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PHYSIOLOGY

Plant Hormones Alter Fiber Initiation in Unfertilized, Cultured Ovules of *Gossypium hirsutum*

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INTERPRETIVE SUMMARY

Increasing crop yield remains a central goal for the U.S. cotton industry. One factor for improving crop yield is to increase the number of fibers produced by each developing seed. Plant hormones, such as indole-3-acetic acid (IAA) (auxin) and gibberellic acid (GA_3), play a vital role in fiber growth and development. This study compares fiber initiation *in vitro* and *in vivo* in two commercial cotton (*Gossypium hirsutum*) cultivars: MD51 and MAXXA GTO. Compared with the MD51 variety, the high-producing MAXXA cultivar exhibited a greater number of fiber initials throughout the study period. Therefore, the high-yielding MAXXA GTO cultivar was used to study the effects of exogenous hormone application on fiber initiation from ovules grown *in vitro*. Previously published work indicated that hormones are essential for fiber development. Our work, however, clearly demonstrates that in the two cotton cultivars studied, unfertilized ovules produced large numbers of fiber initials in the absence of any hormone treatment. Thus hormones and fertilization may be essential for subsequent fiber growth, but they were not essential for the first stage of fiber development, that is, fiber initiation. Exogenous application of either indole-3-acetic acid or gibberellic acid induced significant increases in fiber production, relative to the control treatments in which no hormone was added. Pre-anthesis treatment with indole-3-acetic acid produced the largest increase in fiber initiation. Pre-anthesis treatment with gibberellic acid produced a smaller, yet significant increase. Post-anthesis treatment with gibberellic acid produced the largest increase in fiber initiation. Post-anthesis treatment with indole-3-acetic acid produced a small, but significant increase, relative to control treatments. Considered together, our experiments demonstrated that within a specific cultivar (in this case, MAXXA GTO), physiological changes induced by hormone application can increase fiber initiation. Understanding the factors that control fiber initiation may lead to technologies that improve the yield potential of cotton.

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Abbreviations: GA_3, gibberellic acid; IAA, indole-3-acetic acid

ABSTRACT

Fiber initiation in two commercial cultivars of *Gossypium hirsutum*, MD51 and MAXXA GTO, was compared. MAXXA GTO produced significantly more fiber initials in ovules grown either *in vivo* or *in vitro*. Unfertilized ovules grown in culture without plant hormones exhibited a steady increase in fiber number over the experimental period. Exogenous applications of the plant hormones indole-3-acetic acid or gibberellic acid, either pre-anthesis to flower buds and developing flowers or post-anthesis to ovules, were examined in the MAXXA GTO cultivar. Unfertilized ovules treated pre-anthesis with 0.1 mg L^{-1} indole-3-acetic acid produced a greater number of fibers than did ovules treated with 1.0 mg L^{-1} gibberellic acid or water. Post-anthesis treatment with 1.0 mg L^{-1} gibberellic acid in the culture medium increased fiber production, compared with water or 0.1 mg L^{-1} indole-3-acetic acid applied in the culture medium. Application of either hormone (depending on concentration and time of treatment) resulted in an increase in fiber production, compared with controls. Fiber production in the absence of hormone treatment, either pre- or post-anthesis, suggests that hormones may not be a requirement for fiber initiation. Manipulation of the hormonal levels might cause an increase in the proportion of epidermal cells that differentiated as fibers. On the other hand, the hormone treatments might induce cell division, resulting in more epidermal cells and, consequently, a greater number of fiber initials. These results

indicate that, given an appropriate stimulus, ovules have the capacity to produce a greater number of fibers.

Increasing yield without reducing fiber quality remains a cotton industry priority, but both crop yield and quality are affected by a wide array of genetic and environmental factors. Variability with respect to fiber production exists among and within various cultivars, and this is compounded by the impact of environmental conditions (Harris, 2001). While a number of approaches can be used to increase yield (Lewis, 1992), our work focused on increasing fiber production per ovule.

