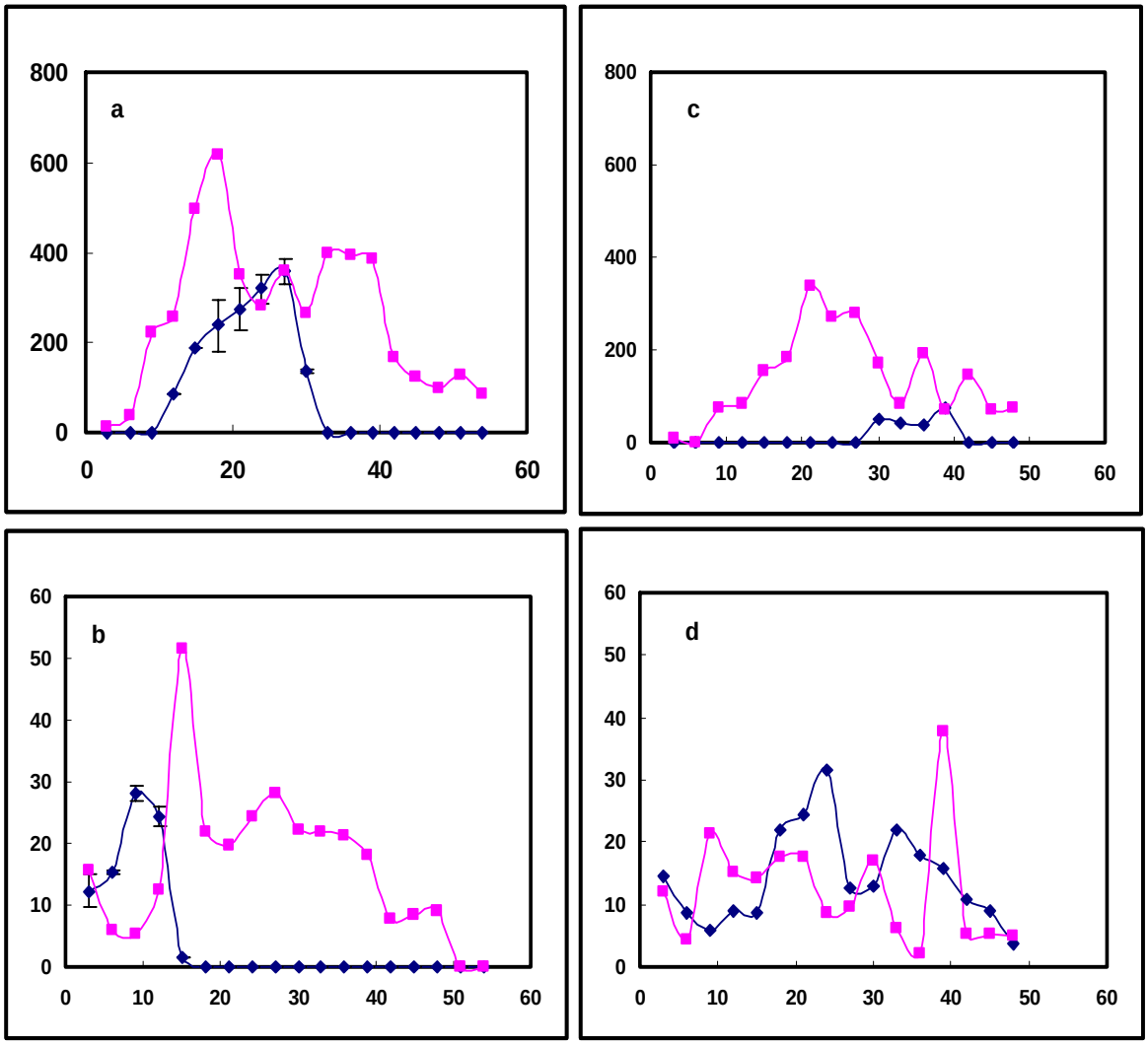

Fig-3: Changes in free (a, b) and conjugate (c, d) zeatin content in boll wall (a,c) and bract (b, d) against boll age in two cotton genotypes H-6 ( ) and G. Cot ( ). Vertical bars represent ±SD or are within the symbol.

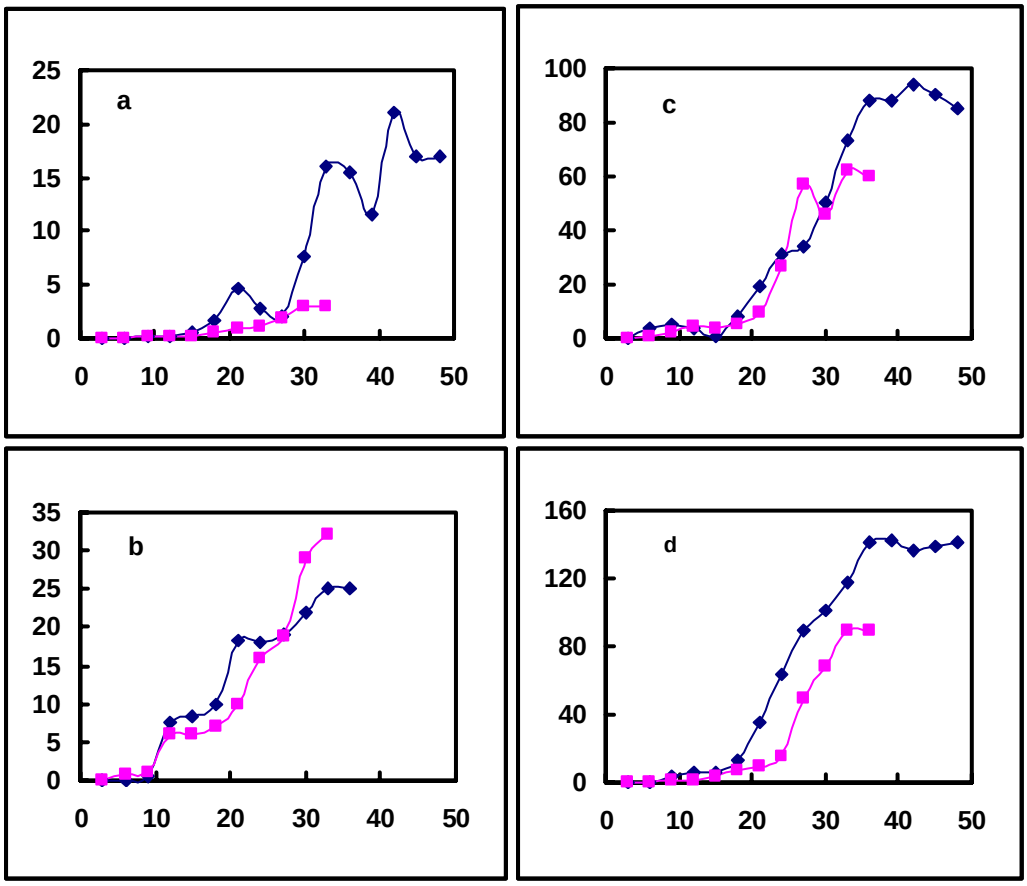

Fig- 4: Changes in dry weight without hormone (a), with KiN (1mg L^{-1}) (b), GA+NAA+KiN (1mg L^{-1}) (c) and GA+NAA (1mg L^{-1}) (d) against days of inoculation in two cotton genotypes H-6 ( ) and G. Cot ( ).

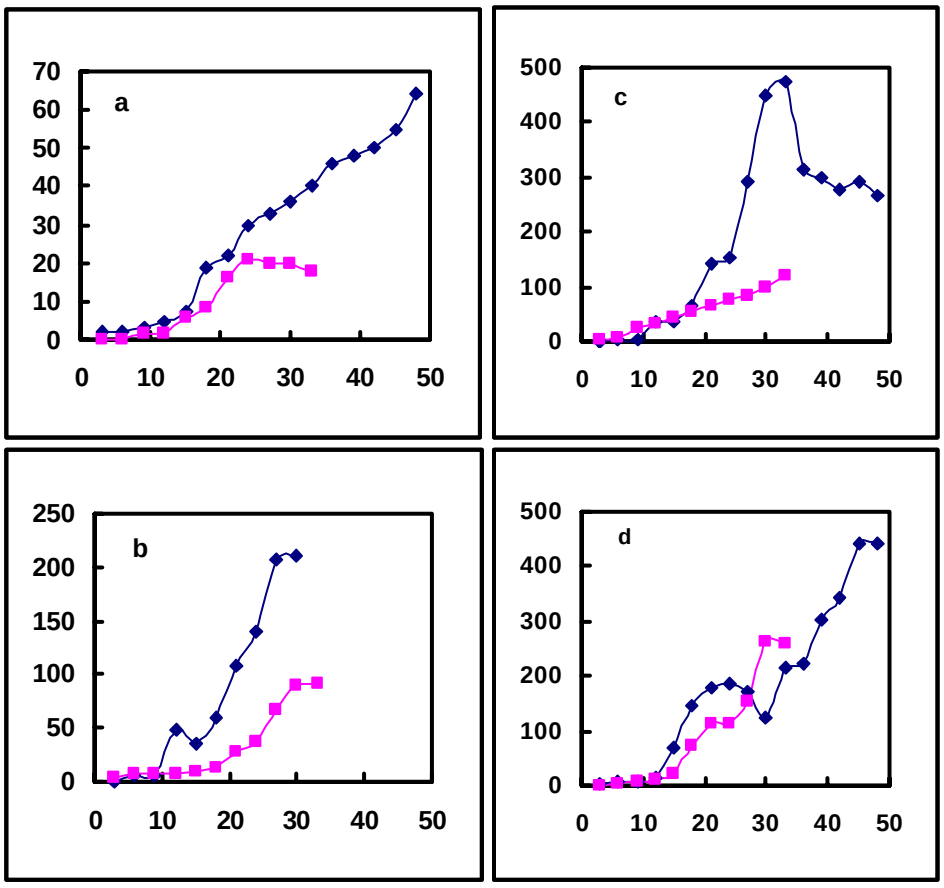

Fig- 5: Changes in water amount without hormone (a), with KiN(1mg L^{-1}) (b) , GA+NAA+KiN (1mg L^{-1}) (c) and GA+NAA (d)(1mg L^{-1}) against days of incoulation in two cotton genotypes H-6 ( ) and G. Cot ( ).

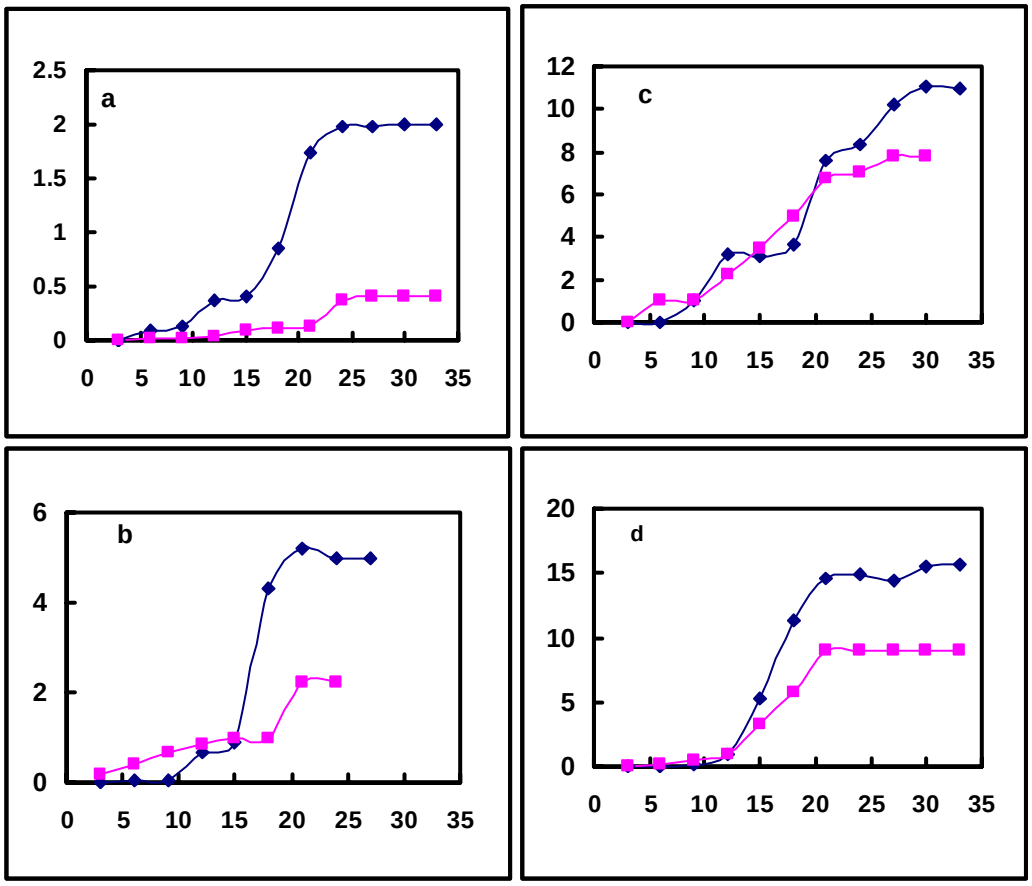

Fig- 6: Changes in fiber length without hormone (a), with KiN (1mg L^{-1}) (b), GA+NAA+KiN (1mg L^{-1}) (c) and GA+NAA (1mg L^{-1})(d) against days of inoculation in two cotton genotypes H-6 ( ) and G. Cot ( ).

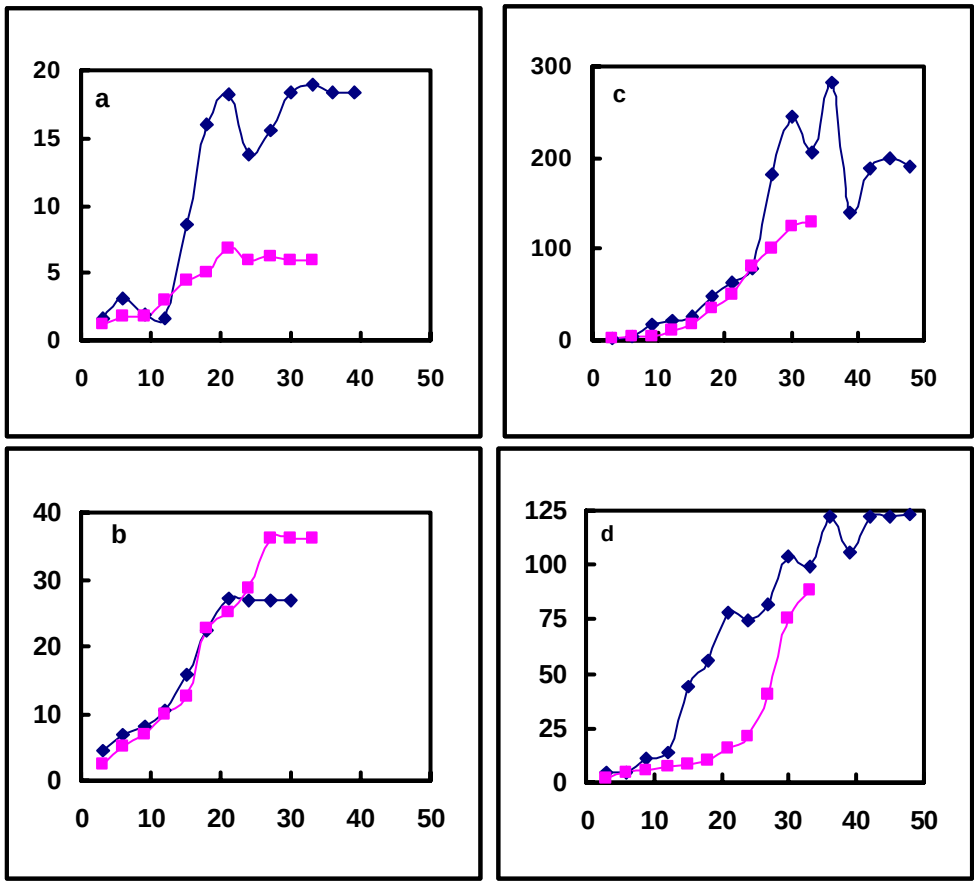

Fig- 7: Changes in seed size without hormone (a), with KiN (1mg L^{-1}) (b) , GA+NAA+KiN (1mg L^{-1}) (c) and GA+NAA (1mg L^{-1}) (d) against days of inoculation in two cotton genotypes H-6 ( ) and G. Cot ( ).

Table-1. ANOVA between two cotton genotypes for dry weight, water amount, fiber length and seed size of cultured ovules supplemented with GA_3+NAA/ GA_3+NAA+KiN/ KiN/ without hormone

Dry weight	Control	GA_3+NAA	GA_3+NAA+KiN	KiN
F-ratio	7.417	5.925	2.21	0.087
p-value	0.01	0.02	0.1	NS
df	26	26	27	27
Water amount	Control	GA_3+NAA	GA_3+NAA+KiN	KiN
F-ratio	8.925	2.951	7.88	3.53
p-value	0.001	0.1	0.01	0.1
df	27	26	26	20
Fiber length	Control	GA_3+NAA	GA_3+NAA+KiN	KiN
F-ratio	10.237	2.653	0.512	2.042
p-value	0.004	0.1	NS	0.1
df	21	22	20	16
Seed size	Control	GA_3+NAA	GA_3+NAA+KiN	KiN
F-ratio	10.64	9.416	4.65	0.246
p-value	0.001	0.01	0.1	NS
df	23	26	26	20

NS- non significant

Table-2. **Percent promotion in dry weight, water amount, fiber length and seed size with GA_3+NAA/ GA_3+NAA+KiN/ KiN applications in two cotton genotypes: H-6 and G. Cot**

Parameter	GA_3+NAA		GA_3+NAA+KiN		KiN	
	H- 6	G. Cot	H- 6	G. Cot	H- 6	G. Cot
Dry weight	829.4	3000.0	502.9	2000.0	147.0	1066.6
Water amount	734.3	1300.0	442.0	600.0	350.0	454.5
Fiber length	787.5	2250.0	500.0	1937.5	250.0	550.0
Seed size	670.7	1466.6	1089.1	2166.6	147.0	600.0

Table-3. **Correlation coefficient between water amount with dry weight and seed size with exogenous applications of GA_3+NAA+KiN or KiN in two cotton genotypes: H-6 and G. Cot**

Dry weight and Water amount	GA_3+NAA+KiN	KiN
H-6	0.806***	0.961***
G. Cot	0.895***	0.976***
Water amount and Seed size	GA_3+NAA+KiN	KiN
H-6	0.927***	0.876***
G. Cot	0.964***	0.895***

Significance level $p \leq 0.001$ = ***, $p \leq 0.01$ = **, $p \leq 0.05$ = *

Chapter VI
Abscisic Acid

SUMMARY

Endogenous contents of free and conjugated ABA was estimated, during the entire period of seed development, in two cotton genotypes varying in their final seed size and fiber length. Free ABA content was low during cell division and cell elongation period in H-6 kernel while G. Cot kernel showed higher content of ABA than that of H-6 during the elongation period. Additionaaly, free ABA content was higher in G. Cot seed coat than H-6. Negative correlation between free ABA level and rate of fiber elongation was observed in both the genotypes. Moreover, in boll wall and bract free ABA content increased with decrease in water amount. Indirect ELISA was performed using antibodies raised against ABA for the estimation of endogenous ABA level. *In vivo* results indicates role of ABA in secondary thickening phase in cotton fiber. *In vitro* studies suggested that ABA supplementation was inhibitory for the seed dry weight, water amount, fiber length and seed size. When media was supplemented with GA_3+NAA+ABA reduction in inhibition was observed for the studied parameters. Maximum promotion in all studied parameters was observed with GA_3+NAA application. From *in vitro* data, it is clear that ABA acts antagonistically with GA and auxin. Further, role of ABA in cotton ovule (seed) and boll development is discussed.

