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# The Effect of Phytohormones on the Dynamics of Protein Biosynthesis and Enzyme Activity in Linted and Naked Cotton Seed

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## ABSTRACT

We determined the effect of exogenous indole-3-acetic acid, α-naphthalene-3-acetic acid and gibberellic acid ($GA_3$) on the enzymatic activity of glucansynthase, peroxidase and cellulase in ovule development of naked L-70 and linted AN-Bayaut-2 cotton (*Gossypium hirsutum* L.) seeds. We isolated a protein–inhibitor of 37 kDa with pI 4.2 from integument tissue of naked cotton seeds. In addition, we studied its inhibitory activity on the biosynthesis of cellulose after $GA_3$ treatment of ovules of linted cottonseed at $10^{-6}$ M. The results help to illuminate our understanding of lint development in cotton.

**Keywords:** cellulose, cotton varieties, glucansynthase, peroxidase

## INTRODUCTION

The study of the regulatory pathways of cotton fiber development has both theoretical and applied relevance. Understanding the developmental mechanism underlying cotton fiber development is not possible without detail knowledge of the expansion of the fiber cell walls structure, mechanisms of synthesis of constitutive components, and regulation of these processes (Polevoy 1982, 1986). Phytohormones, which are organic, low-molecular weight compounds which facilitate the interaction of cell, tissues and organs at very low concentrations, are considered to strongly influence these processes as well as trigger physiologic programs in plants such as seed germination, growth maturation, morphogenesis, and flowering. The mechanism of phytohormone effect on the plant cell includes activation of DNA transcription, biosynthesis of mRNAs, followed by synthesis of enzymes, and leading to the complete activation of cell metabolism (Liao *et al.* 2010). Hence, phytohormone-induced permeability of cell membranes and the increase of ATP-biosynthesis are important. Phytohormones regulate growth and developmental processes in plants as constitutive natural products of metabolism. With the wide-spectrum of physiological activity, exogenous phytohormones have a primary effect on the *in vitro* control of morphogenesis. The physiological action of phytohormones occurs through hormone-receptor complex formation, which determines the phytohormone effect (Polevoy *et al.* 2001; Tarchevchkiy 2001). In the case of cotton fiber, initiation is influenced by physiological changes in the plant. The number of lint fibers per ovule is affected by environmental and agronomic conditions such as planting date (Bowman 2000; Lewis 2000), and mean minimum daily temperature (Lewis 2000).

Studies on the effects of phytohormones on fiber development have been reported (Momtaz 1998; Basra *et al.* 1999; Gialvalis *et al.* 2001; Liao *et al.* 2010). Analysis of hormone content in young ovules and in fibers grown in the plant indicates that auxin levels are high initially (0 d post-anthesis) and drop precipatiously by 8 d post-anthesis (Nayyar *et al.* 1989; John 1994; Chen *et al.* 1996). This research supports previous observations of Jasdanwala *et al.* (1980), which indicated that an increase in indole-3-acetic acid (IAA) levels is important for epidermal cells to differentiate into fibers. There are only a few studies concerning hormonal regulation of cotton cellulose synthesis in recent publications (Tarchevchkiy 2001; Gokani *et al.* 2002; Aleman *et al.* 2008). These studies deal with investigations of the role of endogenous hormones in cell metabolism, but not on the effects of exogenous action of phytohormones. IAA and gibberellic acid ($GA_3$) have a considerable influence on intensification of fiber cellulose formation (Tarchevchkiy 2001; Seagull *et al.* 2004).

It is significant that each phytohormone group influences specific processes in plant morphogenesis. For example, auxins are essential participants in morphogenesis coordination. They have an impact on division, elongation and differentiation of cells. These hormones initiate DNA replication. High concentrations of auxin can cause mitosis in somatic plant cells. Auxin activates synthesis of specific proteins including proteins secreted to cell walls which are not inhibited by actinomycin D. Musaev (1972; 1979) showed that for allopolyploid cotton L-70 cultivars *G. hirsutum* L., four genes exist for lint and naked seed traits. These genes have been separated into different groups: 1) basic genes; 2) additional gene; 3) gene-inhibitor. Dominant alleles of the basic genes control the development of seed linters. The amount of lint depends on the expression of genes in the dominant alleles. The naked seed of *G. hirsutum* L. depends on two factors: first – the presence of a dominant gene-inhibitor in the genotype and second – the presence of recessive genes of seed linters (Du *et al.* 2001). However, investigations of phytohormone action on the ovule development of naked seed cotton have not been performed.