Cotton fibers are single cells derived from the epidermis (Ramsey and Berlin, 1976). Fiber production requires ovule epidermal cells to undergo dramatic changes in growth dynamics to produce the highly elongated fiber cells. Evidence regarding the timing of the fiber initiation event varies, depending on the techniques used to monitor initiation. Ultrastructural studies vary, some work indicating that epidermal cells begin to differentiate into fibers on the day of anthesis and other researchers reporting that initiation begins up to 2 d pre-anthesis (Stewart, 1975; Ramsey and Berlin, 1976; Graves and Stewart, 1988a). Biochemical analyses indicated that changes related to fiber initiation can be detected several days before anthesis (Graves and Stewart, 1988a,b).

Fiber number per ovule varies among species and cultivars (Berlin, 1976) and is influenced by a variety of genetic and physiological factors. The proportion of epidermal cells that develop into fiber varies from 10% (Ryser, 1999) to 25% (Beasley, 1975). This variability may be the result of the aforementioned influences of genetic and physiological factors. For example, environmental changes such as cool night temperatures slow fiber initiation rates (Xie et al., 1993). Plant hormones, such as auxins and gibberellins, are very important during the early stages of fiber development (Dhindsa et al., 1976; Dhindsa, 1978). The auxin, indole-3-acetic acid ($C_{10}H_9NO_2$, Sigma Chemical, St. Louis, MO), and the gibberellin, gibberellic acid ($C_{19}H_{22}O_6$, Sigma Chemical, St. Louis, MO) (gibberellin A_3), appear to play roles in fiber initiation and development (reviewed by Basra and Malik, 1984; Delanghe, 1986; Ryser, 1999). The levels of indole-3-acetic acid in cotton fiber increase during development (John, 1999).

Previously, the role of hormones in fiber development was assessed using a staining method to quantify fiber production (Beasley and Ting, 1973, 1974). This technique measures the total fiber units produced, that is, the combination of the number of fibers produced and the amount each fiber developed. In our studies, we specifically examined the number of fibers produced per ovule. Direct measurements of the effects of hormone levels on the fiber quality (length, strength, and micronaire) indicated that increased levels of indole-3-acetic acid do not alter fiber quality (John, 1999). These experiments did not directly examine possible increases in fiber number (i.e., yield).

In our experiments, fiber initiation in two commercial cultivars of *Gossypium hirsutum* were studied. Also, the effect of gibberellic acid and indole-3-acetic acid on fiber initiation in the high-yielding cultivar, MAXXA GTO, were observed. Our objective was to determine whether altering the physiological environment of the ovule by the exogenous application of hormones, either before anthesis or after anthesis, could improve fiber production, that is, increase the number of fibers generated by culture-grown ovules.

MATERIALS AND METHODS

Plant Growth

Gossypium hirsutum cultivars MAXXA GTO and MD51 were grown in 20-L pots of Pro-Mix potting mixture (Premier Horticulture, Riviére du Loup, Québec). Plants were grown in a greenhouse during the months of June through October with a day-night (16 h-8 h, respectively) temperature cycle of 32°C/26°C. Plants were watered daily and fertilized weekly with Miracle Gro (The Scotts Co., Marysville, OH) general-purpose fertilizer.

Hormone Application

Hormones were diluted to produce final working concentrations of 1.0 mg L^{-1} gibberellic acid or 0.1 mg L^{-1} indole-3-acetic acid and water as the control.Since gibberellic acid stock solutions contained ethanol (C_2H_5OH), and indole-3-acetic