Abbreviations: ABA- Abscisic acid, EDC- N-ethyl-N (3-dimethylamino propyl) carbodiimide hydrochloride, DEAE–cellulose - DiEthyl Amino Ethyl Cellulose

INTRODUCTION

Abscisic acid is a product of the terpenoid pathway of biosynthesis, isolated and identified from the young fruit of cotton (Ohkuma et al. 1963, Ohkuma et al. 1965) and demonstrated to be an abscission-accelerating hormone (Davis and Addicott 1972). ABA regulates many important developmental processes in plants (Pavia and de-Oliveira 1995) viz., carotenoid biosynthesis (Frey et al. 2006), inhibition of internodal elongation (Montague 1997), as well as adaptive responses to imposed environmental stress (Zeevart and Creelman 1988). In addition, during seed development ABA play several important roles including induction of storage protein and lipid synthesis, desiccation tolerance and prevention of precocious germination (Schmitz et al. 2000, Suzuki et al. 2000, Tian and Brown 2000, Silveira et al. 2004).

Developing fruits and seeds have long been known as a rich source of plant hormones (Nitsch 1970). In many species, changes of hormonal content throughout the development of the reproductive parts have been determined. From the accumulated data there appears to be a general pattern of high growth promoter content in early development followed by inhibitor mainly ABA content in later development (Hsu 1979). This late appearance of ABA has been thought to have important implications in fruit maturation (Coombe and Hule 1973), abscission (Davis and Additcott 1972) or seed dormancy (Dure 1975). Supplementary, high ABA content was recorded in those fruits, which were not fertilized and abscised before maturation (Hsu 1979).

Accumulation of ABA during seed development has been reported in many species (Quebedaux et al. 1976, Taylorson and Hendricks 1977, King 1982). Endogenous levels of ABA in *Sechium edule* seeds showed absence of ABA accumulation in coincidence with the maximal seed growth (Ackerson 1984,

Gnagnarinin and Lorenzi 1985). In developing seeds, ABA is synthesized by embryo tissues; however evidences indicate that ABA is also transferred from maternal tissues to the seed when a plant is subjected to water deficit (Ober and Setter 1992, Jeschke et al. 1997), and desiccation terminates into maturation phase (Pavia and Oliveira 1995). Wang and his co-workers (2002) showed the positive relationship between water desiccation and high concentration of ABA in kernel of maize plants. Accumulation of ABA during seed development with high peaks preceding developmental arrest has also been reported in maize (Quatrano 1986) and Cacao (Pence 1991). ABA is believed to regulate many of the activities of late embryogenesis that are associated with developmental arrest (Bradford and Trewavas 1994).

Exogenously supplied ABA prevented germination of excised immature cotton embryos and embryo placed on ABA-free medium exhibited precocious germination (Ihle and Dure 1970). Embryo culture experiments in many other species have also shown that ABA inhibits the synthesis of a set of germination specific proteins while allowing the accumulation of storage proteins and their respective mRNAs (Quatrano et al. 1983, Crouch et al. 1985). Additionally, as the plants become less vigorous, synthesis of the gibberellins may decline permitting the synthesis of relatively greater amounts of ABA (Addicott et al. 1964, Mahouachi et al. 2005). Recent phenotypic and genetic analysis of hormone - mutants suggest that PGRs can influence each other's synthesis and may perhaps share signaling components (Gazzarrini and McCourt 2003). Considering the antagonistic effect between ABA with GA and auxin, *in vitro* experiments were performed. For that different combinations of these hormones were applied to check their effects on dry weight, water amount, fiber length and seed size.

ABA inactivation in plant tissues can occur via two major pathways: oxidation and conjugation (Wang et al. 2002). Conjugation primarily involves formation of ABA glucose ester (Zeevart and Creelmann 1988). Free and conjugated ABA increased during water stress and decreased after irrigation (Guinn and Brummett 1988). ABA conjugates are thought to be stable end products as compared to IAA conjugates. Many workers suggested that conjugated ABA might serve as a reservoir for the release of free ABA (Hiron and Wright 1973, LePage and Bulard 1979).

From the earlier studies on cotton boll development, it was observed that endogenous ABA content correlates inversely with elongation growth of fiber (Yang et al. 2001, Dasani and Thaker 2006) and dry matter accumulation phase of developing cotton seed (Gokani et al. 1998). Moreover, Gokani and Thaker (2001) suggested that the abnormal fiber and seeds accumulated more ABA content than the normal bolls. However, there is no detailed study on the role of ABA in total cotton boll development with respect to its individual organ development. In this study, free and conjugated forms of ABA were estimated from the boll components (i) boll wall, (ii) bracts, (iii) kernel, (iv) seed coat and (v) fiber, in two cotton genotypes.

MATERIALS AND METHODS

(A) Raising of antibodies against ABA

(i) Preparation of ABA-BSA/Casein conjugate

Synthesis of abscisic acid-BSA/casein conjugate was carried out according to the protocol of Gokani and Thaker (2001). Abscisic acid (132 mg) was dissolved in 3 ml of the mixture of water and DMF (2:1) and pH was adjusted to 8.0 with 1 N NaOH. The solution was then added drop wise with gentle stirring to 250 mg of BSA/casein dissolved in water and adjusted to pH 8.5. N-ethyl-N (3-dimethylamino propyl carbodimide hydrochloride (EDC, 210 mg) was added into the preparation in four parts at interval of 30 min and stirred in dark for 19 h at 4 ºC. Conjugate was finally dialyzed against distilled water for 4 days; final volume was made with distilled water and stored at 0 ºC.

(ii) Immunization and (iii) Purification of IgG

As described in Chapter - 3.

(B) Extraction of ABA from the samples

It was performed as described in Chapter- 3.

(C) Indirect ELISA and Calibration curve for ABA

All the steps of ELISA were followed as described in Chapter -3. To test the sensitivity of the assay internal standards (400 ng/well ABA) were mixed with samples. With each plate calibration curve was performed. All estimations were done in triplicates. Calibration curve was prepared with different concentrations of ABA in the range of 100 -700 ng.

(D) *In vitro* ovule culture

For *in vitro* studies, one-day post anthesis bolls were collected from both the genotypes; ovules were separated from the ovary in aseptic condition and floated on the liquid culture media (Beasley and Ting 1973), supplemented with or without ABA (mg L^{-1}). The inoculated flasks were then kept in dark at room temperature. *In vitro* fiber length and seed size measurement was carried out as described in Chapter 3. Callus formation was observed in G. Cot after 36 days and hence experiment was terminated after the period. In addition, as compared to control treatment reduction in the value with application of ABA was calculated as percent inhibition where control result was considered as 100% result. Improvement in dry weight/ water amount/ fiber length/ seed size with addition of GA_3+NAA+ABA or GA_3+NAA was expressed as percent recovery.

RESULTS

In vivo study

Endogenous ABA level

(i) Estimation of ABA in Ovule

In H-6 kernel, free ABA level remained low up to 30 DPA (cell division and elongation phase). Maximum ABA content was recorded on 39 DPA (7.70 μg seed kernel^{-1}) when boll opened. Free ABA level increased during later phases while conjugated ABA content started to decline after 27 DPA (31.02 μg seed kernel^{-1}). Similar, trend was observed for G. Cot kernel, where free ABA content increased after 18 DPA and maximum level was observed on 33 DPA (9.67 μg seed kernel^{-1}, Fig. 6.1a). Conjugated ABA level in G. Cot was also higher during initial period ranging from 18-27 DPA and declined thereafter (Fig. 6.1d).

In H-6 seed coat, free ABA content increased gradually up to 15 DPA (10.83 μg seed coat^{-1}) then declined in subsequent ages. While conjugated ABA level in H-6 seed coat remained higher than free ABA throughout the developmental phases. Maximum conjugated ABA content was recorded on 33 DPA (30.10 μg seed coat^{-1}). Contrary to this, in G. Cot seed coat free ABA level increased rapidly, achieved peak on 24 DPA (13.64 μg seed coat^{-1}) and also remained higher during later stages (Fig. 6.1b). However, conjugated ABA level remained low in G. Cot as compared to H-6 seed coat throughout the developmental period (Fig. 6.1e).

(ii) Estimation of ABA in fiber

Free ABA, in H-6 fiber was significantly higher during 15-39 DPA and declined thereafter. In G. Cot fiber, fluctuation in free ABA content was observed (Fig. 6.1c). Maximum free ABA content recorded was 8.06 μg in H-6 and 4.62 μg in G. Cot fibers respectively. Moreover, conjugated ABA in G. Cot remained significantly higher during 15-36 DPA and maximum content was observed on 24 DPA (15.71 μg seed fiber^{-1}), whereas in H-6 fiber, it was recorded low throughout the fiber development (Fig. 6.1f).

(iii) Estimation of ABA in boll wall and bract

As shown in Figure 6.3a, free ABA level was recorded higher between 12-39 DPA and recorded negligible content in subsequent stages in H-6 boll wall. Whereas, in G. Cot boll wall free ABA level increased gradually up to 33 DPA and declined thereafter. Maximum value was recorded on 27 DPA (608.36 μg boll wall^{-1}) and 33 DPA (188 μg boll wall^{-1}) in H-6 and G. Cot boll wall, respectively (Fig. 6.3a). In addition, conjugated ABA content increased from 3 DPA and remained higher up to 27 DPA in H-6 while in case of G. Cot, conjugated ABA level was higher up to 33 DPA and declined thereafter (Fig. 6.3b).

In H-6 bract, free ABA content was detected during 3-27 DPA and reached to negligible level thereafter. Maximum value was observed on 15 DPA (54.69 μg boll bract^{-1}). Whereas, in G. Cot bract, free ABA content remained low as compared to H-6 throughout the boll development. As shown in figure 6.3c, in G. Cot bract free ABA level remained almost stable up to 42 DPA and declined thereafter. Conjugated ABA levels showed more or less same value in both the genotypes and parallelism in pattern during studied period (Fig. 6.3d).

In vitro analysis

In general, ABA treatment was inhibitory for all studied parameters like dry weight, water amount, fiber length and seed size. When media was supplemented with GA_3+NAA+ABA (1 mg L^{-1}, 1 mg L^{-1}, 3 mg L^{-1}, respectively) improved results were observed. However, excluding the ABA from the media, enhanced results were obtained.

In control treatment, maximum dry weight was observed as 21 mg and 3 mg in H-6 and G. Cot respectively (Fig. 6.4a). When ABA (1 mg L^{-1}) was supplemented to the media, it was found to be inhibitory for the H-6 seed where dry weight reduced to 3.2 mg however; in G. Cot seed slight increase was observed as compared to the control (Fig. 6.4b). As indicated in figure 6.4c, when GA_3+NAA (1 mg L^{-1}) was supplied along with ABA (1 mg L^{-1}), remarkable improvement in dry weight in both the genotypes was observed. Maximum dry weight recorded was 61.7 mg in H-6 and 17 mg in G. Cot, respectively. In addition, when ABA was removed totally and only GA_3+NAA was supplied, utmost dry weight was achieved. This result matched with 141 mg in H-6 and 90 mg dry weight in G. Cot seed (Fig. 6.4d).

In control treatment, water amount recorded was 64 mg in H-6 and 20 mg in G. Cot seed (Fig. 6.5a). Whereas, with 1 mg L^{-1} ABA, maximum water amount in H-6 was 79.6 mg while in case of G. Cot it was stabilized around 19 mg (Fig. 6.5b). As ABA was supplemented along with GA_3+NAA, significant promotion was observed in H-6 and maximum value noted was 171.1 mg while in G. Cot, it was restricted to 38 mg (24 DPA). Moreover, when media was supplemented without ABA (GA_3+NAA, 1 mg L^{-1} both), 6.89 times and 12.69 times higher water amount with respect to control was observed in H-6 and G. Cot seed, respectively. Maximum water amount recorded in H-6 and G. Cot seed was 441.3 mg and 264 mg, respectively (Fig. 6.5d).

Similarly, when fiber length was measured with or without ABA or GA_3+NAA+ABA or GA_3+NAA different levels of inhibitory and promotory effects were observed. In control treatment, final fiber length was 1.99 mm in H-6 and 0.4 mm in G. Cot (Fig. 6.6a). When ovules were cultured with ABA (1 mg L^{-1}), reduction in final fiber length was observed in both the genotypes (Fig. 6.6b). With GA_3+NAA+ABA improved elongation was observed and similar length (5.5 mm) was obtained in both the genotypes (Fig. 6.6c). However, with GA_3+NAA (1 mg L^{-1}) treatment 15.5 mm and 9.0 mm length was recorded in H-6 and G. Cot fiber, respectively (Fig. 6.6d).

In control treatment, final seed size remained 19 mm^2 and 6.87 mm^2 in H-6 and G. Cot, respectively. Seed size measurement showed that ABA treatment was not found to be inhibitory but promotion in seed size was obtained with GA_3 and NAA applications (Fig. 6.7). With GA_3+NAA+ABA, final seed size in H-6 was 50 mm^2 while in case of G. Cot it was restricted to 18 mm^2 (Fig. 6.7c). Whereas, with GA_3+NAA (1 mg L^{-1} both) application 6.42 times increase in H-6 and 12.94 times increase in G. Cot seed size was recorded. The final seed size recorded wad 122 mm^2 in H-6 and 88 mm^2 in G. Cot, respectively (Fig. 6.7d).

DISCUSSION

In vivo analysis

Major findings of *in vivo* study are (i) in H-6 kernel, free ABA was low during cell division and cell elongation phase. Additionally, in G. Cot kernel, free ABA remained higher than H-6 during the same period (ii) After 15 DPA, free ABA content was recorded higher in G. Cot seed coat than H-6 while conjugated ABA level was higher in H-6 seed coat than G. Cot throughout the developmental period (iii) Though, conjugated ABA in protective structures was almost similar in both the genotypes studied but free level was higher in H-6 boll wall and bract than G. Cot.

Free ABA level in H-6 kernel was low during cell division and cell elongation phases and significant level was recorded during dry matter accumulation phase and declined at physiological maturity (Fig. 6.1a). Whereas, in developing kernel of G. Cot, ABA level increased from cell elongation phase and remained higher during DMA and maturation. Earlier work from our laboratory in seed development suggested that ABA content might not have any specific role in dry matter accumulation (Gokani et al. 1998). Further, in H-6 and G. Cot seed coat ABA level increased during initial period and decreased after 24 DPA when kernel tissue started rapid accumulation of ABA. As compared to H-6, G. Cot seed coat has more free ABA level excluding 3-15 DPA while conjugated ABA level was higher in H-6 throughout the developmental period. In fact the concentration of free ABA in ovule (kernel + seed coat) remained at around 12-16 μg/ovule in G. Cot and 8-10 μg /ovule in H-6. From the data presented in this study, it can be speculated that high levels of free ABA in G. Cot seed coat and its accumulation in kernel in early developmental period may be the limiting factor for final seed growth. Hendrix and his co-workers (1987) recorded that immature cotton embryos

accumulate ABA from seed coats by diffusion, apparently against its concentration gradient. Similar results were observed in *Phaseolus* (Hsu 1979) and soybean seeds (Schussler et al. 1991).

Free ABA content measured in the fiber of both the genotypes reached to a peak when rate of cellulose deposition was at maximum (secondary thickening phase); moreover, elevation in the ABA level was noted after completion of elongation phase (Fig. 6.2). Further, free ABA content in fiber increased with rate of DMA in both the genotypes (Fig. 6.2). This leads to the conclusion that elevated ABA level might be a signal for the secondary wall thickening. Further, inverse correlation was obtained between free ABA level and rate of elongation in both the genotypes H-6 and G. Cot ($r = -0.699$ in H-6 and $r = -0.402$ in G. Cot, respectively). Patel and Thaker (2007) have reported similar result during internode development in *Meremmia*. Many workers have indicated that ABA inhibits fiber elongation as ABA has an opposite role to that of IAA and GA (Beasley and Ting 1973, Yang et al. 2001, Dasani and Thaker 2006). ABA is regarded as the inhibitory hormone, which counteracts the promotive effects of GA and terminates the elongation by inhibiting the IAA biosynthesis (Pilet and Saugy 1987).

In addition, in the present study, free and conjugated ABA levels were also measured from boll wall and bract of both genotypes throughout the boll development. From the growth data (Chapter-2, Fig. 2.13) and fluctuation in boll wall ABA level (Fig. 6.3), it is clear that free ABA content increased after water amount started to decrease. Similar observation was noted for bract tissues in both the genotypes suggesting that desiccation leads to the high level of ABA even in the protective structures. Water deficit leading to more accumulation of ABA in maize plants has been reported (Ober et al.1991, Artlip et al. 1995, Setter et al. 2001).

In vitro analysis

It has been suggested that balance ratio of growth promotory and inhibitory hormones decides the final yield (Millborrow 1970, Patel and Thaker 2007). As ABA treatment decreased the water amount and fiber length with respect to control (Figs. 6.5, 6.6), it is clear that ABA inhibits the water uptake and cell elongation that leads to restrict the sink size. Moreover, when GA_3 and auxin (NAA) was added to the media, inhibitory effects of ABA were reduced in all the studied parameters *i.e.* dry weight, water amount, fiber length and seed size. And elimination of ABA from the media showed the maximum promotion in dry weight, water amount, fiber length and seed size (Table-1). From the *in vitro* study, it is proposed that ABA has antagonistic effect against GA and auxin.

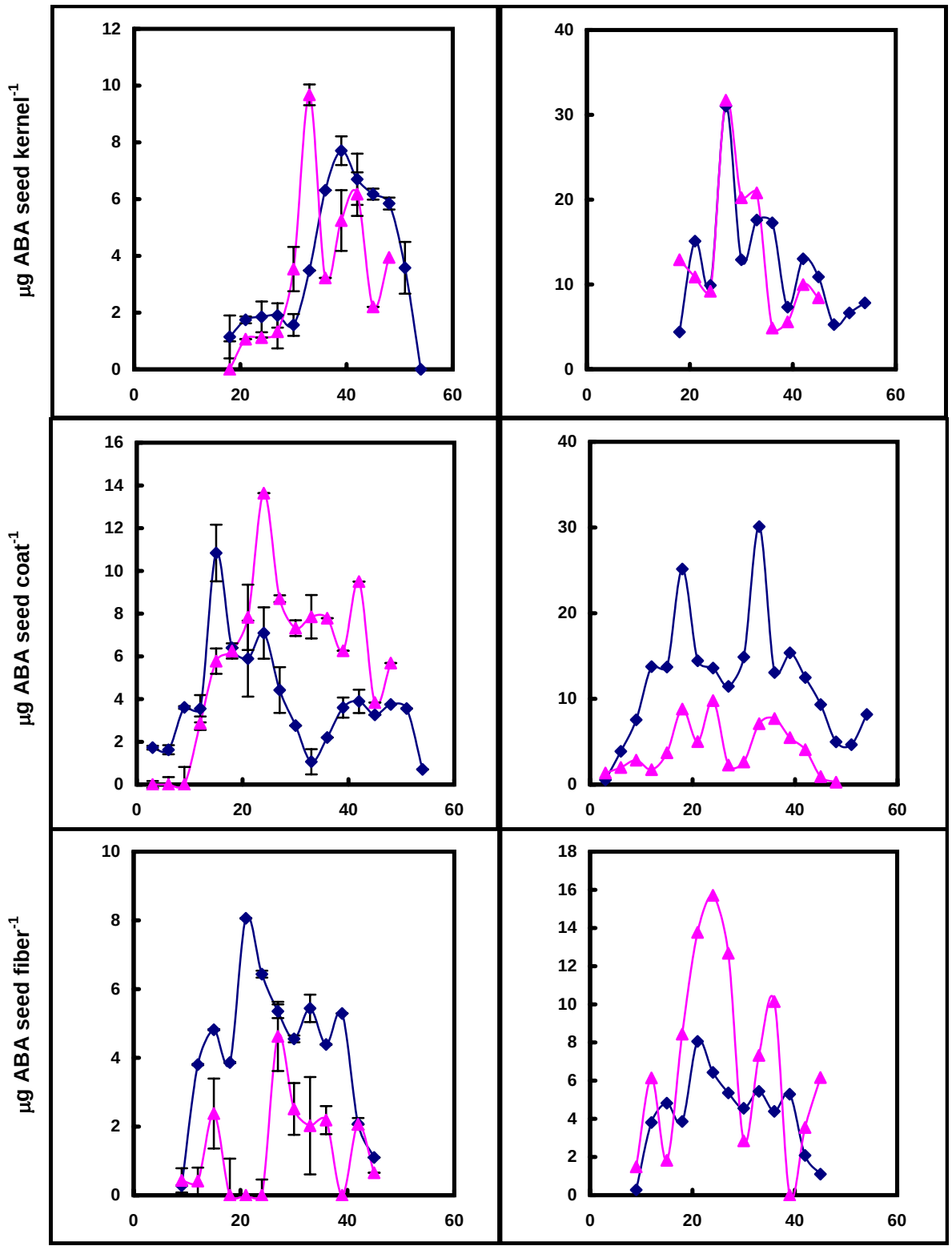

Fig-1 Changes in endogenous freeABA level (a,b,c) and conjugated ABA level (d,e,f) in kernel (a,d), seed caot (b,e) and fiber (c,f)in two cotton genotypes H-6 and G. Cot.

