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Role of Abscisic Acid in Cotton Fiber Development[1]

S. H. Dasani* and V. S. Thaker**

**Department of Life Sciences, K.C. College, Dinsha Watchha Road, Churchgate, Mumbai 400020, India*
***Department of Biosciences, Saurashtra University, Rajkot 360 005, India*
e-mail: vsthakar@hotmail.com; vrindathaker@yahoo.co.in

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Abstract—Fibers of three cotton cultivars varying widely in their final fiber length, i.e., long staple (*Gossypium hirsutum* H-4), middle staple (*G. hirsutum* H-8), and short staple (*G. arboretum* G. Cot-15) were analyzed to study the role of ABA in fiber elongation and dry matter accumulation, *in vivo* and *in vitro.* The fibers were analyzed for different growth parameters and endogenous ABA content during the entire period of their development using indirect ELISA by raising the antibodies against ABA. From growth analysis, cotton fiber development was divided into four distinct phases, (i) initiation, (ii) elongation, (iii) secondary thickening, and (iv) maturation. An inverse correlation between final fiber length and ABA content was observed in all the cultivars. In long staple cultivar (H-4), rapid ABA accumulation started after fiber had attained peak elongation growth while, in short staple cultivar (G. Cot-15), ABA accumulation was observed even during elongation growth. Significant inhibition in length of short and middle staple cultivars as compared to long staple cultivar was observed in *in vitro* grown fibers when media were supplemented with ABA (1, 3, and 5 mg/l). The addition of growth promoters like NAA and GA, along with ABA, has reduced the inhibition in fiber elongation in all the cultivars. These results suggest a regulatory role of ABA in cotton fiber elongation along with auxins and gibberellins.

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Key words: Gossipium - abscisic acid - ELISA - in vivo - in vitro - fiber growth

INTRODUCTION

Cotton is one of the important cash crops, providing five basic products, i.e., lint, linters, seed oil, seed meal, and seed hull. Cotton fiber length and its dry weight per seed are two important parameters of commercial importance. Fiber length is the best criterion for spinning value and determines the finest yarn size into which cotton can be spun [1]. Fiber dry weight per seed on the other hand, is an important determinant in fiber yield, and any advancement in this component is likely to improve productivity [2, 3].

In addition to its commercial importance, the developing cotton fiber has several attributes that recommend it as an experimental system of choice for investigation of physiological and biochemical changes accompanying cell elongation and/or maturation. Advantages of the cotton fiber as a model system includes (i) their synchronous growth as single cell masses on the epidermal layer of the seed, which is free from the process of cell division, (ii) genotypic variations in the final staple length present in different cotton cultivars, (iii) distinct primary and secondary growth phases make it one of the most suitable systems to study cell elongation and dry matter accumulation, and (iv) ovules in a suitable culture medium undergo normal morphogenesis, including fiber production. Thus, cotton fibers provide an excellent model system to study regulation of cell development *in vivo* and *in vitro.*

The phytohormone ABA plays regulatory roles in physiological processes in all higher as well as lower plants [4, 5]. The ability of ABA to induce tuberization and to promote flowering in some species [6] appears to be related to its ability to inhibit elongation growth. Inhibitory effects of ABA on elongation in many systems, such as coleoptiles, hypocotyls, and radicles are well known [7, 8].

Earlier studies from this laboratory, using three cotton cultivars varying in their final fiber length, regulation by auxin [9, 10] and GA [11] has been reported. In this paper, role of ABA in regulation of cotton fiber development is discussed from *in vitro* and *in vivo* studies.

MATERIALS AND METHODS

Seeds of three cotton cultivars varying widely in their final fiber length, *Gossypium hirsutum* Hybrid-4

[1] The text was submitted by the authors in English.

Abbreviations: DEAE cellulose—diethyl amino ethyl cellulose, DMF—dimethylformamide, DPA—days post anthesis, EDC—ethyl-N(3-dimethylaminopropyl)carbodiimide hydrochloride, HSA—human serum albumin, IgG—immunoglobulin G, PAA—phenylacetic acid.