acid stocks contained KOH, all treatment solutions (including the water control) were adjusted with appropriate amounts of ethanol and KOH to yield a final concentration of 0.02% ethanol and 5×10^{-5} *M* KOH. Pre-anthesis treatment started as soon as the squares were detected. Developing squares were treated every other day with the specified single-hormone solution until the day of anthesis. Five drops of the appropriate treatment were applied directly to the developing square and/or flower. Sepals were gently spread and solutions were applied directly to the developing flower. Any excess treatment solution was allowed to drip off the plant. Each treatment was applied to 10 flowers. At 0 d post-anthesis, the pistil and anthers of each flower were removed. At 1 d post-anthesis, each flower was harvested and the ovules cultured, using the media and techniques of Beasley and Ting (1974). The ovules from 10 flowers were combined into one population in a large plate. Ten to twelve undamaged ovules were then randomly chosen for culturing in 30-mm petri dishes with 10 mL of medium containing no hormones, again using the techniques of Beasley and Ting (1974). Cultures were incubated in the dark at 30°C and collected at specified days between 2 and 5 d post-anthesis.

In post-anthesis treatments, no hormones were applied while the square was on the plant. At 0 d post-anthesis, the pistil and anthers of the flower were removed. At 1 d post-anthesis, the ovules from 10 flowers were cultured as described above, except that media were supplemented with the appropriate hormone (1.0 mg L^{-1} gibberellic acid or 0.1 mg L^{-1} indole-3-acetic acid). Cultures were incubated at 30°C in the dark and collected at appropriate days from 2 to 5 d post-anthesis.

Fiber Counts

Three random samples of two ovules each from each day post-anthesis were collected and fixed in methanol (CH_3OH)/acetic acid (CH_3COOH), 3:1 (v/v) for at least 1 h. To facilitate fiber counting, ovules were macerated using the technique of Seagull (1998). Briefly, the ovules were rinsed with water and placed in 5 *M* HCl for 45 to 60 min. They were then rinsed with water and soaked in Schiff's reagent ($C_{19}H_{18}N_3Cl$, Sigma Chemical, St. Louis, MO) for 1 h. Schiff's reagent was originally used to stain nuclei (Van't Hof, 1999), but because we were not monitoring nuclei, we omitted this step without affecting the overall maceration of the cotton ovules. After another rinse with water, the ovules were stored in 45% acetic acid.

Ovules were separated into individual cells in Eppendorf tubes (VWR Scientific, Westchester, PA) with 500 mL of 45% acetic acid, using a Pellet Pestle tissue grinder (VWR Scientific, Bridgeport, NJ). Fibers were counted using a Fuchs-Rosenthal counting chamber (VWR Scientific, Bridgeport, NJ). Observations were made using bright field microscopy, with an Olympus CHT or KHC microscope (Olympus Optical, Tokyo, Japan). Twenty-four random samples from three sets of two ovules each were counted for each treatment. Statistical analysis was performed using a two-way analysis of variance. All pairwise multiple comparisons of factors (days post-anthesis and treatment) were performed using the Student-Newman-Keuls Method (alpha set at $P < 0.05$). Regression analyses and graphical representations illustrating means and standard errors were prepared using Sigma Plot (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Comparison of the cultivars MD51 and MAXXA GTO showed that both exhibited similar rates of fiber initiation on unfertilized ovules grown *in vivo* and *in vitro* in hormone-free medium (Fig. 1).

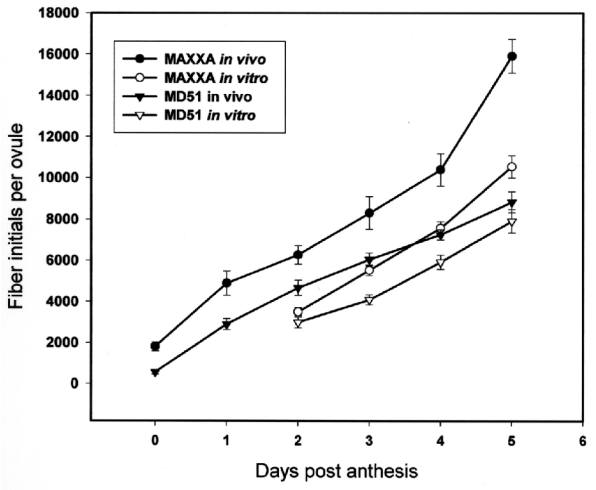

Fig. 1. Fiber initiation on unfertilized ovules of *Gossypium hirsutum* cultivars MD51 and MAXXA GTO, grown *in vivo* and *in vitro* in hormone-free medium.