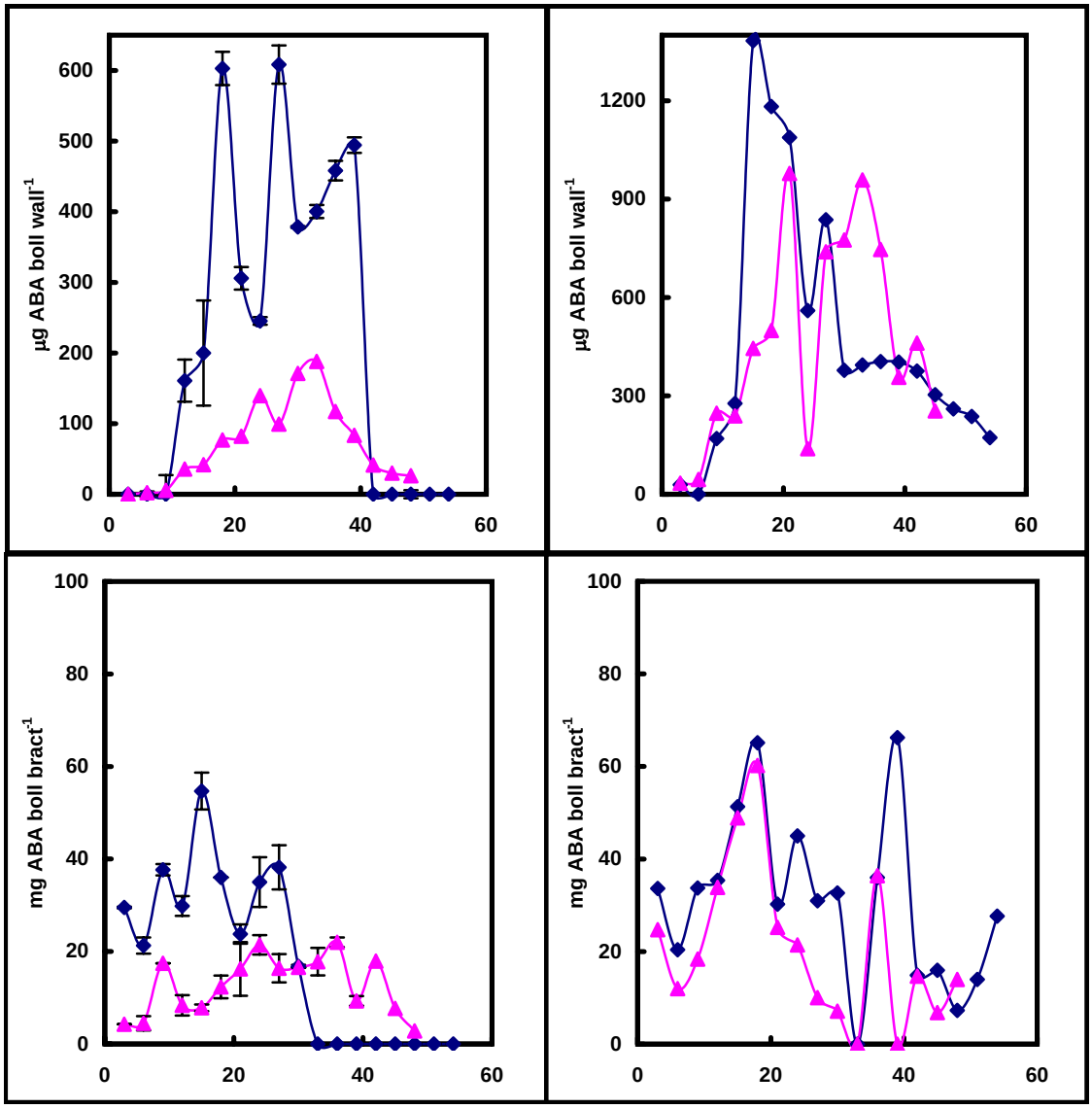

Fig-2 Changes in endogenous freeABA level (a,b) and conjugated ABA level (c,d) in boll walll (a,d), bract (b,d) in two cotton genotypes H-6 ()and G. Cot ()

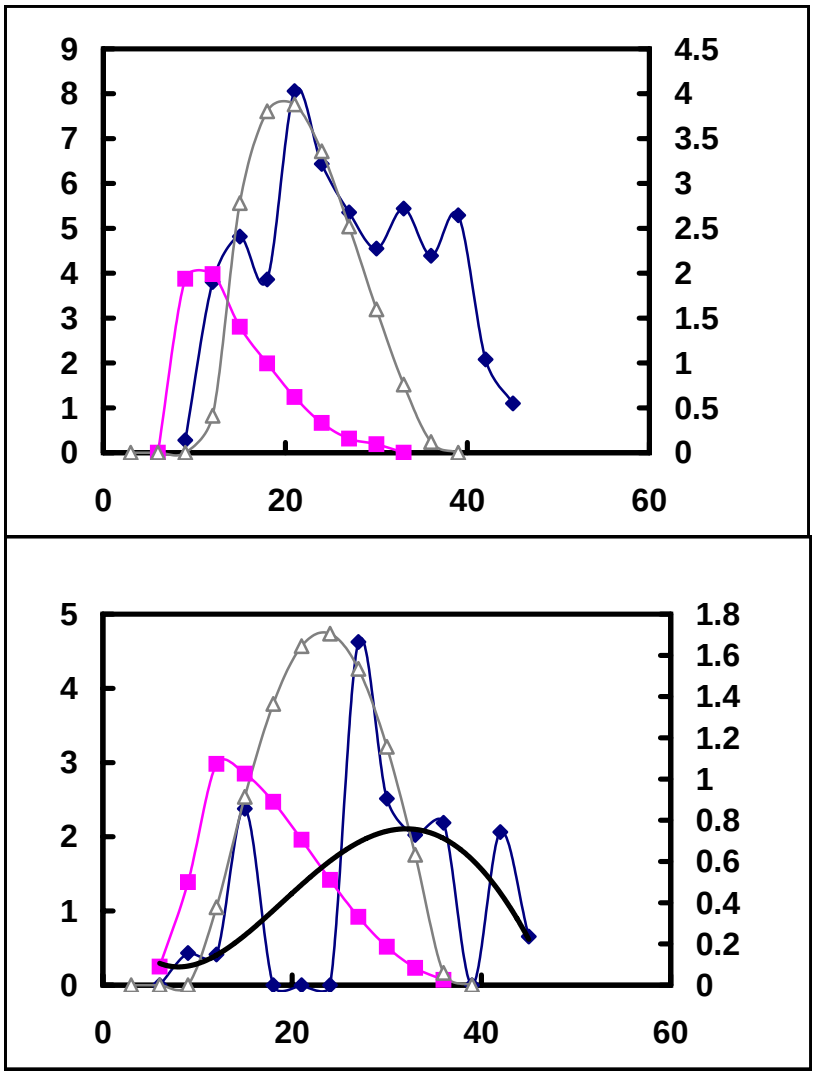

Fig-3: ABA content with rate of DMA and rate of water uptake against boll age.

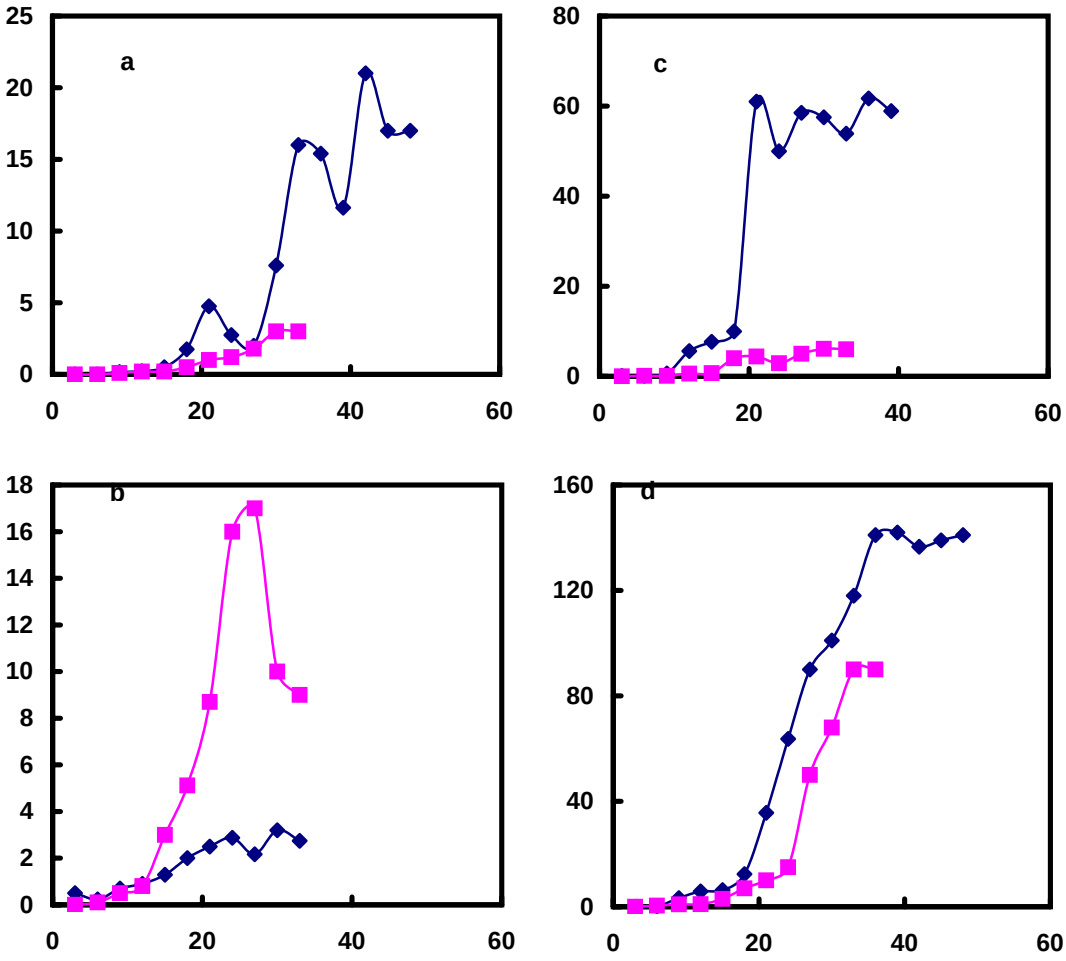

Fig-4: Changes in *in vitro* dry weight , without hormone supplement (a), with ABA [1mg L^{-1}](b), with GA+NAA+ABA [mgL^{-1}, Mg L^{-1}, 3mg L^{-1}] (c), GA+ NAA [mg L^{-1}] (d) against age in two cotton cultivars H-6 (◊) and G. Cot ( ) respectively. Vertical bars represent SD or are within the symbol

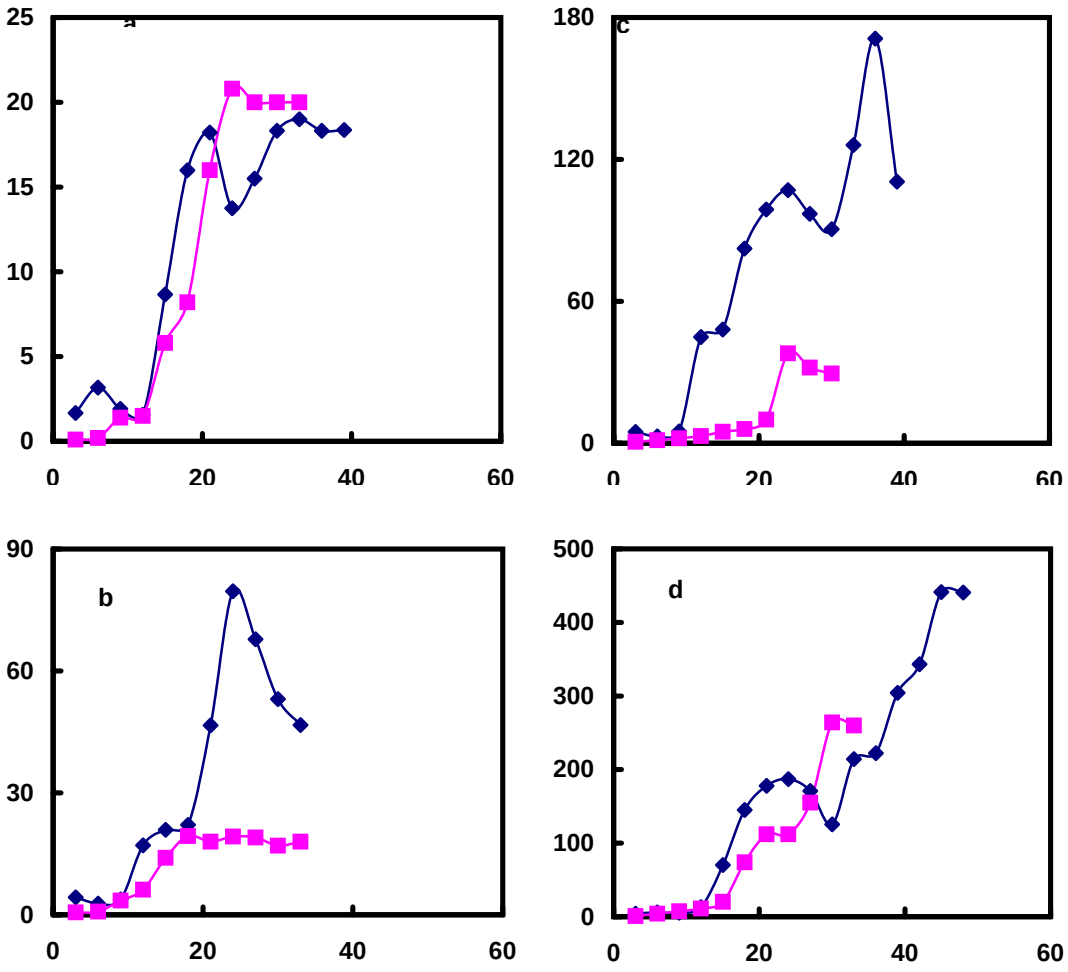

Fig-5: Changes in *in vitro* water amount , without hormone supplement (a), with ABA [1mg L^{-1}](b), with GA+NAA+ABA [mgL^{-1}, Mg L^{-1}, 3mg L^{-1}] ©, GA+ NAA [mg L^{-1}] (d) against age in two cotton cultivars H-6 (◊) and G. Cot ( ) respectively. Vertical bars represent àSD or are within the symbol

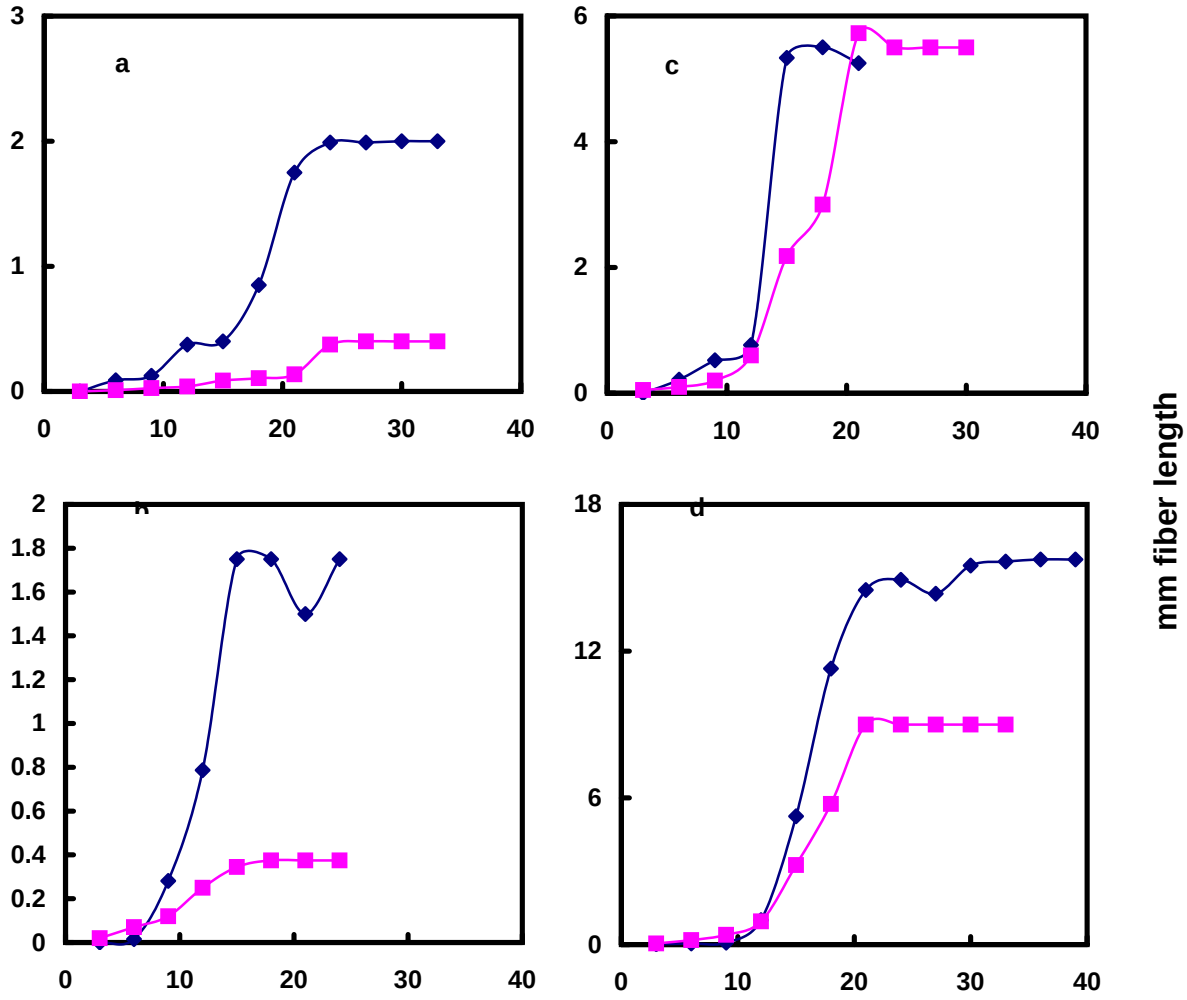

Fig- 6: Changes in *in vitro* fiber length , without hormone supplement (a), with ABA [1mg L^{-1}](b), with GA+NAA+ABA [mgL^{-1}, Mg L^{-1}, 3mg L^{-1}] ©, GA+ NAA [mg L^{-1}] (d) against age in two cotton cultivars H-6 (◊) and G. Cot ( ) respectively. Vertical bars represent àSD or are within the symbol