(H-4, long staple), Hybrid-8 (H-8, middle staple) and *G. arboretum* Gujarat Cotton-15 (G.Cot-15, short staple) were grown in the field. The seeds were obtained from Cotton Specialist, Cotton Research Center, Gujarat Agricultural University, Junagadh, Gujarat, India. Seeds of uniform size were selected and sown initially in polythene bags (8 × 12 cm) filled with finely powdered farm soil and farmyard manure in the 3 : 1 ratio. Seeds were soaked in water and sown at the depth of 1 cm. The experimental plots were mechanically ploughed. Farmyard manure was added to the field. Healthy seedlings were transferred from the polythene bags and planted in rows at 90 × 60 cm space. Cultural practices including irrigation, application of fertilizers and insecticides, etc., were conducted to optimize the lint yield. On the day of anthesis, each individual flower was tagged and healthy bolls were harvested for estimation of endogenous ABA content and fiber length and dry weight. Plants were harvested within the plot bordered by two rows on each side. Although boll size is genetically controlled, environmental conditions play a major role in growth and development of the bolls in cotton plant [12]. Duration of different growth phases greatly depends upon genotypes [13] and environmental conditions [14]. Therefore, to minimize the effect of environmental variations, flowers that had bloomed during a narrow period were selected. Bolls of different developmental stages (i.e., with three-day intervals), until opening, were used for analysis.

Dry weight measurements. Fibers were removed manually with the help of forceps from the seed without removing the seed coat. Fibers from the different locules from three bolls were used for dry weight measurement (9–10 replicates). Separated fibers were weighed after oven drying to a constant weight at 70°C to obtain data on dry weight.

Fiber length measurements. Fiber length was determined at three-day intervals throughout the developmental period, by the method of Gipson and Ray [15]. Three locules from three bolls were placed in boiling water to allow the seeds to separate from each other, and each seed was placed on a convex surface of a watch glass. Fibers were streamed out with a jet of water. Length of the fiber was measured to the nearest millimeter from the rounded side of the seed, adjacent to the chalazal end. The final values represent the mean of 20–25 replicates.

Raising of antibodies against ABA. ABA is a heptane (low molecular weight compound); therefore, it is nonimmunogenic. To make ABA immunogenic, it was conjugated with a larger protein molecule (BSA).

Preparation of ABA–BSA conjugate. The synthesis of ABA–BSA conjugate was carried out according to Weiler [16]. ABA (132 mg) was dissolved in 3 ml of the mixture of water and DMF (2 : 1, v/v), and pH was adjusted to 8.0 with 1 N NaOH. The solution was then added dropwise with gentle stirring to 250 mg of BSA dissolved in water and adjusted to pH 8.5. N-ethyl-N(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 210 mg) was added into the preparation in 4 portions during 90 min and stirred in the dark for 19 h at 4°C. Conjugate was finally dialyzed against tap water for 4 days, Final volume was made with distilled water and stored at 0°C.

Immunization. Two rabbits were immunized by intramuscular and intravenous routes [17]. The antigen was prepared by mixing ABA–BSA conjugate with equal volume of Freund's complete adjuvant and injected to the rabbits. Booster doses of injections were given to all the rabbits by intramuscular route at every 20 days in order to increase antibody titer. Animals were bled, and serum was separated. IgG was collected by passing the serum through DEAE-cellulose column pre-equilibrated with 10 mM phosphate buffer (pH 8.0). The purified IgG was concentrated to the original volume of serum and used for the ELISA test after appropriate dilution.

ELISA assay. ELISA was performed as a conformation test by indirect assay as described by Gokani *et al.* [18]. Color development procedure was modified as Kara *et al.* [19].

Preparation of conjugate for ELISA. In indirect ELISA assay, the antigen was absorbed by the wells of the microtiter plate. Immunization was done with ABA–BSA conjugate; hence, showed the reaction with BSA and also showed cross-reaction with human serum albumin (HSA). To avoid artifact of assays and to facilitate binding of the antigen molecule to the ELISA plate, ABA was conjugated with casein. The procedure was same as ABA–BSA conjugate.