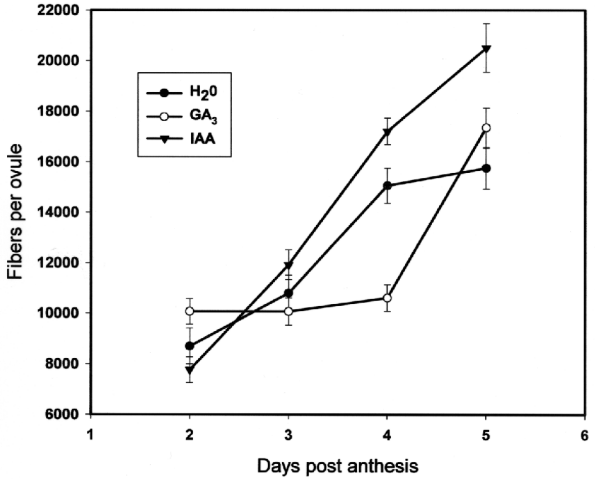

Fig. 2. The effects of pre-anthesis treatment with indole-3-acetic acid (IAA) or gibberellic acid (GA_3) on fiber initiation on unfertilized ovules of *Gossypium hirsutum* cultivar MAXXA GTO, grown *in vitro*.

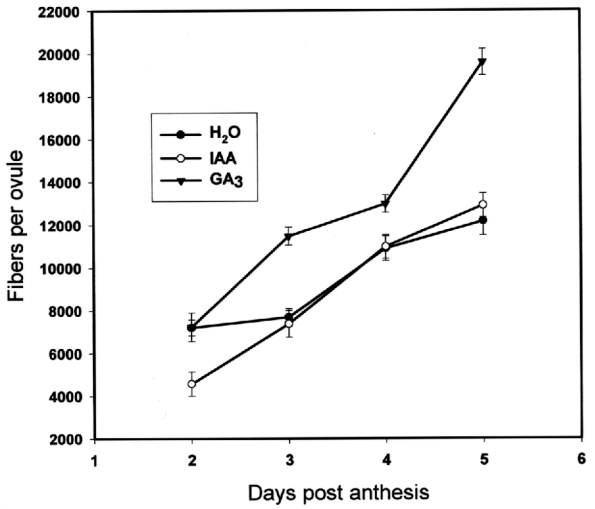

Fig. 3. The effects of post-anthesis treatment with indole-3-acetic acid (IAA) and gibberellic acid (GA_3) on fiber initiation on unfertilized ovules of *Gossypium hirsutum* cultivar MAXXA GTO, grown *in vitro*.

The greater number of fibers on ovules from MAXXA GTO grown *in vivo* appears to be due to a greater number of fiber initials at the start of the experiment (Fig. 1). The increased fiber production in MAXXA GTO was also evident in ovules grown *in vitro* (Fig. 1). In both MD51 and MAXXA GTO, ovules grown *in vitro* produced fewer fibers than ovules grown *in planta* (Fig. 1). This decrease appeared to be due to reduced fiber production early in the cultured-ovule experiments (0 to 2 d post-anthesis). These data indicated that the culturing procedure might inhibit fiber initiation for a short time (2 d), after which initiation proceeds at a rate comparable to ovules grown *in planta* (Fig. 1). Comparisons of fiber production in MAXXA GTO and MD51 indicated that the increased yields observed in MAXXA GTO may be at least partly due to higher fiber initiation. Because MAXXA GTO produced more fibers than MD51, MAXXA GTO was used for subsequent experiments.