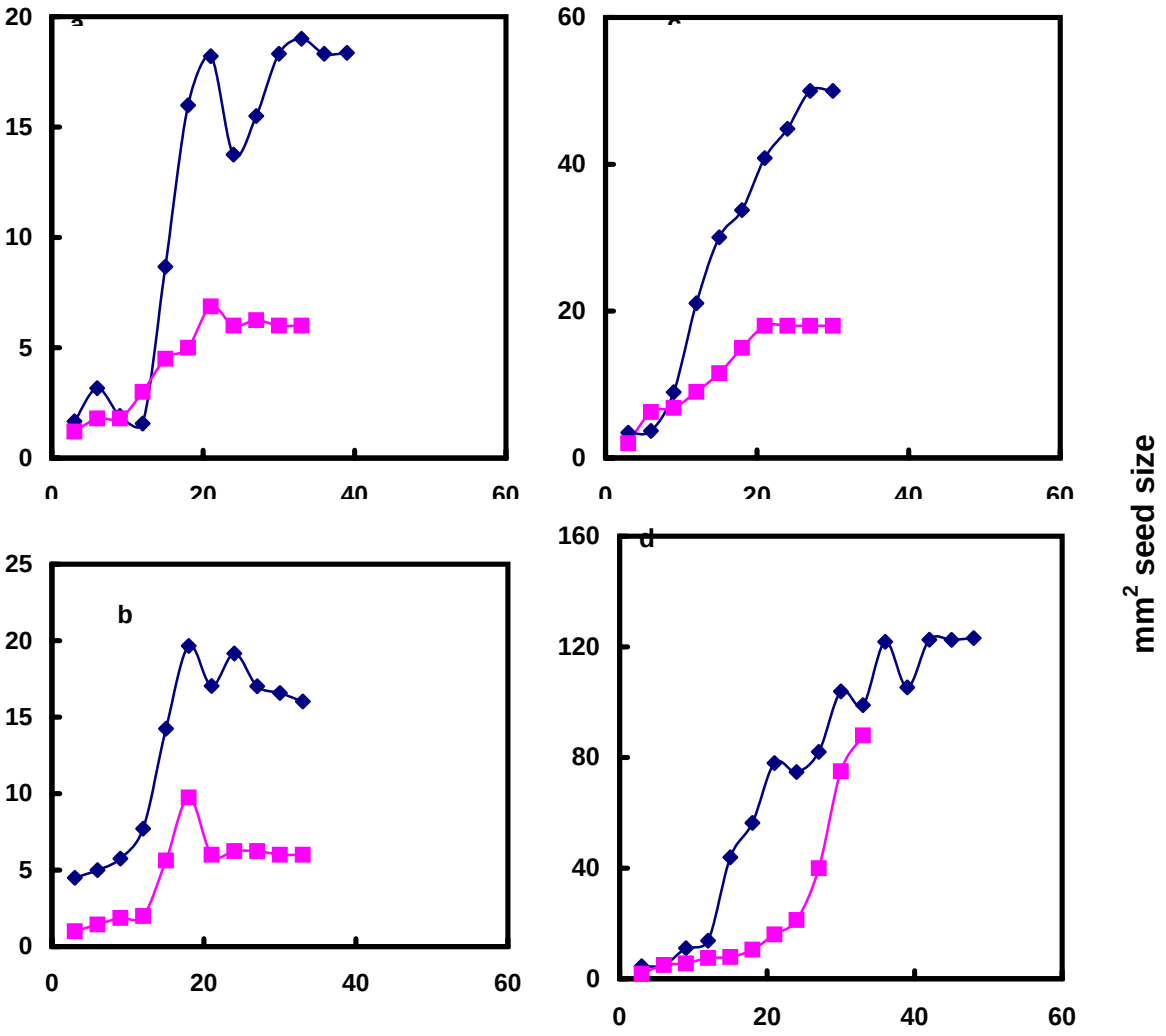

Fig-7: Changes in *in vitro* seed size , without hormone supplement (a), with ABA [1mg L^{-1}](b), with GA+NAA+ABA [mgL^{-1}, Mg L^{-1}, 3mg L^{-1}] ©, GA+ NAA [mg L^{-1}] (d) against age in two cotton cultivars H-6 (◊) and G. Cot ( ) respectively. Vertical bars represent àSD or are within the symbol

Table-1. Percent recovery / inhibition in dry weight, water amount, fiber length and seed size with exogenous hormonal treatments in two cotton genotypes: H-6 and G. Cot.

Parameter	ABA % Inhibition		GA_3+NAA+ABA % Recovery		GA_3+NAA % Recovery	
	H-6	G. Cot	H-6	G. Cot	H-6	G. Cot
Dry weight	5.29	300.0	346.0	200.0	829.41	3000.0
Water amount	78.0	90.0	184.0	147.5	734.0	1300.0
Fiber length	87.5	93.75	300.0	314.0	900.0	2400.0
Seed size	84.0	100.0	260.0	300.0	642.0	1466.0

Chapter VII
Peroxidase Activity

SUMMARY

Changes in cytoplasmic and wall bound peroxidase activity in kernel and seed coat were analyzed in two cotton genotypes H-6 and G. Cot. Growth analysis was worked out throughout the development period, in terms of dry weight and water amount in seed kernel and seed coat. Cytoplasmic and wall bound peroxidase fractions were assayed with guaiacol as hydrogen donor. In general, cytoplasmic peroxidase activity was recorded higher in H-6 ovule whereas wall bound peroxidase activity was higher in G. Cot. Cytoplasmic as well as wall bound activity was recorded many folds higher in seed coat than kernel of both the genotypes. The role of peroxidase in sink size development in cotton ovule is discussed.

Key words: cytoplasmic, guaiacol, kernel, peroxidase, seed coat, sink development, wall bound

INTRODUCTION

Peroxidases encompass a group of specific enzymes and are known for their vital role in biological systems by way of catalytically oxidising a wide variety of electron donor substrate species bearing phenolic, amine or acid functions aided by H_2O_2 or organic peroxides. Peroxidases are classified into three classes, which comprises of class I enzyme from mitochondria, chloroplast and bacteria, class II from fungi and class III, classical plant peroxidases (Welinder 1992). The best-studied plant peroxidase is horseradish peroxidase (HRP), which is present in high concentrations in the roots of the horseradish plant (Vinodu and Pdmanabhan 2001) though they are present in all higher plants (Lagrimini 1990).

Peroxidases produced by plants are present in multiple isozymic forms. According to the electrophoretic data isoperoxidases in a given species are separated into two distinct groups cationic and anionic (Miller et al. 1990). Plant peroxidases are monomeric, heme-containing proteins that are usually glycosylate with four-conserved disulphide bridges and two calcium ions. Peroxidases have various physiological roles in plant cells, including lignifications (Dean and Kolattukudy 1976, Quiroga et al. 2000), cross-linking of cell wall proteins (Bradley et al. 1992), xylem wall thickening (Hilaire et al. 2001), oxidation of indole 3-acetic acid (Lagrimini et al. 1997, Thaker 1998) and regulation of cell elongation and phenol oxidation (Campa 1991, Gaspar et al. 2002). It is clear that peroxidases play a key role in the stiffening of the cell wall and in processes associated with plant growth through the formation of phenolic cross-link (Fry 1986). Involvement of peroxidases in termination of elongation phase was reported in wheat (Chanda and Singh 1997) and in maize root (Liszkay et al. 2004).

Peroxidases also play an important role in disease resistance or in wound healing (Sasaki et al. (2004), Gijzen et al. (1993). Peroxidases have also been implicated in metabolic processes such as ethylene biogenesis, cell development and membrane integrity (Haard 1977) and cell differentiation (Walter and Gordon 1975). Increases in peroxidase activity during incompatible plant pathogen/elicitor interactions are often associated with a progressive incorporation of phenolic compounds within the cell wall (Reimers et al. 1992, Milosevic and Slusarenk 1996).

Role of peroxidases in the process of fiber cell elongation was reported earlier (Jasdanwala et al. 1977, Rama Rao et al. 1982a, 1982b, Thaker et al. 1986). Rabadia et al. (2006) suggested that peroxidases affect dry matter accumulation in cotton seed. The final weight of the seed at physiological maturity, play an important role in determination of yield. Localization of peroxidases in developing of sink-cotton ovule *i.e.* coat and kernel is yet unclear. Cotton ovule can be divided into kernel and seed coat. The cotton ovule is a useful model for studying growth related changes in cytoplasmic and wall bound peroxidase pattern and their relationship with physiological processes because its growth occurs in four well defined development stages. The present investigation was designed to study the localization of cytoplasmic and wall bound peroxidases activity in cotton ovule parts *i.e.* kernel and seed coat in two cotton genotypes H-6 (*Gossypium hirsutum*) and G. Cot (*Gossypium arboreum*) during total developmental period.

METHODS AND MATERIALS

(A) Growth analysis

Growth analysis was done in terms of fresh weight, dry weight and water amount throughout the seed development. Freshly harvested bolls were opened with a sharp scalpel and fibers were separated out from the seeds. Seeds were dissected in to seed coat and kernel. The mean dry weight and water amount per seed kernel and seed coat with ± SD were calculated. Sets of data on dry weight and water amount of seed coat and seed kernel were fitted to an appropriate polynomial equation by computer curvilinear method. Rate of dry matter accumulation and water amount were worked out.

(B) Enzyme extraction and purification

Randomly harvested bolls of different ages were dissected and seeds were separated to kernel and seed coat. Dissected kernel tissues and seed coat tissues were weighed (500 mg) and frozen in liquid N_2. Since cotton plants are well known for its rich phenolic compounds, therefore extraction buffer was prepared with addition of 8% polyethyleneglycol (PEG) and Na-EDTA (1mM) to Na- acetate buffer (1mM, pH 5.0). Frozen material was crushed in a cooled mortar with sterilized sand in pre-chilled extraction buffer. The homogenates prepared of all ages (3-54 days) were centrifuged at 10,000 g for 15 min. The resulting supernatant was passed through column of Sephadex G-25 that was pre equilibrated with Na-acetate buffer (1 mM, pH 5.0). The desalted preparations were then used for the assay of all cytoplasmic enzymes. To avoid possible loss of enzyme activities, crushing and desalting were carried out rapidly in a cold room (4 ºC).

(C) Preparation of wall bound enzyme

The pellet was washed thoroughly with distilled water for several times and centrifuged till the supernatant was free from the peroxidase activity. To the washed pellet 1N NaCl was added and kept overnight at room temperature to extract ionically wall-bound enzyme. On the next day, treated pellet was centrifuged at 10,000 g for 15 min and supernatant was collected. It was washed twice with 1N NaCl to extract trace of the enzyme. The pulled supernatant was used as a source of wall bound enzyme.

(D) Peroxidase assay

Continuous spectrophotometric assay was used for measurement of peroxidase activity, described by Mahesh and Thaker (2004). The reaction mixture consisted of 1.5 ml of Na-acetate buffer (1 mM, pH 5.0), enzyme extract 0.1ml for cytoplasmic peroxidase and 0.5 ml for wall bound peroxidase, 0.5 ml substrate (32 mM) and 10 µl H_2O_2. Both cytoplasmic and wall bound peroxidases fractions were assayed with guaiacol. The reaction was triggered by addition of H_2O_2 and increase in the absorbance was monitored at 470 nm. The linear phase of each reaction was considered for the calculation of mean values.

(E) Protein estimation

The protein content of the enzyme extract was estimated according to Bradford (1976) using coomasie brilliant blue dye with dye binding method. The complete enzyme analysis was done in triplicate from which the mean was calculated. The activity was expressed as ΔA_{470} mg protein^{-1} min^{-1}.

RESULTS

Growth analysis

As shown in Figs. 2.5 to 2.11, (Chapter 2) H-6 kernel and seed coat shown approximately double rate of water uptake as well as rate of dry matter accumulation than G. Cot kernel and seed coat. After an initial lag period of 15 days in H-6 kernel and 21 days in G. Cot, water amount continued to increase, attained a peak when rate of dry matter accumulation was maximum, then declined gradually and reached to negligible level at maturity.

Water amount

In H-6 and G. Cot kernel, water amount increased up to 36 (29 mg) and 39 DPA (26 mg) respectively. Maximum rate of water amount in these genotypes was recorded at 15 DPA (4.56 mg seed kernel^{-1} day^{-1}) and 33 DPA (2.59 mg seed kernel^{-1} day^{-1}) respectively. In H-6 seed coat, initial lag phase was recorded short only up to 3 days, water amount increased steadily up to 18 DPA (84.4 mg water amount seed coat^{-1}), then declined gradually and reached to negligible level at maturity (Fig. 2.5b). Similarly, in G. Cot initial lag phase was recorded up to 6 days, water amount increased gradually, attained a peak at 27 DPA (65 mg seed coat^{-1}) and declined thereafter (Fig. 2.7b). Maximum rate of water uptake in these genotypes was recorded at 9 DPA (6.80 mg seed coat^{-1} day^{-1}) and 15 DPA (4.43 mg seed coat^{-1} day^{-1}) respectively.

Dry weight

In H-6 kernel, dry weight was initially low up to 21 DPA (7.37 mg seed kernel^{-1}), increased up to 48 DPA (57.35 mg seed kernel^{-1}) and declined thereafter slightly (Fig. 2.5a). In G. Cot, similar trend was observed; dry

weight remained low up to 30 DPA (5 mg) then increased up to 45 DPA (34.5 mg seed kernel^{-1}) and declined thereafter at maturity (Fig. 2.7d). Maximum rate of dry matter accumulation in H-6 and G. Cot kernel was achieved on 33 DPA (3.11 mg seed kernel^{-1} day^{-1}) and on 36 DPA (2.56 mg seed kernel^{-1} day^{-1}) respectively. However, the time taken by the kernel to attain its maximum dry weight (duration of dry matter accumulation) and its increase per day (rate of dry matter accumulation) varies in both the genotypes (Chapter-2, Table-3). In H-6 maximum dry weight was recorded at 48 DPA (57.35 mg seed kernel^{-1}), whereas in G. Cot it was recorded at 45 DPA (34.5 mg seed kernel^{-1}).

In H-6 seed coat, dry weight increased slowly up to 27 DPA, achieved peak on 48 DPA (33.6 mg seed coat^{-1}) and stabilized there after (Fig. 2.5b) Whereas, in G. Cot dry weight increased up to 24 DPA (31 mg seed coat), slightly decreased and stabilized in maturation phase as shown in Fig-7b. Maximum rate of dry matter accumulation was recorded on 9 DPA (2.08 mg seed coat^{-1} day^{-1}) and 15 DPA (1.80 mg seed coat^{-1} day^{-1}) in H-6 and G. Cot respectively (Chapter-2, Table-3).

Cytoplasmic peroxidase activity

As shown in figure. 7.1a, cytoplasmic peroxidase activity in H-6 ovule was negligible initially, increased after 18 DPA with maximum activity on 30 DPA and declined thereafter. From the growth data, it is clear that sharp increase in cytoplasmic peroxidase activity coincide with decrease in water amount. In case of G. Cot ovule, considerable cytoplasmic peroxidase activity recorded during cell division phase and cell elongation phase (Fig. 7.1b). Further, there was sharp increase during maturing phase in G. Cot.

Cytoplasmic peroxidase activity in H-6 kernel increased after 18 DPA, achieved peak on 39 DPA (22.83 ΔA_{470} mg protein^{-1} min^{-1}) and declined thereafter (Fig. 7.2a). Whereas in H-6 seed coat, activity was negligible

during 0-15 DPA, followed by considerable increase in activity between 18-39 DPA and rapid fall was observed in subsequent ages (Fig. 7.2b). G. Cot kernel showed rapid increased in cytoplasmic peroxidase activity after 24 DPA followed by higher activity till maturity. Maximum activity was recorded on 33 DPA (95.70 ΔA_{470} mg protein^{-1} min^{-1}). In developing G. Cot seed coat activity remained low up to 27 DPA followed by continuous increased till maturation phase (Fig. 7.2c, d).

Wall bound peroxidase activity

Wall bound peroxidase in H-6 ovule (kernel + seed coat) showed almost similar trend to cytoplasmic activity. In H-6, Wall bound peroxidase activity was noticed during cell division phase achieved peak on 30 DPA (148.67 ΔA_{470} mg protein^{-1} min^{-1}) and declined gradually after 39 DPA. While in G. Cot, wall bound peroxidase activity remained low up to 27 DPA followed by gradual increase till maturation (Fig. 7.1b). It was interesting to note that in H-6 ovule both cytoplasmic and wall bound peroxidase activities were negligible after 39 DPA when boll opened (Fig. 7.1a).

Wall bound peroxidase activities from kernel and seed coat were measured in both genotypes through out the developmental period. As shown in figure 7.3a, ionically extracted wall bound peroxidase activity was higher in H-6 kernel as compared to cytoplasmic fraction. Wall bound peroxidase activity increased up to 33 DPA (38.45 ΔA_{470} mg protein^{-1} min^{-1}) and declined in subsequent phases (fig. 7.3a). While in case of H-6 seed coat fluctuating pattern was noticed up to 27 DPA than achieved a sharp peak on 30 DPA (140.08 ΔA_{470} mg protein^{-1} min^{-1}) and declined thereafter (Fig. 7.3b).

Like that of H-6, when wall bound peroxidase measured from G. Cot kernel and seed coat, it was found to be many fold higher than H-6 kernel and seed coat. G. Cot kernel showed rapid increase in enzyme activity up to 33

DPA (219.06 ΔA_{470} mg protein^{-1} min^{-1}) followed by gradual decrease in later phase (Fig. 7.3c). In G. Cot seed coat enzyme activity was almost stable during 3-24 DPA followed by rapid increased up to 45 DPA than declined (Fig. 7.3d). Maximum activity was obtained on 45 DPA as 997.84 ΔA_{470} mg protein $^{-1}$ min $^{-1}$.

Changes in cytoplasmic and wall bound peroxidase activity in kernel and seed coat in both the genotypes along with their water uptake rate and dry matter accumulation rate are presented in Figs. 7.4 to 7.7. In general, it was observed that when water uptake rate recorded higher, cytoplasmic and wall-bound activity remained low. It was interesting to note that in G. cot kernel showed partial parallelism with cytoplasmic and wall bound peroxidase activity and water uptake.

DISCUSSION

Major findings of the present investigation are: (i) seed coat has higher cyto plasmic and ionically wall bound peroxidase activity than kernel in both the genotypes H-6 and G. Cot (ii) in kernel tissue wall bound peroxidase activity found to be higher than cytoplasmic in both the genotypes studied (iii) G. Cot kernel and seed coat showed many folds higher activity (both cytoplasmic and wall bound) than H-6 (iv) ANOVA between two genotypes suggests that both the varieties were significantly different in their wall bound peroxidase activity (Table-1a). (v) ANOVA between the ovule components i.e. kernel and seed coat for their cytoplasmic and wall bound peroxidase activity has shown significant difference (Table-1b).

The cell wall is also considered the site of primary action of plant peroxidases (Saroop et al. 2002). Earlier work from this laboratory on cotton fiber suggested that ionically wall- bound peroxidases have inverse correlation with cell elongation (Rama Rao et al. 1982a, b, Thaker et al. 1986) and recent study on cotton seed development and peroxidase activity suggested that there is inverse correlation between water uptake and enzyme activity (Rabadia et al. 2006). In the present work, similarly insignificant or negative correlation between enzyme activity and water uptake have been observed (Table-2).

From the growth data, it is clear that when seed coat completed dry matter accumulation (24 DPA); in seed kernel it is started (18 DPA) in H-6 while in case of G. Cot higher degree of overlapping between dry matter accumulation phase of kernel (24-42 DPA) and coat (3-30 DPA) was observed. Thus, in G. Cot both kernel and seed coat compete for the available photosynthates. Regulation of water uptake by the seed organs is an important determinant of seed weight and therefore seed development. The increase in seed size is primarily a resultant of cell expansion. Rabadia

et al. (1999) suggested that slower dry weight accumulation of seed or seed fiber occurred during the period of declining water content, indicating thereby that the capacity for growth was limited by resistance to cell and/or tissue expansion.

In the present study, in general cytoplasmic and peroxidase activity remained low when rate of water uptake was significantly higher (Figs. 7.4 to 7.7). There was sharp increase in both cytoplasmic and/or wall-bound activity, with decrease in water uptake in both kernel and seed coat, thus showed inverse relationship with sink development. The present results support the earlier work of our laboratory (Rama Rao et al. 1982a, b, Thaker 1986,1998) and elsewhere (Higuchi 1985, Reinecke and Bandurski 1988, Gaspar et al. 1991,) that the peroxidase plays an important role in regulation of elongation process. The individual results of kernel and seed coat coincide with the results of Rabadia et al. (2006) that suggest cotton ovule ontogeny shows parallelism with seed ontogeny. Similarly, inverse correlations between peroxidase activity and sink development and dry matter accumulation have also been reported (Chanda and Singh 1997, Thaker 1998, Saroop et al. 2002). Further, G. Cot kernel has shown higher cytoplasmic and wall bound activity during cell elongation phase that may restrict its growth (Fig. 7.6, 7.7). It has been postulated by several authors that the action of peroxidase would be to confer rigidity to the cell wall and prevent later expansion involved in growth (Palmieri et al. 1978, Fry 1979, Lamport 1986, Goldberg et al. 1987).