Preparation of sample from plant material. From equal sized bolls of each developing stage, fibers were separated from seed carefully without removing seed coat, crushed with liquid nitrogen, and stored at –20°C prior to use. 100 mg of fiber samples were suspended in triplicate in 1 ml 80% methanol (containing 0.01% ascorbic acid as an antioxidant). The samples were then incubated for 48 h at 4°C and centrifuged at 5000 *g* for 10 min at 0°C. The supernatant was collected, and residues were re-suspended twice for complete extraction. All three supernatants were collected and air-dried in darkness. Final volume (5 ml) was adjusted by 80% methanol. The solution was used as a sample source in subsequent experiments.

Preparation of calibration curves and determination of the antigen concentration. ELISA plates were coated with ABA–casein conjugate at an optimum dilution, incubated for 3 h at 37°C and overnight at 4°C. For determination of ABA content in the plant extract or for calibration curves of ABA, samples reacted *in vitro* with antibody before coating the ELISA plate. Sample (400 µl, with or without internal standard) reacted with an equal volume of antibody in a microcentrifugal tube. It was allowed to react for 3 h at 37°C and overnight at 4°C. Sample (350 µl) was then added to each well of antigen coated plate. The next step was coating the

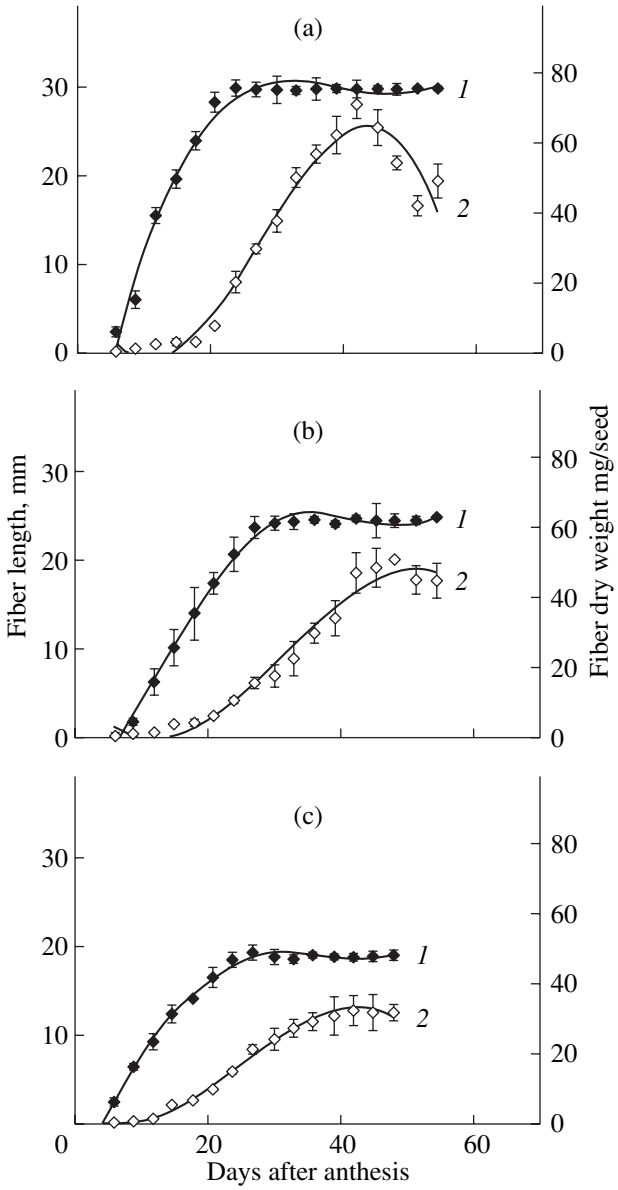

Fig. 1. (*1*) Fiber length and (*2*) dry weight in dependence of boll age in three different cotton genotypes: (a) H-4, (b) H-8, and (c) G. Cot-15.

Standard deviations are marked by vertical bars or are within the symbol.

plate with antirabbit goat IgG conjugated with peroxidase. After each coating, ELISA plate was washed thoroughly with phosphate buffered saline containing 0.05% Tween 20. Color was developed using *o*-phenylenediamine, and the reaction was terminated by addition of 6 N sulfuric acid.