Unfertilized ovules grown *in vitro* produced large numbers of fibers in the absence of hormone in the media (Figs. 2, 3). These observations appear to contradict those of Beasley and co-workers (Beasley and Ting, 1974; Beasley, 1977), who suggested that hormones are essential for fiber production in unfertilized ovules. The Beasley experiments measured fiber production after 14 d, using a stain/de-stain method. This approach measures the total amount of fiber produced, that is, a combination of initiation (number of fibers) and elongation (length of fiber). It is possible that this technique cannot accurately detect fiber initials and thus fiber initials were produced but not detected. In our experiment, we specifically and directly measured fiber initiation. The data presented (Figs. 2, 3) clearly indicate that neither fertilization nor hormone application is essential for the fiber-initiation event.

Application of indole-3-acetic acid to the squares pre-anthesis, followed by culturing in a hormone-free environment, resulted in a higher final number of fiber initials than did treatments with gibberellic acid or water (Fig. 2). This increase was rapid without leveling off, from 2 d through 5 d post-anthesis, and resulted in a greater rate of increase in fiber initiation than in water-treated ovules (Table 1). Since levels of indole-3-acetic acid are low before anthesis and fertilization (Naithani et al., 1982), addition of indole-3-acetic acid before anthesis might result in higher residual levels of auxin on the day of anthesis and during the early stages of fiber development. Thus external application of hormones might result in accumulation of the hormone in the ovule, leading to higher numbers of fibers produced in the early stage of initiation. Pre-anthesis treatment with gibberellic acid resulted in a gradual increase in fiber production between 2 and 3 d post-anthesis, followed by a significant increase in fiber production between 3 and 5 d post-anthesis (Fig. 2; Table 1). At the termination of the experiment, pre-anthesis

Table 1. Statistical analysis of the effects of indole-3-acetic acid (IAA) and gibberellic acid (GA_3) on fiber initiation rates using the Student-Newman-Keuls Method for all pairwise multiple comparisons, $P < 0.050$. The effect of age on fiber initiation was assessed using regression analysis.

	Pre-anthesis treatments						
	Within treatment comparisons (regression analysis)			Between treatment comparisons (means)†			
	Control	IAA	GA	DPA	Control	IAA	GA
Intercept	3699	-862	4210	2	8710b‡	7774c	10078a
Slope	2539	4348	2234	3	10809b	11932a	10154c
R^2	0.938	0.993	0.659	4	15063b	17209a	10617c
				5	15754c	20508a	17345b
	Post-anthesis treatments						
	Within treatment comparisons (regression analysis)			Between treatment comparisons (*P* values)			
	Control	IAA	GA	DPA	Control	IAA	GA
Intercept	3126	-1054	-701	2	7192a	4563b	7219a
Slope	1816	2858	3861	3	7676b	7374c	11469a
R^2	0.929	0.988	0.943	4	10894b	10975b	12977a
				5	12174c	12893b	19587a

† Within treatment analysis, all DPA (days post-anthesis) means are significantly different at $P < 0.05$ and the regression line equation is $y = y_0 + bx$.

‡ Between treatment comparisons means in rows followed by the same letter are not significantly different at the 0.05 probability level.

application of gibberellic acid had smaller, yet significant, effects on fiber initiation (Fig. 2; Table 1). The R^2 value for this treatment is lower than for either the water-treated or the indole-3-acetic acid-treated samples (0.659 compared with 0.938 and 0.993, respectively), suggesting that fiber initiation rates for gibberellic acid-treated ovules might be less linear. Delanghe (1986) showed that gibberellic acid acts as a stimulus of fiber growth 48 h after anthesis. Our data were consistent with a delayed response to gibberellic acid since treatment with gibberellic acid results in an initial slow rate of initiation (2 to 3 d post-anthesis), followed by a rapid increase in fiber number (3 to 5 d post-anthesis) (Fig. 2). Because the ovules were removed from the ovaries and placed in culture 24 h after anthesis, a delay in the gibberellic acid response might have occurred.