In addition, changes in cytoplasmic peroxidase activity in kernel and seed coat in both the genotypes during total developmental period showed that seed coat has many fold higher activity than kernel tissue. Similar observations are obtained for the inocially extracted wall-bound peroxidase activity in developing sink organs of both the genotypes

(Figs. 7.2, 7.3). Many workers have proposed that peroxidases are major components of seed coat which particularly activate during seed coat development and promoting impermeabilization by polymerization of soluble phenolics (Egli et al. 1983, Mullin and Xu 2000). Thus, seed coat may restrict further expansion and seed size.

Cytoplasmic and wall bound peroxidase activities for ovule in both the genotypes H-6 and G. Cot (kernel + seed coat) showed clear distinct results. In H-6 ovule, cytoplasmic peroxidase remained negligible during initial phases of ovule development (3-15 DPA), whereas wall bound activity was present. Cytoplasmic peroxidase activity in H-6 was higher than wall bound peroxidase activity excluding initial phase. However, in G. Cot wall bound peroxidase remained many times higher than cytoplasmic activity throughout the total development.

From the present study, it can be concluded that cytoplasmic and wall bound peroxidases may be responsible for the controlling the site for cessation of elongation growth in cotton ovule. In G. Cot, high level of peroxidase accumulation in seed coat may be a constitutive defense reaction against infection by microorganisms (Schopfer 2001). Further, growth result and distinct peroxidase activity in seed coat suggest its role in restricting the sink development.

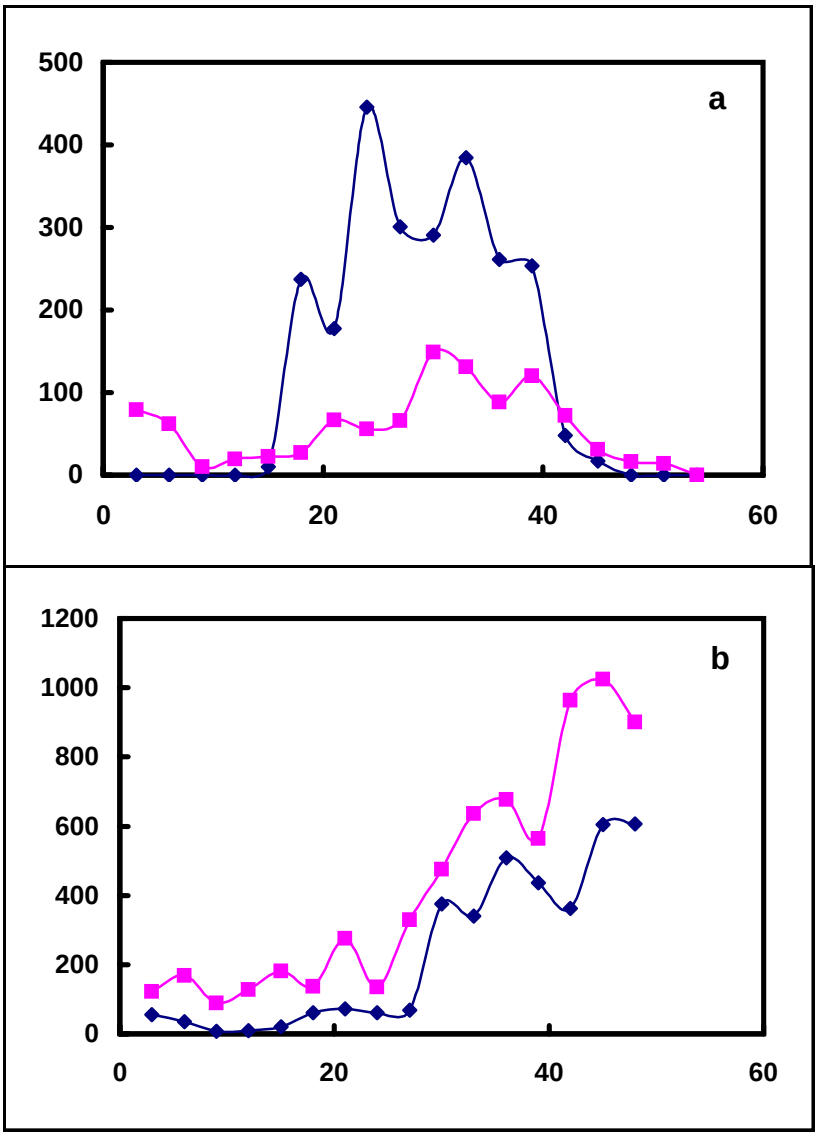

Fig-1: Changes in cytoplasmic ( ) and wall bound ( ) peroxidase activity against age of developing ovule of two cotton genotypes- H-6 (a)and G. Cot (b)

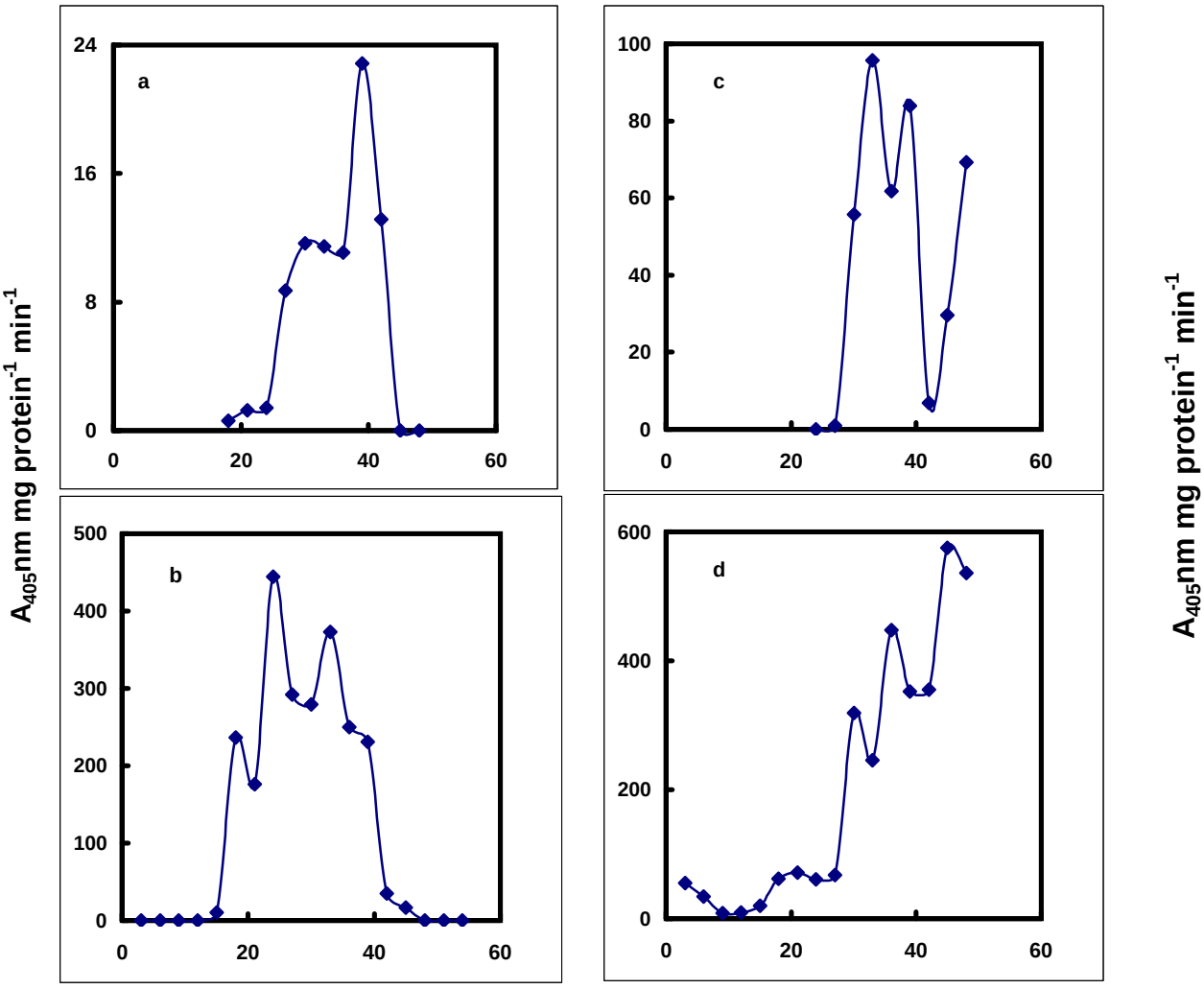

Fig-2: Changes in cytoplasmic peroxidase (activity/mg protein/min) in kernel (a, c) and seed coat (b, d) in developing ovule of two cotton genotypes H- 6 (a, b) and G. Cot (c, d). Vertical bars represent SD

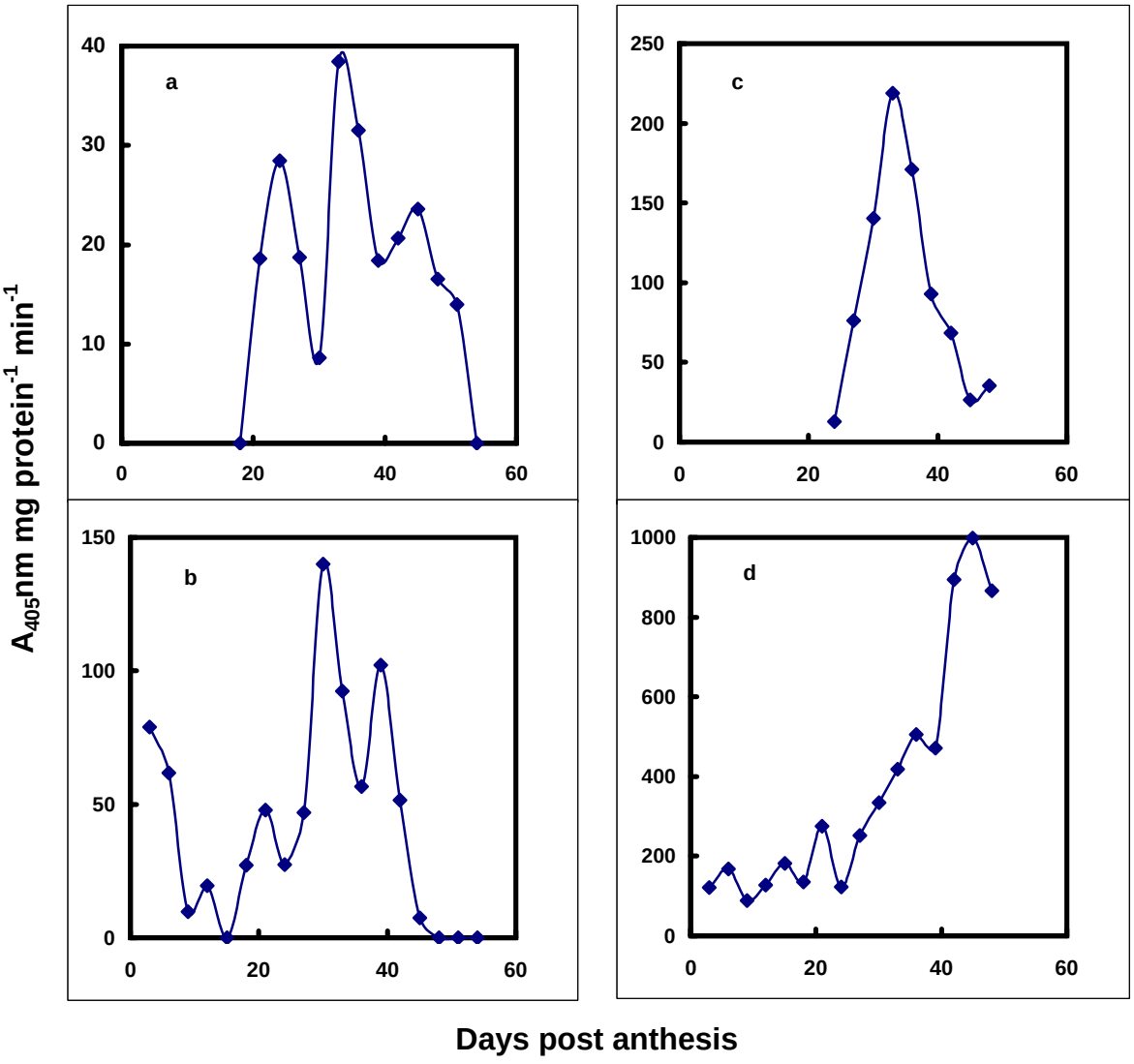

Fig-3: Changes in wall-bound peroxidase (activity/mg protein/min) in kernel (a, c) and seed coat (b, d) in developing ovule of two cotton genotypes H- 6 (a, b) and G. Cot (c, d). Vertical bars represent SD

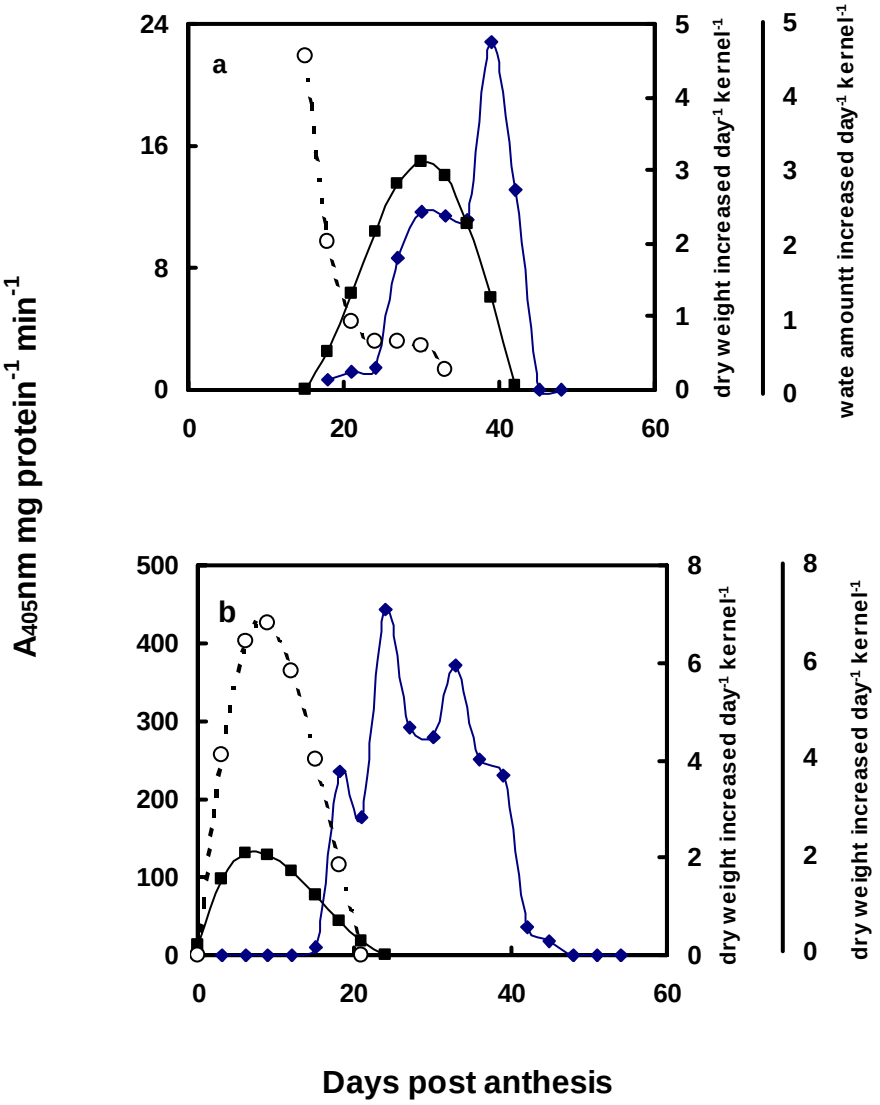

Fig-4: Changes in cytoplsmic peroxidase activity/μg protein/min) against age in developing seed kernel (a) and seed coat (b) of H-6 along with rate of dry matter accumulation () and rate of water amount uptake () .

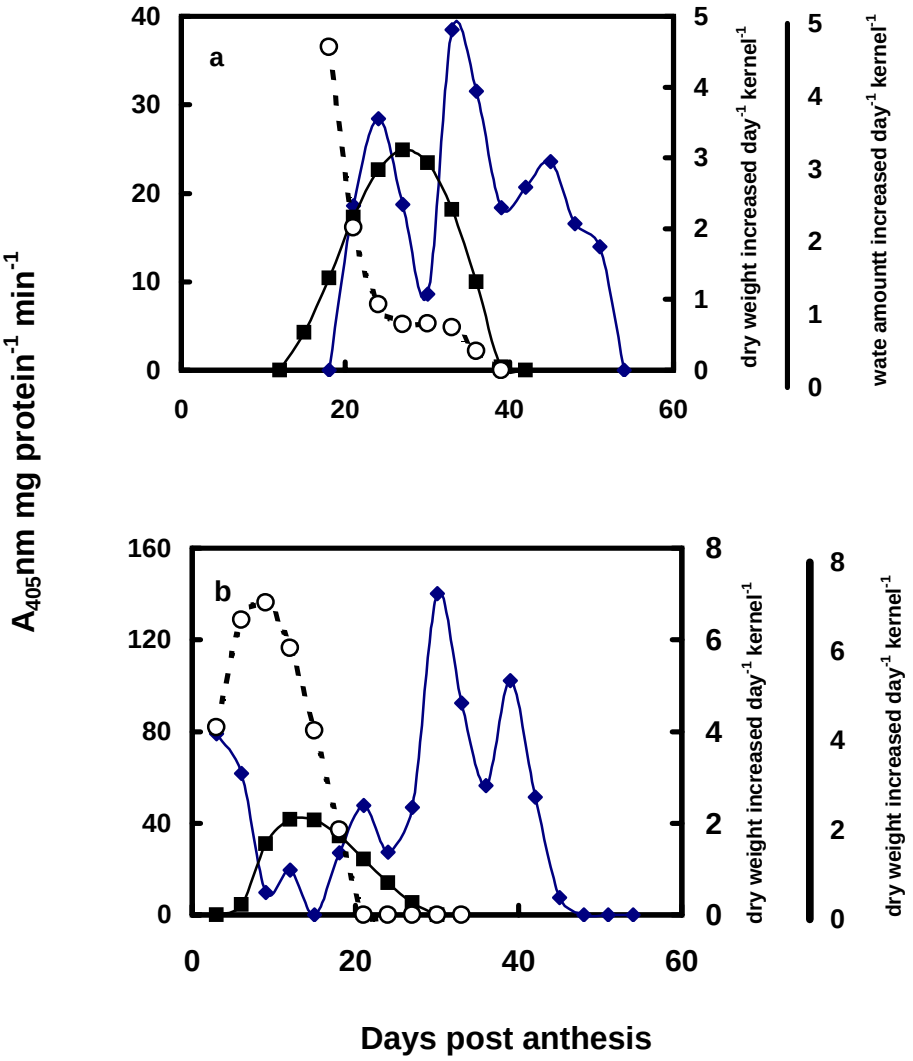

Fig-5: Changes in wall bound peroxidase activity/μg protein/min) against age in developing seed kernel (a) and seed coat (b) of H-6 along with rate of dry matter accumulation () and rate of water amount uptake () .