Assay values were obtained as the mean of nine replicates. Relative binding values were calculated as B/B_0, where, B and B_0 are the values of absorbance in the presence and absence of internal hormone standard or sample, respectively. To test a sensitivity of the assay, each sample was mixed with a known concentration of ABA as an internal standard before reacting with the antibodies. A standard curve was prepared for each particular plate.

In vitro ovule culture. For *in vitro* studies, one day post anthesis (DPA), bolls were collected, ovules were separated from the ovary under aseptic condition and floated in the liquid culture media [20], with or without ABA (1, 3, and 5 mg/l). The inoculated flasks were then kept in dark at 28 ± 2°C. In preliminary experiments, each cultivar was tested for optimum hormone concentration and it was observed that the concentrations higher than 1 mg/l of GA_3 or NAA had no significant effect.

In vitro fiber length measurement. From cultured ovules (control or treated with 1, 3, and 5 mg/l ABA), cross sections were taken and nearly 150–200 cells were measured with an ocular micrometer during 10 days. During the subsequent periods, the length of the fiber was measured with a scale. The experiment was continued until the rate of fiber elongation declined.

Statistical analysis. Growth analysis and estimation of endogenous ABA content with fiber age was performed during 2000 and 2001. However, shift in the duration and thus peak values were observed, may be due to environmental variations. Therefore, only the data of 2000 were considered. Polynomial equations were performed to smooth the growth curve. *In vitro* experiments were also repeated during the same years and data of 2000 are presented. ANOVA was performed to evaluate the influence of ABA in all three cultivars.

RESULTS

Data of fiber length and dry weight of three cotton cultivars (viz. *G. hirsutum* H-4, H-8, and *G. arboreum* G. Cot-15) were fitted to polynomial equations of different degrees and 'best-fit' equation was determined statistically by performing 't-test' for different R2 values. Fiber initials started elongation soon after anthesis, entered a linear phase of elongation, and attained a plateau with no further significant change in fiber length. In H-4, H-8, and G. Cot-15 maximum fiber length was observed at 33 (30.5 mm), 33 (25.6 mm) and 30 days post anthesis (19.0 mm), respectively (Fig. 1). In these cultivars the maximum rate of fiber elongation was recorded on 12 (2.35 mm/day), 15 (1.46 mm/ day) and 9 DPA (1.4 mm/day), respectively (Fig. 2).

In all the cultivars studied, fiber dry weight showed a typical sigmoidal curve. After an initial lag, the fiber enter into a linear phase of dry matter accumulation, up to plateau level, and then dry matter declined slightly at maturity or remained unchanged (Fig. 1). In H-4 fiber, dry weight was low up to 15 DPA (1.3 mg/seedfiber), the time when fiber has already attained its maximum rate of elongation (2.35 mm/day, 12 DPA, Fig. 2a). It increased sharply up to 42 DPA (65 mg/seedfiber), and declined slightly thereafter (Fig. 1a). The highest rate of dry matter accumulation