Post-anthesis application of gibberellic acid in the culture medium resulted in an increase of fiber initials, compared with cultures treated with either indole-3-acetic acid or water (Fig. 3). Ovules treated with gibberellic acid or water had similar numbers of initials at 2 d post-anthesis. However, by 3 d post-anthesis, the initial counts of hormone-treated ovules exceeded the controls (Fig. 3). Regression analyses indicated that ovules treated with gibberellic acid exhibited the highest rate of fiber initiation (Table 1). R^2 values were consistent with all treatments producing a linear increase in fibers over time (Table 1). Because gibberellic acid stimulates fiber growth 48 h after anthesis (Delanghe, 1986), the presence of gibberellic acid in the culture media might have aided the accumulation of the hormone in the ovule, producing a higher number of fiber initials after the ovules were cultured for a few days. Alternatively, the difference between gibberellic acid and water treatments might result from a reduction in initiation rates in the water treatments. Between 2 and 3 d post-anthesis, water-treated ovules exhibited a small, yet significant, increase in fiber number, whereas gibberellic acid-treated ovules exhibited a large increase in fiber number (Fig. 3; Table 1).

Our observations are consistent with those of Kosmidou-Dimitropoulou (1986), who reported a requirement for gibberellic acid in the days following anthesis, rather than on the day of anthesis. Applications after anthesis of indole-3-acetic acid in the culture media resulted in a significantly reduced number of initials in the early part of the experiment, that is, lower than the water control without growth regulators (Table 1; Fig. 3). This observation indicated that either the timing of the application or the level of indole-3-acetic acid (0.1 mg L^{-1}) was inhibitory.

We are unable to explain why pre-anthesis treatment with water, compared with the corresponding post-anthesis treatment, resulted in an increase in fiber production (15,754 and 12,174

fibers per ovule, respectively). An increase in moisture around the developing square might be beneficial to the development of the flower bud and ovules. Pre-anthesis treatment, compared with post-anthesis treatment, with indole-3-acetic acid resulted in nearly twice as many fiber initials (20,508 fibers per ovule compared with 12,893) by 5 d post-anthesis (compare Figs. 2, 3; Table 1). This increase was present at 2 d post-anthesis (7774 fibers per ovule compared with 4563), and the pattern continued through 5 d post-anthesis. Application of gibberellic acid to the culture media, rather than upon pre-anthesis ovules, resulted in a higher number of fiber initials (19,587 compared with 17,345 fibers per ovule).

In this study, exogenous hormone application clearly had an impact on fiber production. Previous research indicated that changes in hormone levels do not affect fiber-quality parameters (John, 1999). Transgenic fibers with increased levels of indole-3-acetic acid did not exhibit improved fiber-quality traits (John, 1999). However, possible increases in fiber number were not examined. Future research with indole-3-acetic acid, as well as with gibberellic acid, should focus on hormone concentration and/or application time in culture-grown ovules. To more closely mimic field conditions, this work will be extended to ovules grown *in planta*. We hope to determine the minimal "window of opportunity" for increasing fiber production with respect to both hormone concentration and application. Such observations may help identify specific cellular processes that control fiber production.

CONCLUSIONS

The growth of fiber initials on unfertilized ovules cultured in hormone-free medium indicated that initiation does not appear to require either fertilization or hormone application. The involvement of plant hormones in fiber initiation is complex. The response of ovules to hormone application depended on the hormone applied (indole-3-acetic acid or gibberellic acid) and on the timing of the application (pre- or post-anthesis). Pre-anthesis treatments with indole-3-acetic acid resulted in the largest number of fibers per ovule; yet post-anthesis treatment with gibberellic acid produced the most fibers per ovule.

These observations indicate that fiber numbers on ovules are controlled not only by the cultivar, but also by the physiological conditions under which the ovules develop. Understanding the types of stimuli that are important for fiber production and the timing of those stimuli may provide clues as to the breeding or biotechnology approaches needed to increase fiber productions on ovules.

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