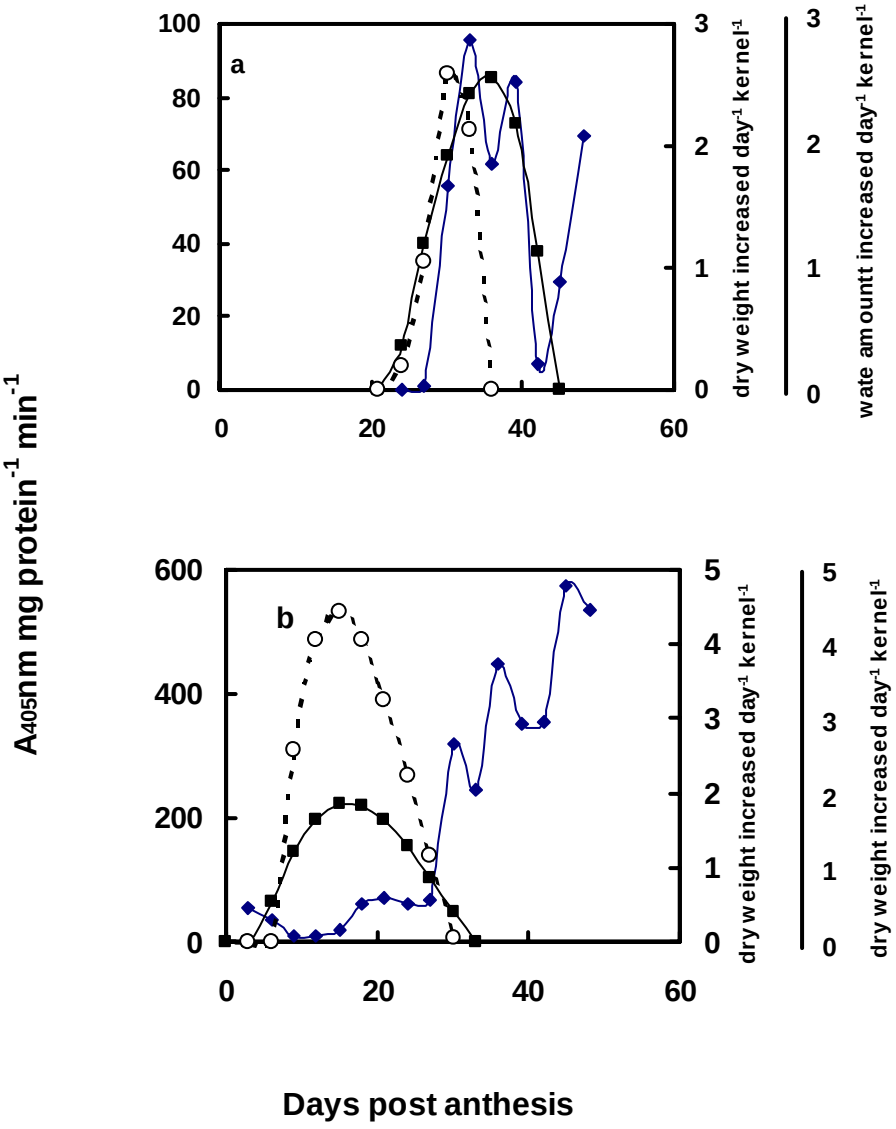

Fig-6: Changes in cytoplasmic peroxidase activity/μg protein/min) against age in developing seed kernel (a) and seed coat (b) of G. Cot along with rate of dry matter accumulation () and rate of water amount

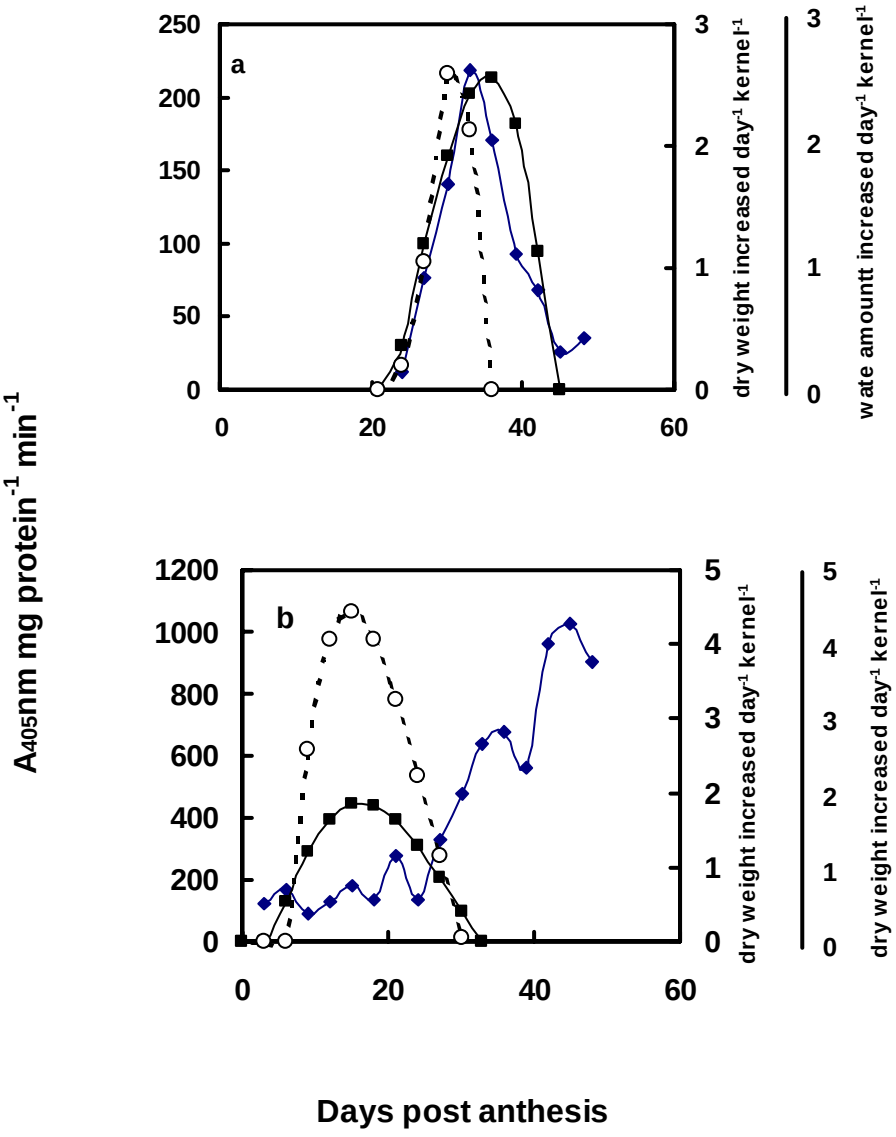

Fig-7: Changes in wall-bound peroxidase (activity/μg protein/min) against age in developing seed kenel (a) and seed coat (b) of G. cot along with rate of dry matter accumulation () and rate of water amount uptake () .

Table-1a. ANOVA between two cotton genotypes for their cytoplasmic and wall bound peroxidase activity in kernel and seed coat

Source		Cytoplasmic	Wall bound
Kernel	F-value	0.805	15.549***
	df	1,33	1,33
Seed coat	F-value	1.076	2.922**
	df	1,33	1,33

Significance level p ≤ 0.001= ***, p ≤ 0.01=, p ≤ 0.1=***

Table-1b. ANOVA between kernel and seed coat for their cytoplasmic and wall bound peroxidase activities in two cotton genotypes: H-6 and G. Cot.

Source	Peroxidase activity	F-value	df
H-6	Cytoplasmic	8.492***	1,35
	Wall bound	3.666**	1,35
G. Cot	Cytoplasmic	9.913***	1,33
	Wall bound	2.829*	1,33

Significance level p ≤ 0.001= ***, p ≤ 0.01=, p ≤ 0.1=***

Table-2. Correlation between rate of water uptake and peroxidase activity along with rate of dry matter accumulation and peroxidase activity in kernel and seed coat in two cotton genotypes: H-6 and G. Cot

Cytoplasmic activity	Kernel		Seed coat	
	Rate of Water uptake	Rate of DMA	Rate of Water uptake	Rate of DMA
H-6	- 0.513	0.533	- 0.865	- 0.801
G. Cot	0.268	0.743	- 0.447	- 0.425
Wall bound activity	**Kernel**		**Seed coat**	
	Rate of Water uptake	Rate of DMA	Rate of Water uptake	Rate of DMA
H-6	- 0.627	0.425	0.036	- 0.145
G. Cot	0.205	0.613	- 0.411	- 0.373

Chapter VIII
General Discussion

Cotton well known as “white Gold” grown worldwide in tropical and subtropical regions as an important cash crop, which gives five basic needs i.e. lint, linters, oil, meal and seed hull. India ranks first for cotton cultivation (about 8.5 to 9.0 million hectors). Government of India has launched ‘The technology Mission on cotton’ since 2000 with a view to improve production and quality of cotton (Deosarkar et al. 2007).

Cotton fibers represent an agricultural commodity of major economic importance and so extensively studied by numbers of the workers in India and abroad. Comparatively, little is known about the development of seeds and other parts of the boll (Rabadia et al.1999). Cotton seeds are extracted for its oil and protein content. Cotton fiber is a single epidermal cell of seed coat; composed of 97% of cellulose while seeds accumulate high concentrations of protein and lipid. Therefore, cotton seed is an important system to study metabolic changes during seed development.

In developing cottonseed, kernel, seed coat and fibers function as a competitive sinks throughout the seed development. They are imported assimilates for growth, maintenance and storage. Sink strength has been considered as a product of sink size and sink activity. Sink size is defined as the physical constraint and sink activity as the physiological constraint (Wareing and Patrick 1975). For maximum yield potential both, capacity of the seed to continue to utilize assimilates (sink capacity) and the ability of the maternal tissue to continue to supply assimilates are the major factors that could influence final seed weight. For maximum yield when photosynthates supply is not limiting, sink capacity plays a very important role (Thaker 1999).

In this thesis, the objective of selection of two cotton genotypes differing in their growth and final yield is to find out the relationship between in vivo hormonal content and growth phases to establish the source-sink relation. According to the “competing sinks” hypothesis, fruit growth can be limited with concentration of nutrients as they are needed for the building of new tissues and that the higher concentration of hormones in seed are necessary in order to create a strong sink. Many workers have talked about source-sink relationship, however it remains to be cleared if source restricts seed development as among the selected genotypes, dramatic difference in seed components weight and fiber length was observed. The difference can be ascribed to different ability of sink organs in mobilizing carbohydrates (Mohpatra and Sahu 1991, Wang et al. 2006). Similar results have been observed in the present study, on the basis of dry matter allocation, boll wall has high negative correlation with kernel and fiber and not with seed coat that suggests its role in kernel and fiber development. However, in G. Cot bract has high negative correlation with seed coat, this might have restricted import of assimilates to kernel and hence kernel growth (Fig. 2.2). Moreover, in H-6 rate of DMA and rate of water uptake was higher in all boll components along with rate of fiber elongation than G. Cot (Table-3, Chapter-2). Data presented showed that all the boll components of both the genotypes are significantly distinct on the basis of their dry weight (Table-1).

In agriculture, crop analysis is done at three different levels (i) In first approach the crop performance is with the help of molecular markers i.e. screening of genes of interest using genome analysis (ii) in another approach the morphological traits are used by the breeders to identify the promising cultivar and (iii) in the third approach, key metabolites like hormones are studied. Phytohormones have regulatory effects on both the other approaches and its influence on enhancement in yield is well known.

Plant hormones regulate plant growth and development from germination to senescence, several plant growth regulators interfere with the endogenous balance of hormones (Fletcher et al. 2000). Understanding the hormonal control should offer opportunity to modify plant growth for better agricultural practices. Auxins and gibberellins are known to promote cell elongation in higher plants (Demason 2005, Le et al. 2005). However, there is a great deal of controversy for mechanism of their action in cell elongation. Abscisic acid is known as an effective inhibitor of growth in various plant species (Ravishankar et al. 1995, Ramirez-Parra et al. 2005) and an effective promoter of fruit abscission (Gomez-Cadenas et al. 2000). It is well documented that Cks play an important role in cell division and maintenance of cell cycle (Swarup et al. 2002, Nordstrom et al. 2004).

In general, phytohormones are present in three different forms (i) free form that is readily available form for plant growth and development (ii) conjugated forms, which are considered as storage form and (iii) oxidized form. Any changes in the free endogenous concentrations of these phytohormones reflect as changes in morphological, physiological, anatomical and at gene expression level. Conversion of free to conjugated forms or visa versa balances the physiology of the developing sink organ. As it is reported in the present study that contents of free and conjugated forms of different hormones i.e. IAA, PAA, GA, zeatin and ABA vary at different physiological growth phases (Table-2). Comparative analysis of phytohormones in two cotton genotypes is presented in chapters 3 to 7.

In the present study, free IAA and PAA have positive and significant correlation with fiber elongation in both the genotypes and thus they play an important role in fiber length determination (Table-3, chapter-3). There is positive relationship between water amount and endogenous

PAA level suggesting its role in seed development through water uptake (Table-4, chapter-3). Further, in H-6 PAA content was distinctly higher in kernel, fiber and boll wall in contrast to G. Cot where PAA content was recorded higher in seed coat and bract; different distribution pattern of PAA content amongst the boll components might be reason for different yield. In the present study, many times higher endogenous GA content in H-6 fruit (boll) as compared to G. Cot coincides with the higher weight and larger size boll. The observation of endogenous GA estimations lead to two probabilities (i) higher GA level participates in controlling seed size and finally high yield of the genotype (ii) higher GA level is responsible for the longer fiber length.

In H-6, kernel and seed coat free zeatin level remained higher during cell division and expansion phases than G. Cot. It is assumed that the lower level of zeatin during early development phase is the limiting factors for seed development in G. Cot. It has been believed that plant hormones are closely associated with seed development with their regulation on the sink size, either by mediating the division and elongation or controlling the import of assimilates to seed (Wang et al. 2006). Further, it was observed that in vivo zeatin has no effect in fiber length determination because G. Cot fiber possesses more zeatin content than H-6. Though there was not much difference in seed (kernel+seed coat+fiber) zeatin content but it was distinctly different in boll because in H-6 boll, 28 seeds are present while in G. Cot there are only 18 seeds. This observation made it clear that seeds/ boll is an important factor in yield determination as productivity changed due to both weight per seed and number of seeds per boll. In this thesis, higher level of free ABA in G. Cot seed coat and its accumulation in early period of kernel development revealed the fact of limited seed growth in G. Cot as compared to H-6. ABA level increased after completion of fiber elongation in both the genotypes suggesting that elevated free ABA level may be a signal for initiation of secondary wall thickening in fiber.

Plant growth and development are regulated by the interactions between the endogenous factors, especially hormones (Novakova et al. 2005). Marked variation in hormonal levels between both the genotypes along with all developing boll components were recorded with accurate and sensitive immunoassay (Tables-1, 2). Distinct differences in the endogenous concentrations of PGRs from all the boll components between these two genotypes justified their growth differences and proved the proposed hypothesis in this thesis that changes in endogenous phytohormone regulate growth and development of cotton ovule/ seed development. Fiber length and seed index varies among species and genotypes and is influenced by a variety of genetic and physiological factors. It is considered that endogenous hormonal levels control the physiology of developing cotton fruit (Gialvalis and Seagull 2001).

Plant hormones are defined as traveling messengers that provide signals to responsive cells after perception of a developmental or environmental condition at the sensing site. Endogenous hormone concentration is a balance between synthesis, degradation, transport, and conjugation (Taylor and Cowan 2001). Sometimes they act synergistically in promoting the growth; sometimes they substitute for one another and sometimes they act antagonistically. In the present thesis work, ratio has been worked out between promotory to inhibitory hormones (Figs. 8.1 to 8.10).

IAA to ABA

As shown in figure 8.1, free IAA to ABA ratio in H-6 kernel remained more than one throughout the development period while in G. Cot it was higher during 21-36 DPA (elongation phase) and declined thereafter. In seed coat of both the genotypes the ratio was less than one but in G. Cot it was negligible during cell division phase (Fig. 8.1b). In case of H-6

fiber, during elongation phase free IAA/ABA ratio was near to 2.8 to 1.0 while in G. Cot it was less than 0.4 during elongation phase (Fig. 8.1c). Yang et al. (2001) showed that the ratio of ABA to IAA increased rapidly after 30 DPA. It has been suggested that the ratio of ABA to IAA in fibers could have a regulating role on cellulose accumulation and secondary wall thickening. However, some investigators reported that GA3 and IAA had no effect on cellulose synthesis, kinetin inhibited it slightly, but ABA was stimulatory (Jaquet et al. 1982, Francey et al. 1989). IAA to ABA ratio was more than 1 in G. Cot boll wall during total period studied while in H-6 for the same period it was less than 1. For bract, it remained less than 1 in both the genotypes but was higher in G. Cot.

Conjugated IAA to ABA level in H-6 kernel was near to 1 throughout its development and ratio was also higher during later phase (Fig. 8.1d). In G. Cot kernel, ratio was less than one during elongation phase. In H-6 seed coat ratio was less than 1 during total period, while in G. Cot it was less than 1 during cell division and cell elongation phases but remained 1 or more than 1 during dry matter accumulation and maturation phase (Fig. 8.1e). In case of H-6 fiber, ratio was 4.5 to 1.5 during elongation phase (9-21 DPA), near to one during secondary thickening phase and declined at maturation phase while in G. Cot it was less than 1 throughout the development period (Fig. 8.1f). Continuous decrease in IAA to ABA ratio suggests that IAA might be utilized in fiber elongation. In H-6 boll wall, ratio was near to 0.8 to 1.8 till 39 DPA (boll opened) and declined thereafter while in case of G. Cot; it was less than 1 throughout the period (Fig. 8.2c). In case of bract in both the genotypes, no clear trend was observed. Although during 15-36 DPA ratio was higher in G. Cot than H-6 bract (Fig. 8.2d).

PAA to ABA

In H-6 kernel, PAA to ABA ratio was in the range of 1 to 9 throughout the kernel development. In case of G. Cot, it was less up to elongation phase, increased during DMA phase (Fig. 8.3a). In seed coat, PAA to ABA ratio was 1 or more than 1 during total period studied in H-6. Whereas, in G. Cot it was negligible during cell division phase then it increased and remained more than 1 till boll opened and declined thereafter (Fig. 8.3b). In H-6 fiber, ratio was more than one throughout fiber development whereas in G. Cot, it was negligible up to 24 DPA (till elongation phase) and remained more than 1 during DMA phase (Fig. 8.3c). Kosmidou-Dimitropoulou (1986) considered that auxin is necessary for secondary wall thickening. In G. Cot bract, ratio was more than 2 till boll opened then declined while it was distinctly less in H-6 bract than G. Cot throughout the study period (Fig. 8.3b) but no clear trend was observed in case of boll wall.

GA to ABA

In H-6 kernel, GA/ABA ratio was more than 1 up to 36 DPA (elongation phase) while in G. Cot, ratio was near to 1 or more than 1 during 27-36 DPA, rest of the ages it was les than 1 (Fig. 8.5a). In H-6 seed coat, ratio was nearly 1 or more than 1 during 30-39 DPA and rest of the time it was around 0.6 whereas, in G. Cot seed coat, ratio was nearly 0.5 or less than it (Fig. 8.5b). Ratio of GA/ABA was more than 1 throughout the developmental period in H-6 fiber while in G. Cot fiber, it was less initially (9-24 DPA; elongation phase), and remained more than 1 during secondary thickening and maturation phases (27-45 DPA, Fig. 8.5c). Up to 15 DPA, GA/ABA ratio was more than 1 in G. Cot boll wall then it was declined up to 21 DPA and increased gradually. However, up to 15 DPA it was negligible in H-6 boll wall. In bract of both the genotypes GA to ABA ratio was high up to 20 DPA and then declined (Fig. 8.6a). But in G. Cot it was ranging from 5.5 to 1 while in H-6 it was nearly 0.7-0.8 only (Fig. 8.6b).

Zeatin to ABA

In H-6 kernel, free zeatin/ABA ratio increased from 0.6 to 1.2 during 18-30 DPA then declined while in G. Cot kernel fluctuating trend was observed (Fig. 8.7a). In H-6 seed coat, zeatin to ABA ratio was more than 1 during cell–division phase. In contrast to this, it was negligible in G. Cot (Fig. 8.7b). Moore-Gordon et al. (1998) proved in small fruit phenotype in avocado that imbalance in Ck to ABA ratio through reduced Ck synthesis or accumulation of ABA, might have impact on cell division cycle activity and final fruit size. In H-6 fiber, ratio was nearly 0.5 throughout the developmental period while in G. Cot it was more than 1 up to 18 DPA and then declined (Fig. 8.7c). In H-6 boll wall, ratio was negligible up to 9 DPA then it increased and remained between 0.4-1.4 during 12-30 DPA. In G. Cot boll wall, it was negligible for longer period up to 27 DPA (Fig. 8.8a). In H-6 bract, ratio was less than 1 and moreover, it was in range of 0.5-0.8 initially then it was negligible while in G. Cot bract GA/ABA ratio was near to 1 or more than 1 throughout the total period (Fig. 8.8b).