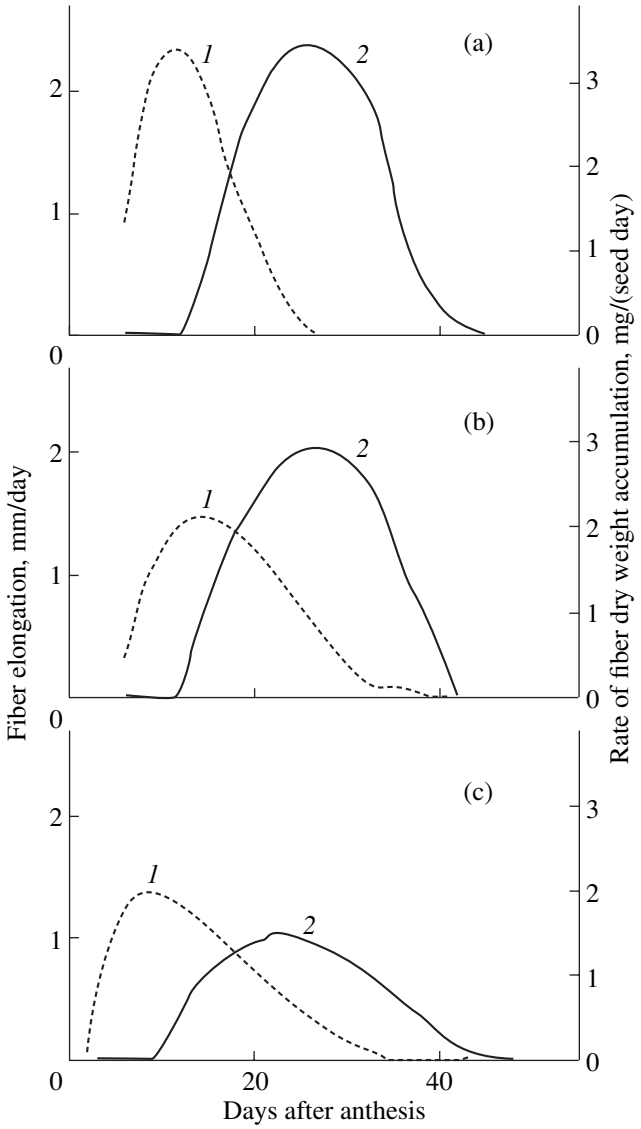

Fig. 2. The rate of (*1*) fiber elongation and (*2*) dry matter accumulation in dependence of boll age in three cotton genotypes: (a) H-4, (b) H-8 and (c) G. Cot-15.

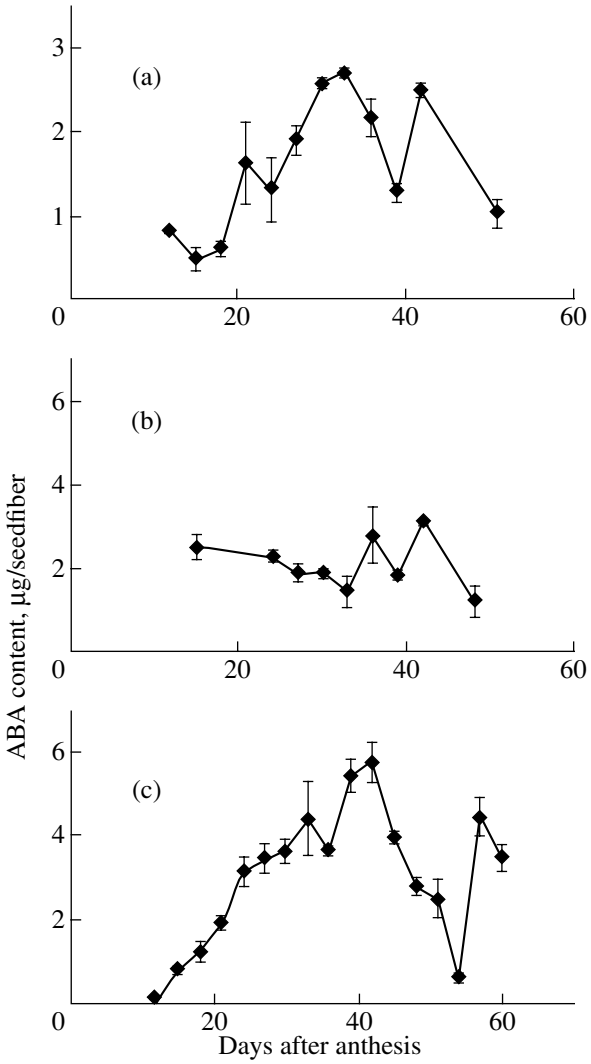

Fig. 3. Endogenous ABA content in dependence of boll age in three cotton genotypes: (a) H-4, (b) H-8 and (c) G. Cot-15. Standard deviations are marked by vertical bars or are within the symbol.

was recorded on 27th DPA (3.43 mg/(seedfiber day), Fig. 1a), when the rate of fiber elongation has already declined and approaches its minimum level (Fig. 2a).