Conjugated zeatin to ABA ratio was less than 1 in kernel of both the genotypes where G. Cot kernel has still less value than H-6 (Fig. 8.7d). In contrast to this, in seed coat of G. Cot has higher value than H-6 though in both the cases it was less than 1 (Fig. 8.7e). In fiber of G. Cot, ratio was higher (more than1) initially up to 15 DPA and during later stage (27-45 DPA). While in H-6 fiber, value of zeatin/ABA ratio was less than 0.5 throughout the period (Fig. 8.7f). In case of boll wall, zeatin/ABA ratio was less than 0.5 in G. Cot and it was 0.5 to 1.0 in H-6. Moreover, zeatin/ABA ratio in bract was fluctuating (in the range of 0.5 to 1.0) throughout the period of study in both the genotypes (Fig. 8.8d).

Since IAA is thought to act synergistically with Ck in the control of cell division, the decision by a cell to divide might be mitigated by the digital output of analogous phytohormone signals that stimulate and inhibit cell cycle activity (Grill and Himmelbach 1998). Ck stimulate the oxidative

catabolism of ABA (Cowan et al. 1999). Together with Ck and ABA, IAA is also known to play a role in cell division (Jacobs 1995) and the highest level of auxin is found in regions of active cell division. In Bromeliad, Ck zeatin/IAA levels could be thought of as coordinated signals, controlling the final phase of vegetative and reproductive development (Mercier and Endres 1999). Nefed'eva and his co-workers (2005) have explained from impulse-pressure treatment on buckwheat seeds that IAA+zeatin/ABA ratio characterizes the suitability of conditions for the growth of vegetative organs of embryo. Moreover, they showed that an increase in the GA_3/ABA ratio within 3 h after soaking turned out to be necessary for normal seed germination.

Promotory to inhibitory hormone ratio

In H-6 kernel tissue, promotory to inhibitory ratio (IAA+PAA+GA+zeatin/ABA) was more than 5. Additionally, it was declined during maturation phase. In G. Cot, the ratio was more than 1 but less than that of H-6 (Fig. 8.9a). In seed coat, ratio was more than 1 in H-6, throughout the developmental period while in G. Cot seed coat it was negligible during cell division phase and remained more than 1 during rest of the three developmental phases (Fig. 8.9b). In H-6 fiber, it was more than 3 during total period of study while in G. Cot it was 1-3 initially then declined to negligible level and increased from 3-7.5 during secondary thickening phase, declined at maturation though it was more than 1 (Fig. 8.9c).

In protective organ like boll wall ratio of promotory to inhibitory hormones was more than 2 in H-6. However, during initial period and after boll opening it was negligible. In case of G. Cot, it was more than 2 throughout the period of study (Fig 8.10a). In H-6 bract, ratio was more than 1 till 33 DPA then it reached to negligible level while G. Cot bract has clearly higher ratio as compared to H-6 (Fig. 8.10b).

In general, in seed components promotory hormone content or hormonal ratio of promotory to inhibitory was higher in H-6 and in contrast to these results, G. Cot showed higher ratio in protective organs. These results led to the speculation that transport of promotory hormones from protective organs like boll wall and bract (source) to sink organs (kernel, seed coat, fiber) is restricted in G. Cot, which may regulate sink size development.

From the present study, it is proposed that the relatively higher ratio of promotory to inhibitory hormone might be beneficial for better seed index and long staple length in H-6. The study of individual boll components and *in vivo* and *in vitro* hormonal metabolism allowed a detailed picture of balance hormone theory in cotton boll.

Endogenous growth regulators work in unison to control the development of the seed/plant is widely accepted among the plant physiologist and so the effects of combined application of GA, NAA and Kinetin on cultured ovules (one day post anthesis) of two cotton genotypes was studied. To test the *in vivo* results and synergistic or antagonistic effects of hormones on different growth parameters of cotton ovules *in vitro* experiments were performed with different combinations of promotory and inhibitory hormones.

With exogenous application of auxin (IAA, PAA and NAA) seed size and dry weight increased that leads to conclusion that auxin is involved in cell division and dry matter accumulation processes. In present *in vitro* study addition of auxin in media does not affect the water uptake in the seed, although, marked promotion was observed in seed size. This lead to the assumption that water uptake may be an independent parameter in the process of cell elongation.

When gibberellic acid was supplied to media percent promotion was higher in G. Cot than that of H-6 for all four parameters studied proved the hypothesis of *in vivo* experiments that G. Cot may be deficient in endogenous GA content. Initial difference in water amount and seed size in control was nullified with GA_3 treatment. These results led to conclusion that GA_3 control seed size by regulating water amount. However, statistical analysis showed that GA_3 alone was not effective on seed dry weight (Table-3a, chapter-4). *In vivo* and *in vitro* experiments have proved that GA and auxin has an important role in fiber elongation and seed size determination. Different results observed here for auxin and GA provides compelling evidence that the two growth regulators act by different mechanisms. When GA was supplemented with NAA, maximum promotion in all studied parameters like dry weight, water amount, fiber length and seed size was observed. It can be speculated that GA and auxin act synergistically or additively. Similar results have been observed by Sata et al. (2002) and Ross et al. (2003).

Likewise, with KiN application initial difference in dry weight and seed size was nullified while significant difference in water amount was reduced with it (Table-1, chapter-5). As it was observed with GA_3 application, percent promotion was many folds higher in G. Cot suggesting that it may be deficient in endogenous zeatin level. There was close correlation obtained between water amount with seed size and dry weight by exogenous application of KiN or GA_3+NAA+KiN. These results led to conclusion that Ck has synergistic relationship with GA and auxin (Iqbal et al. 2006).

Further, cultured experiments showed that ABA application was inhibitory for water amount and fiber length. When GA and NAA were added along with ABA recovery was observed in all studied parameters. Moreover, complete elimination of ABA from media, showed maximum promotion suggesting its antagonistic effects with GA and auxin (Nayyar et al. 1989, Mahouachi et al. 2005).

It is well documented that cell wall from fibers cultured *in vitro* are remarkably similar to those derived from the fiber of field grown cotton plant, in terms of composition during development (Yang et al. 2001). Although final length of the cultured fiber was shorter than that of the field grown plant; entire developmental sequences of both *in vivo* and *in vitro* fiber were quite similar.

It has been suggested that the effects of exogenous hormone depend on the variable endogenous hormone levels and the changes in hormonal sensitivity over time. In short, from the *in vivo* and *in vitro* results it is proposed that plant hormones work in unison and final physiology depend upon their balance ratio. Similar results have been reported in *Merremia emarginata* (Patel and Thaker 2007). Control of plant hormone homeostasis is crucial for normal organ development in plant.

One way to minimize the limitation of the localization of the site of hormonal metabolism is to measure the activity of key enzymes involved in phytohormone metabolism (Taylor and Cowan 2001). Earlier work in our laboratory suggested that peroxidase has inverse correlation with sink size development; therefore in the present work, cytoplasmic and wall bound peroxidase activities were checked in developing cotton ovule parts (kernel and seed coat) as they are considered as strong sink**.** Role of peroxidase and elongation process, especially in cotton fiber is well known therefore it was eliminated in the present work. It was observed that seed coat has higher cytoplasmic and wall bound activities than kernel in both the genotypes. Therefore, it is concluded that seed coat is limiting factor for seed size development and on an average higher peroxidase activity in G. Cot as compared to H-6 may restrict its sink size.

The uncertainties of interpretation arised from the unknown (or unmeasured) amount of growth substances absorbed with its exogenous

application, its distribution and the extent to which endogenous levels are altered by these treatments. The second important point is treated tissues are composed of variety of cells which may vary in their developmental phase. Thus, it is suggested that isolated system is required to compare and study the response of endogenous hormonal concentrations with respect to physical or physiological treatment. The biological effects of stress conditions in plants are a widely studied topic (Zaka et al. 2002) though effects of abiotic stress (radiation) on hormonal regulation are rather scarce.

Cotton fiber offers many advantages for irradiation study as it is an isolated system including (i) it is a single-cell epidermal trichome on the seed coat and provides large populations of nearly synchronously growing cells (ii) cotton genotypes show wide variation in the staple length which can help us to understand the possible correlation with endogenous concentration of hormone with cell elongation and (iii) whether the difference is hormone-induced or due to some physical factor(s) can be tested *in vitro*. In order to develop a framework for assessment of the impact of irradiation, it is necessary to establish the relationship between exposure and accumulated dose in the plant samples, which can be facilitated with the single cell system. Therefore, in the present study cotton fiber was selected to study the influence of radiation on endogenous hormonal levels. These results can help to understand the role of irradiation on endogenous free phytohormones in unicellular system, which further can be, exploited for GMO production.

In the present study, after ^{7}Li irradiation (45 MeV, fluence 10^{7} p/cm^{2}), released endogenous levels of IAA, PAA, GA and ABA were measured from G. Cot fiber. Zeatin content was not measured as *in vivo* study suggested that it has no direct influence on fiber development especially

in fiber elongation. In the present work, hormonal content was expressed as μg PGR released per gram fresh weight sample.

Endogenous IAA content measurement showed that with respect to control, it was increased in treated 24, 27 and 33 DPA samples and dose-dependent response was also observed in 27 and 30 DPA. In case of 24 DPA, there was no additional increase in free IAA level when dose was increased from 1 Gy (31.9 μg) to 4 Gy (30.8 μg) (Fig. 8.11a). In 30 DPA, only 4 Gy (42.3 μg) dose was found to be effective. As compared to control, gradual increase in PAA content was noted in 30 and 33 DPA samples with 1 Gy (16.67 μg in 30 DPA and 19.09 μg in 33 DPA) and 4 Gy (18.21 μg in 30 DPA and 22.5 μg in 33 DPA) doses. However, there was no distinct difference between control and treated sample of 24 DPA (Fig. 8.11b). Thus, endogenous PAA level is more radiosensitive as compared to IAA level.

Similarly, when GA content was measured after irradiation, a distinct increase in GA level in 30 and 33 DPA was recorded. Though, in 24 DPA this difference was less but increase with 1 Gy (12. 87 μg) and 4 Gy (13.34 μg) treatment was observed with respect to control (12.18 μg) (Fig. 8.12a). Further, in 30 DPA GA content in control sample recorded was 5.93 μg while with 1 Gy dose it was increased up to 8.97 μg and with 4 Gy nearly double value was obtained (10.76 μg). In case of 33 DPA sample, with increasing dose many times increase in GA content was observed. In control, GA level was 4.17 μg, which increased to double value with 1 Gy dose (9.87 μg) and with 4 Gy released GA content (12.04 μg) was three times higher than the control. Thus, dose dependent response was clear in the samples of later stages. The probable reason for these observations during earlier period of fiber development may be that the free content remained higher while in later stages conjugation of free forms takes place.

Therefore, radiation treatment was not much effective in 24 DPA as compared to 30 and 33 DPA.

When ABA level was measured from different age samples, it remained almost same in control and treated samples excluding 27 DPA (Fig. 8.12b). These results suggest that radiation has no clear effect on *in vivo* ABA content in cotton fiber. This data led us to propose that some different mechanisms or responses might be operating endogenous hormonal regulation at cellular level.

In the present study, an attempt made with monolayer, which may help to understand cellular metabolism with dose-response effect precisely. Major observations of this study are (i) compared to control, irradiated fiber samples showed higher amount of endogenous hormonal level (ii) dose dependent RBE (relative biological effectiveness) was observed.

In a multicellular system, different types of cells have different physical status and biochemistry and therefore the radiation doses applied to the system may vary insensitivity according to their growth condition (biological variability). In addition, there are two probabilities for radiation effects on increased level of endogenous hormones (i) radiation cause de novo synthesis of free hormonal level to overcome the physical stress and (ii) due to radiation effects, conjugated forms converted to free form. Additional research is needed to quantify the effect of irradiation on *in vivo* hormonal regulation and improvement of short staple cultivar for cotton economy. Data presented in this thesis, suggests thereby that *in vivo* hormonal levels are radiosensitive at least in cotton fiber. Heavy ion radiation states an objective, which helps in understanding the endogenous hormonal changes or receptor level changes. Thus, it is an important technique for plant biologist or plant biotechnologist to improve low yielding variety through increasing

endogenous hormonal level and perhaps may be useful in plant breeding programme.

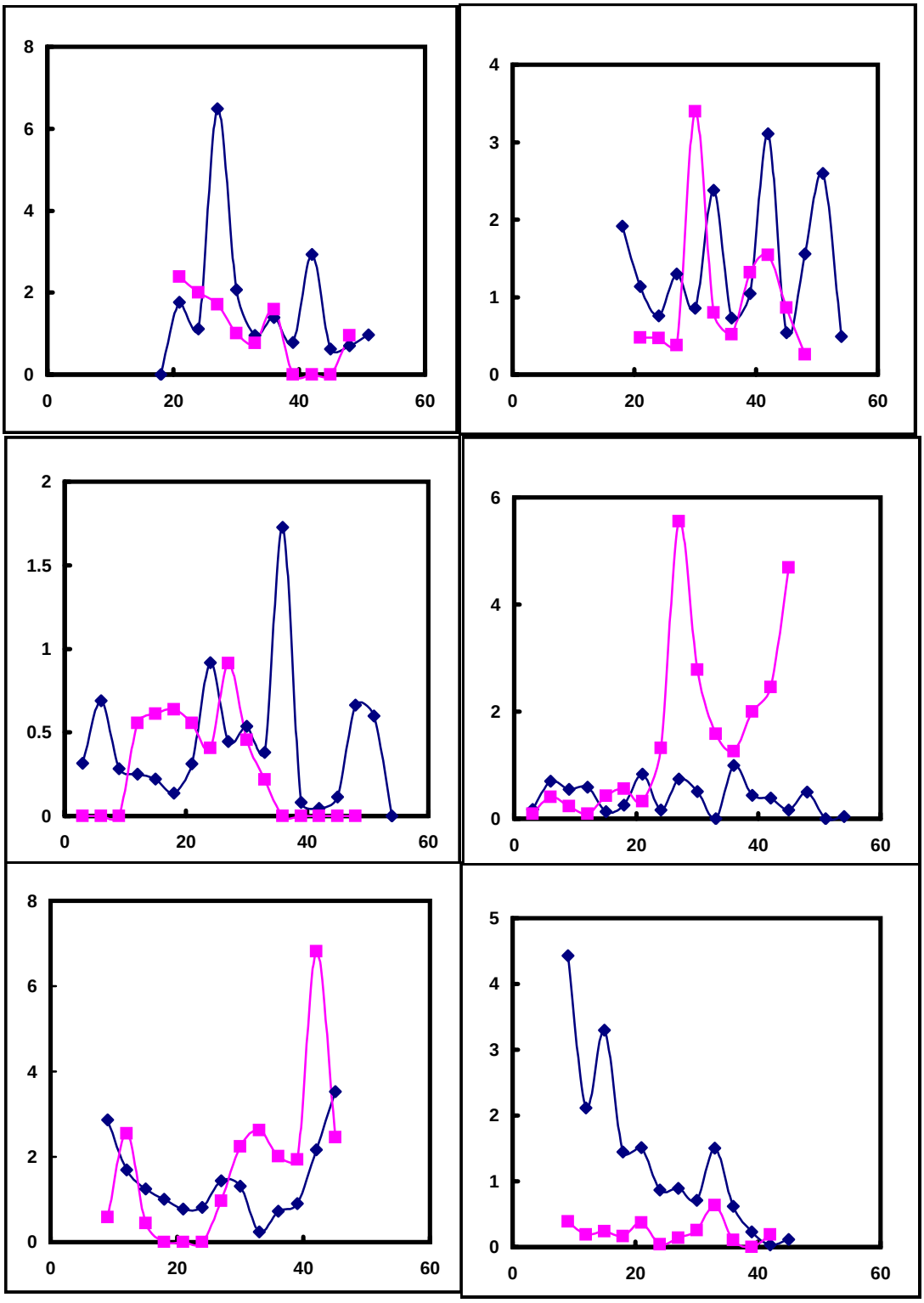

Fig-1: IAA : ABA ratio

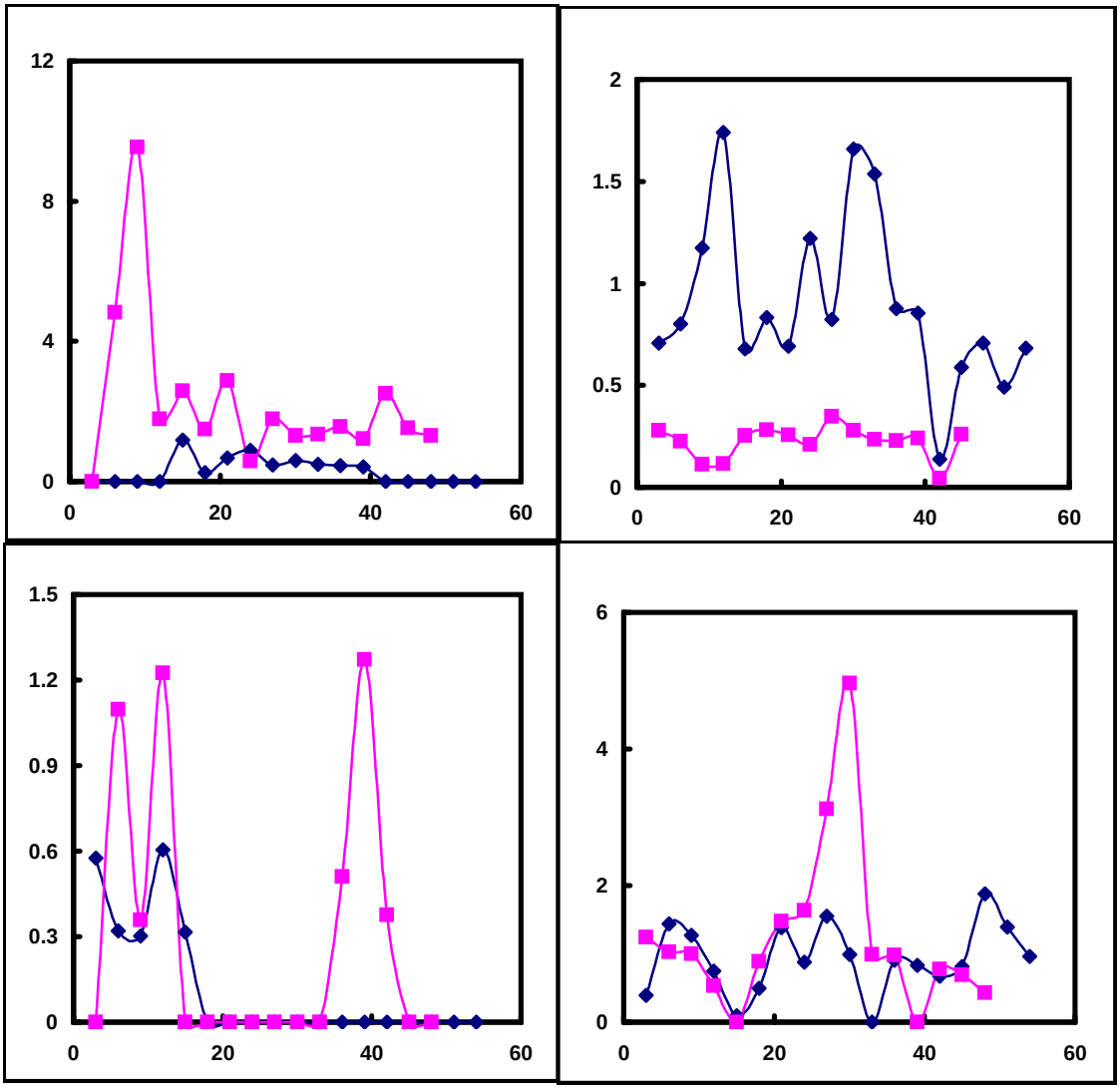

Fig-1: IAA : ABA ratio

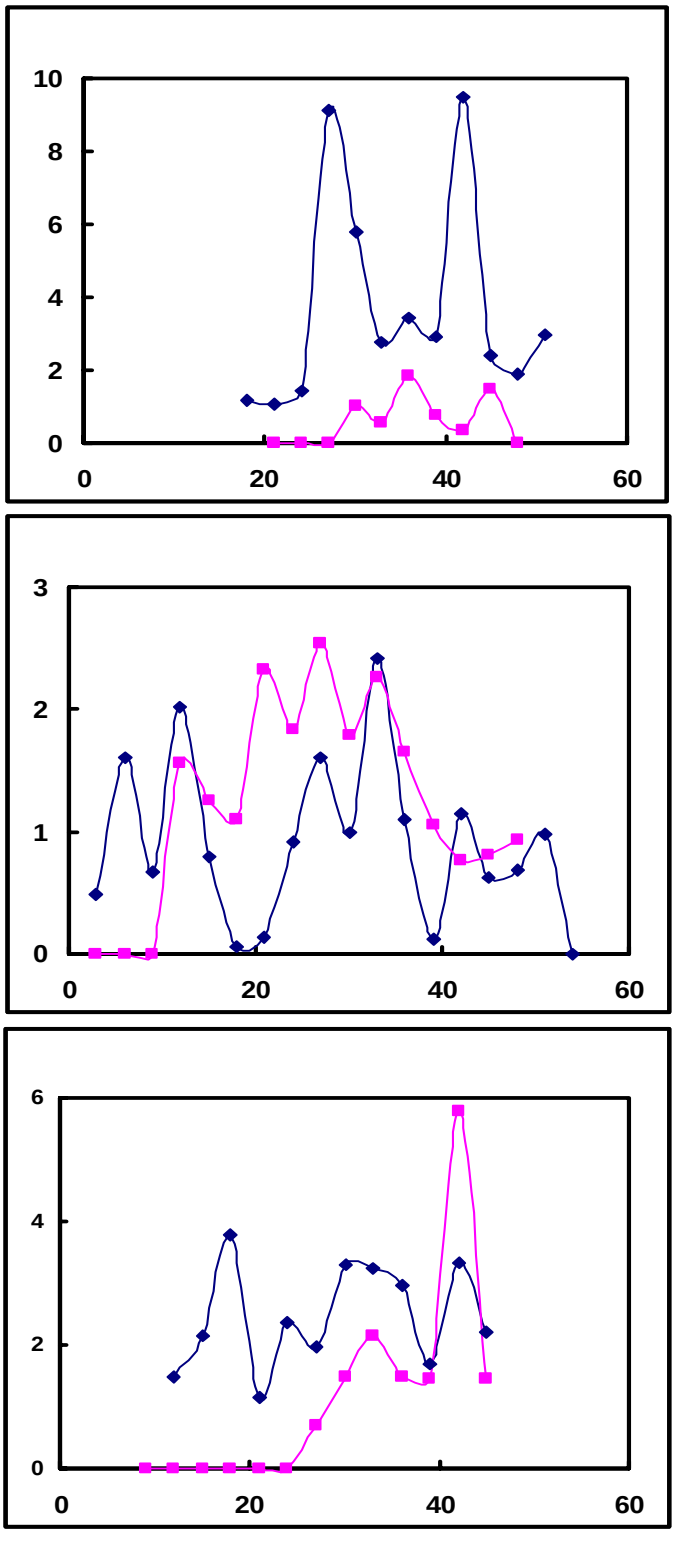

Fig-3: PAA : ABA ratio

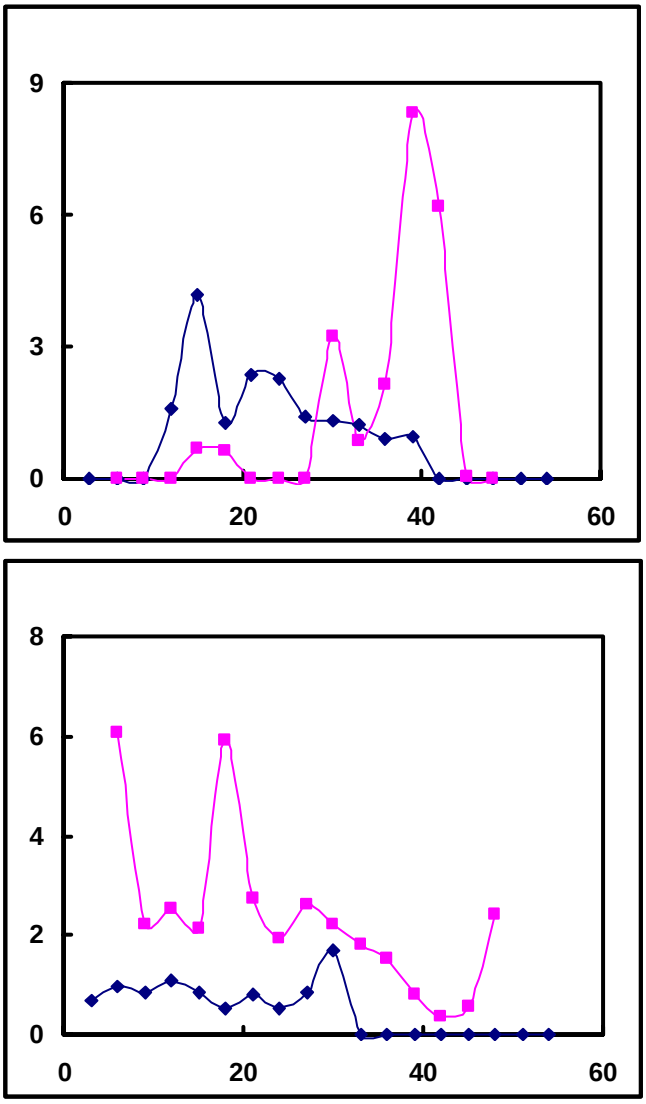

Fig-4: PAA : ABA ratio

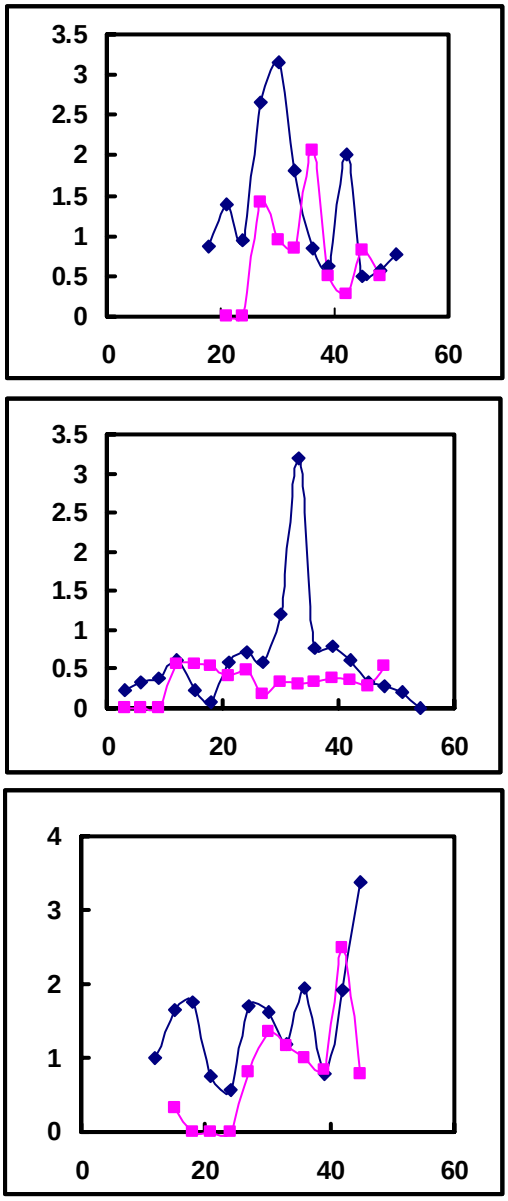

Fig-5: GA : ABA ratio

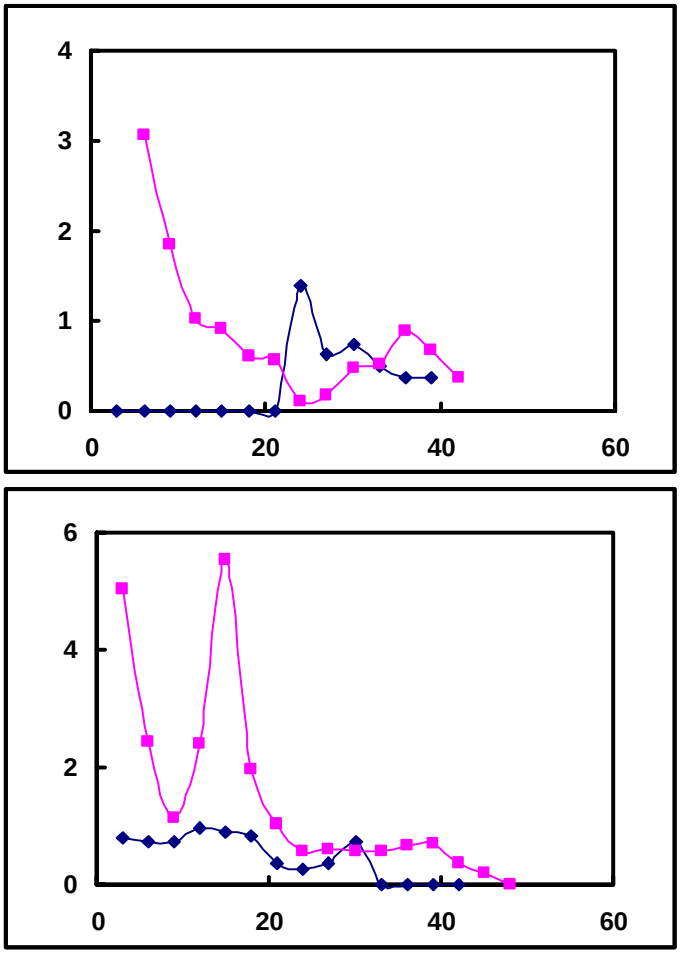

Fig-6: GA : ABA ratio

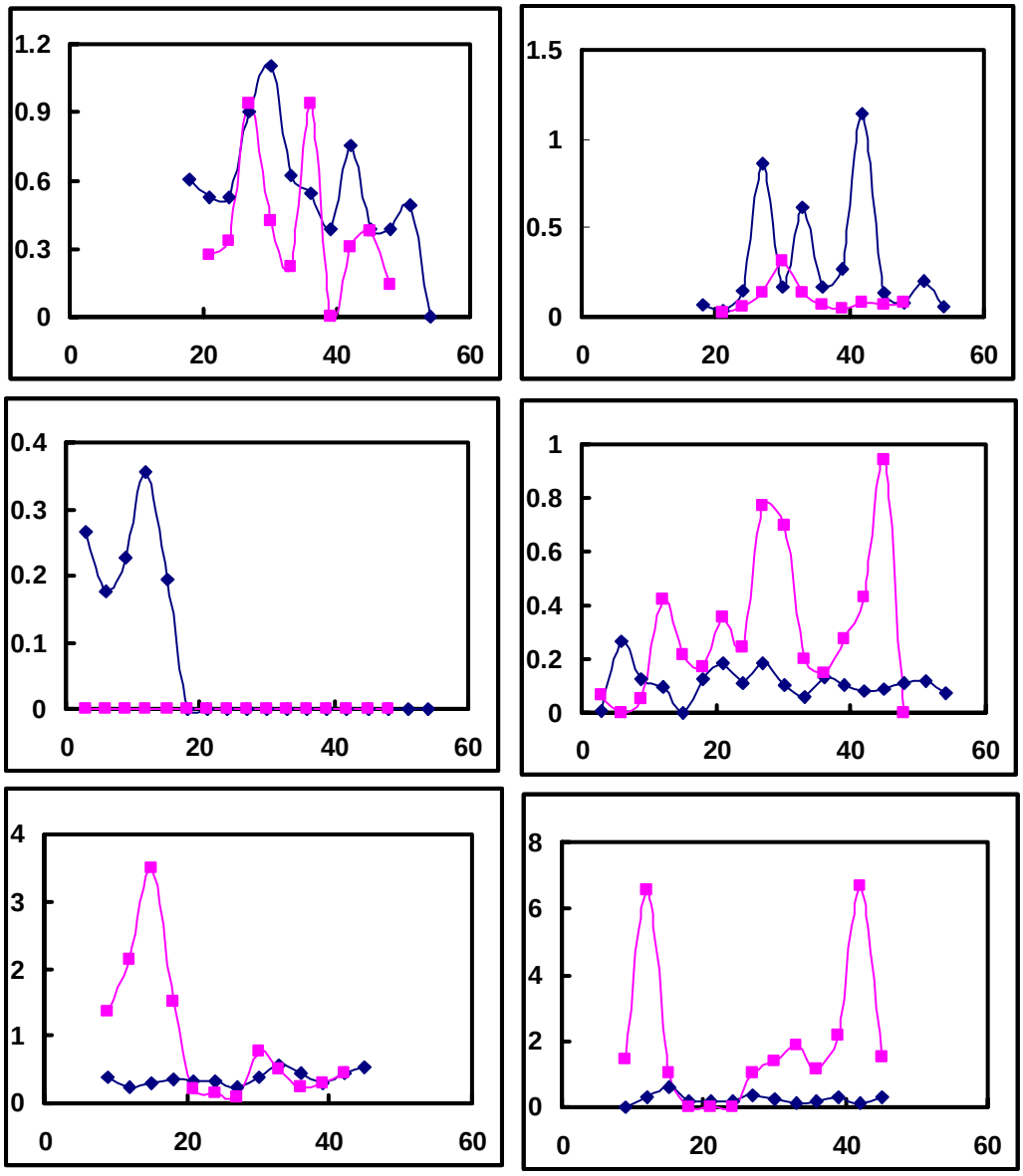

Fig-8: Zeatin : ABA ratio

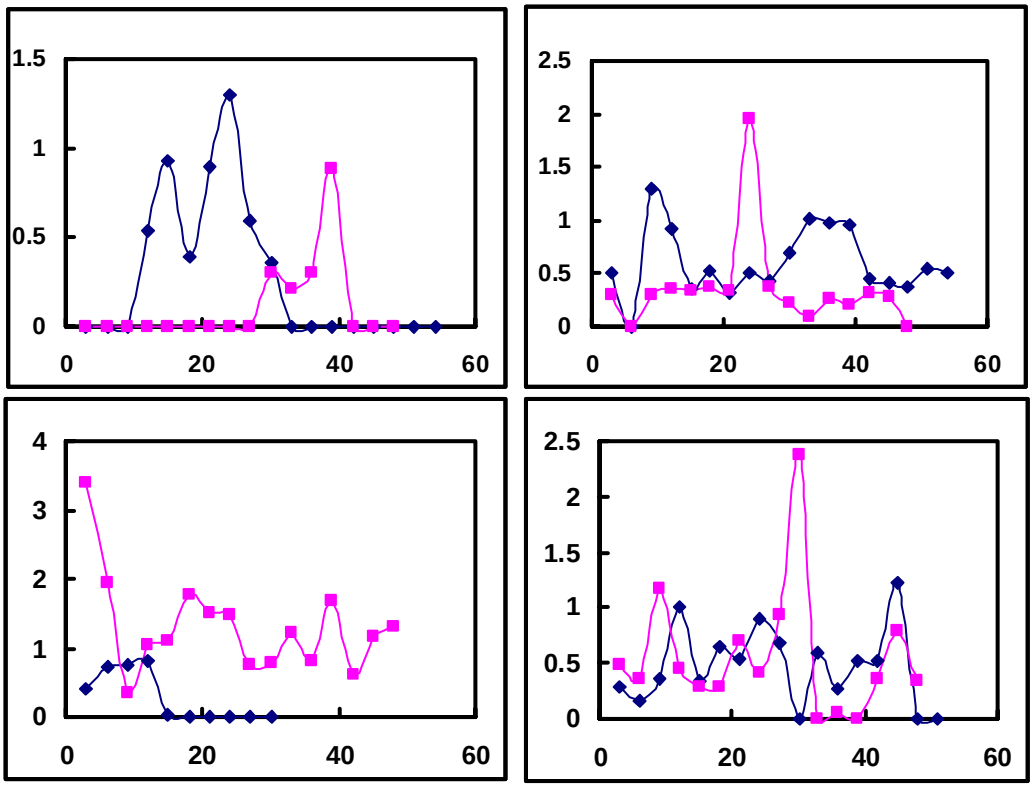

Fig-8: Zeatin : ABA ratio

TAble-1. p level significance between two cotton genotypes in different boll components for mean value

Parameter	Boll components				
	Kernel	Seed coat	Fiber	Boll wall	Bract
Dry weight	0.01	NS	0.001	0.001	0.001
Water amount	NS	NS	0.05	0.01	0.1
Free IAA	0.1	NS	0.1	NS	NS
Conjugate IAA	0.01	0.1	0.001	0.001	0.1
PAA	0.001	0.001	0.001	0.01	0.01
GA	NS	NS	0.001	0.01	0.1
Free ABA	NS	0.1	0.001	NS	0.01
Conjugate ABA	NS	0.001	NS	NS	0.1
Free zeatin	0.05	0.1	0.001	0.01	0.001
Conjugate zeatin	NS	NS	NS	0.001	NS

Table-2. Hormonal analysis in different parts of cotton boll in two cotton genotypes: H-6 and G. Cot

Hormone	Organ	H-6		G. Cot	
		Free	Conjugate	Free	Conjugate
IAA (μg/organ)	Kernel	12.34 (27)	9.16 (33)	7.49 (33)	14.59 (30)
	Seed Coat	6.50 (24)	11.33 (21)	7.93 (27)	12.87(24)
	Fiber	9.24 (21)	16.69 (21)	6.02 (24)	5.14 (21)
	Boll wall	283.13 (27)	983.75 (18)	254.53 (33)	303.19 (24)
	Bracts	18.03 (12)	54.85 (39)	10.23 (12)	54.44 18)
	Organ	H-6		G. Cot	
PAA (μg/organ)	Kernel	22.40 (39)		6.02 (36)	
	Seed Coat	8.54 (15)		25.0 (24)	
	Fiber	17.71 (33)		4.33 (33)	
	Boll wall	864.12 (27)		551.44 (30)	
	Bracts	47.037 (15)		72.97 (18)	
	Organ	H-6		G. Cot	
GA (μg/organ)	Kernel	6.28 (33)		8.22 (33)	
	Seed Coat	5.11 (24)		6.57 (24)	
	Fiber	7.90 (15)		4.59 (24)	
	Boll wall	385.25 (27)		104.4 (36)	
	Bracts	48.44 (15)		43.15 (15)	
	Organ	H-6		G. Cot	
		Free	Conjugate	Free	Conjugate
ABA (μg/organ)	Kernel	7.70 (39)	5.53 (39)	9.67 (33)	20.79 (36)
	Seed Coat	10.83 (15)	19.52 (33)	13.64 (24)	9.79 (24)
	Fiber	8.06 (21)	13.88 (27)	4.62 (27)	15.712(24)
	Boll wall	608.36 (27)	1383.14 (15)	171.0 (30)	978.24 (21)
	Bracts	54.69 (15)	66.23 (39)	21.99 (36)	60.20 (18)
	Organ	H-6		G. Cot	
		Free	Conjugate	Free	Conjugate
Zeatin (μg/organ)	Kernel	3.423 (36)	2.381(30)	3.03 (36)	1.962(30)
	Seed Coat	2.1 (15)	0.38 (6)	2.304 (18)	2.417 (24)
	Fiber	2.819 (15)	6.200 (36)	4.778 (27)	13.177 (12)
	Boll wall	358.44 (27)	617.495 (18)	74.227 (39)	335.88 (21)
	Bracts	28.157 (9)	51.619 (15)	31.536 (24)	37.885 (39)

Parentheses showed the ages at which maximum value was obtained.

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