In H-8 and G. Cot-15, the maximum dry weight was recorded on 48 and 42 DPA (50.98 mg/seedfiber and 32.9 mg/seedfiber, respectively, Figs. 1b, 1c), whereas the maximum rate of dry matter accumulation was 2.9 mg/seedfiber/day on 27 DPA in H-8 and 1.5 mg/(seedfiber day) on 24 DPA in G. Cot-15 (Figs. 2b, 2c).

Changes in the endogenous ABA levels in three cotton cultivars showed variations (Fig. 3). In H-4, ABA levels remained low until 18 DPA, increased gradually up to 33 DPA, and declined thereafter. The maximum ABA content in H-4 was recorded on 33 DPA (5.37 μg/seedfiber). In the case of H-8, ABA levels were very low as compared to H-4 throughout the period of fiber development with a peak at 36 DPA (2.76 μg/seedfiber). In G. Cot-15, ABA levels increased gradually from 12 to 42 DPA (5.44 μg/seedfiber) and declined thereafter (Fig. 3).

In culture experiments, fibers grown with or without NAA and GA_3 showed clear ABA-induced inhibition in the fiber length (Fig. 4). The fertilized ovules grown *in vitro* showed that, in control experiment without hormones, H-4 had a maximum length followed by H-8 and G. Cot -15. 1 mg/l ABA was sufficient enough to inhibit fiber growth in culture media without application of NAA and GA_3 in all cultivars. In the media supplemented with NAA and GA_3 (1 mg/l), the addition of 1, 3, and 5 mg/l ABA to the culture media showed a remarkable inhibition in all three cultivars. However,

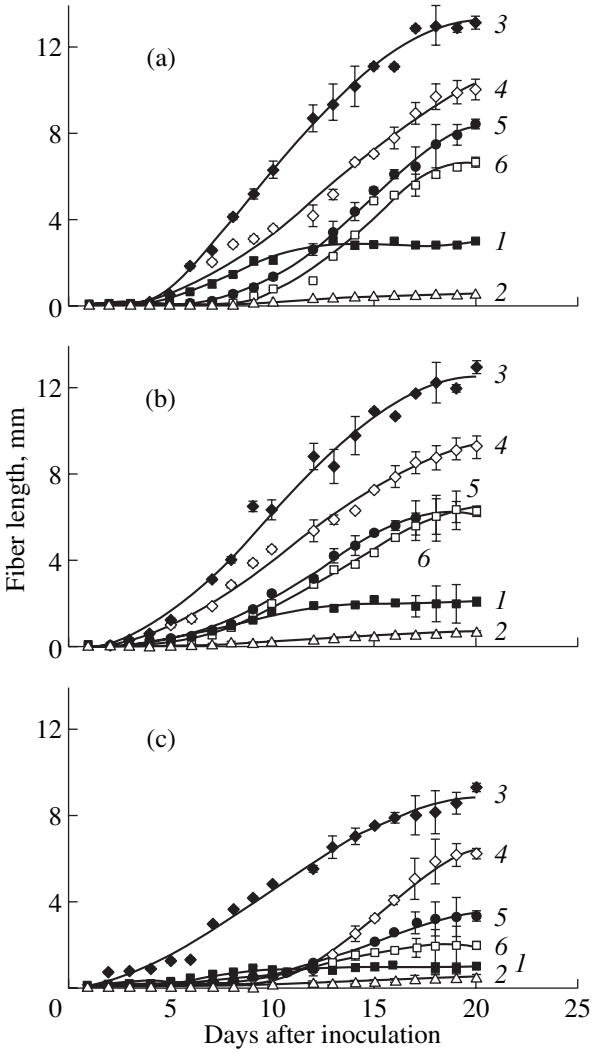

Fig. 4: Changes in *in vitro* fiber length, when ovules were cultured in the media (*1*) without any hormone supplement, (*2*) without NAA+GA_3 but with 1 mg/l ABA, (*3*), with NAA+GA_3, (*4*) with NAA + GA_3 + 1 mg/l ABA, (*5*) with NAA + GA_3 + 3 mg/l ABA, and (*6*) with NAA + GA_3 + 5 mg/l ABA in dependence of boll age in three cotton genotypes: (a) H-4, (b) H-8, and (c) G. Cot-15.

Standard deviations are marked by vertical bars or are within the symbol.

inhibition was more prominent in G. Cot-15 as compared to H-8 and H-4 (Fig. 4, table).

DISCUSSION

The total length of the fiber and final fiber weight are the result of the rate and duration of fiber elongation and the rate of dry matter accumulation, respectively [9, 21, 22]. The epidermal cells of cotton ovule elongate soon after anthesis and for about half of their elongation phase they remain bounded by a thin primary wall. As the rate of elongation diminishes, the rate of dry matter accumulation and secondary wall thickening increases. Thus, elongation and secondary thickening phases appear as two distinct phases. From growth analysis, cotton fiber development is divided into (i) initiation (0–3 DPA), (ii) elongation (3–24 DPA), (iii) secondary thickening (18–42 DPA), and (iv) maturation (40–48 DPA) phases ([9, 22, 23], Figs. 1, 2). It is a general observation from the number of the cotton cultivars studied that a duration of these phases showed genotypic and environmental variations [14].

In the present study, the duration of fiber elongation and dry matter accumulation was almost similar in all three cultivars (Fig. 1). However, the rate of elongation and dry matter accumulation differed among the cultivars. The long staple cultivar (H-4) had the highest rates followed by medium and short staple cultivars (Fig. 2). The data support an earlier hypothesis that the final fiber length and dry weight is a product of their increments and duration [9, 14, 22]. When the fiber attained the maximum rate of elongation, dry matter accumulation starts (Fig. 2). The results suggest that these two independent phases overlap with each other considerably.

The pattern of ABA accumulation showed that the maximum amount of ABA accumulated was nearly 5.4 μg/seedfiber in both cultivars, H-4 and G. Cot-15. However, in G. Cot-15, ABA levels remained higher throughout the fiber development. A comparison of ABA levels (Fig. 3) with the rates of fiber elongation of these cultivars (Fig. 2) indicates that ABA levels were relatively low in H-4 and G. Cot-15 until the fiber has attained its maximum rate of elongation, they increased with a decrease in the rate of fiber elongation and achieved their peaks when the fiber had reached its maximum length. However, in contrast to that in H-4, in the short staple cv. G. Cot-15, the IAA, PAA, and GA levels were significantly lower [10, 11], and, therefore, this must change the ratio between different plant growth regulators, which affect the fiber development. The middle staple cultivar, H-8 showed a lower rate of elongation than that of H-4, but a higher one as compared to G. Cot-15 (Fig. 2). However, in this cultivar, a low ABA level was observed during elongation phase, which increased after the fiber has attained the peak of elongation growth. These results suggest that ABA might have played an inhibitory role in cotton fiber elongation along with IAA, PAA, and GA.

An inverse relationship between internal ABA levels and cell elongation in roots and coleoptiles of anaerobically grown rice seedlings indicates that ABA is an inhibitor of growth in rice seedlings [8]. In deep water rice, internal ABA levels were inversely correlated with the rapid elongation of internodes [24]. There was a negative correlation between ABA levels in the elongation zone of maize roots and the growth rate of the root [25].

If ABA plays an inhibitory role in cotton fiber elongation, then exogenously applied ABA should inversely correlate with elongation growth. A marked inhibition of the fiber length by addition of ABA to the cultured medium was observed (Fig. 4). When various concen-

trations of ABA were applied to all three cultivars, variation in inhibition was observed (Fig. 4). When media were supplemented with NAA and GA_3 in addition to ABA, the inhibition was more prominent in G. Cot-15 followed by H-8 and H-4.

Existing literature on growth and development suggests that regulation may be controlled by a balanced ratio between hormones, by opposing effects between hormones, and by alteration of the effective concentration of one hormone by another [26]. Earlier studies from our laboratory [9, 10] reported that long staple cultivars have more auxins and GA_3 [11] then middle and short staple cultivars.

In this experiment with three cotton cultivars, the addition of equal amount of ABA to the culture media showed variations in the inhibition of fiber length. These results suggest that the levels of endogenous ABA and differences in the amount of endogenous growth promoters like IAA, PAA, and GA provide regulation of fiber elongation in these cultivars.

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