Thus, our study is focused on the investigation of biochemical characteristics of cottons differing by seed lint, fiber maturating enzyme activity, protein composition of cotton fiber and integument tissue of naked and linted seed cotton. Thus, we investigated specific proteins and modula-

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tion of glucansynthase, peroxidase and cellulase activity by α-naphthylene-3-acetic acid (NAA), IAA, and $GA_3$ in the integument of naked seed cotton, and the fiber and the integument tissue of linted cotton.

## MATERIALS AND METHODS

### General

The subject of research is proteins from 20 dpa ovule integument of naked seed cotton (line 'L-70') and fiber and integument of linted seed cotton (*Gossypium hirsutum* L. cv. 'AN-Bayaut-2'; *Malvaceae*) (Institute of Cotton Breeding, Republic of Uzbekistan), which were treated with IAA, NAA and $GA_3$. Cotton plants were grown on the experimental field of Institute of Bioorganic Chemistry and divided into four groups (for experiments with each phytohormone and control variant). Stamens and pistils were sprayed with a solution of phytohormone in the first day of flowering after full disclosure of the flower. The volume of one spraying was 10 mL (per one flower) of $1\times10^{-6}$ M solution by hand using a syringe.

### Protein isolation from cotton integument and fiber (line 'L-70' and cv. 'AN-Bayaut-2')

The method of Xu *et al.* (2006) was followed. Plant material (fiber or integument) was ground with liquid nitrogen. Total protein was extracted with 0.05 M Tris-HCI (pH 7.8), containing 1M NaCl (1:4 buffer), for 30 min at 4°C, and centrifuged for 5 min at 1500 × *g*. The supernatant was collected. The precipitate was suspended in a small volume of the same buffer and centrifuged for 5 min at 1500 × *g*. The supernatants were combined and centrifuged for 30 min at 5000 × *g*. The total protein was precipitated by addition of 5 volumes of cold acetone, centrifuged (15 min at 6000 × *g*), lyophilized, dissolved in a small volume (2 ml) of the same buffer, and desalted on a column with Sephadex G-10 (2.5 × 80 cm) in distilled water and lyophilized in a freeze drier (Labconco, USA). All experiments were repeated at least three times.

### Electrophoresis

Electrophoretic analysis of proteins was performed in gradient (10 to 15%) polyacrylamide gel (PAG) by the method of Laemmly (1971) using a vertical apparatus (Himifil, Estonia) with Sigma reagents. Isoelectrofocusing was performed using ampholins with pI 3.5 to 9.6 (Sigma).

### Protein chromatography on TSK-gel column

Lyophilized total protein was dissolved in 1 mL of 0.05 M sodium phosphate (pH 7.0) and loaded on a TSK HW-55F (Toyopearl, Japan) column (0.7 × 30 cm) in the same buffer. Flow rate was 2 mL/h.

### Ion-exchange chromatography of proteins

Fractions collected from the TSK gel were dissolved in 0.7 ml of 0.001 M sodium acetate (pH 4.6) and put on a column (1.2 × 4 cm) with DEAE-TSK (Toyopearl, Japan) using the same acetate buffer. Elution was performed using a linear gradient of sodium chloride in the same buffer. Flow rate was 20 mL/h. Obtained fractions were dialyzed against distilled water and lyophilized.

### Determination of enzyme activity

The glucansynthase (GS, E.C. 2.4.1.34), peroxidase (POX, E.C. 1.11.1.7) and cellulase (Ce, E.C. 3.2.1.4) activity was determined by the method described in Akhunov *et al*. (2001).

#### *1. Determination of glucansynthase activity*

Seedlings obtained from crude membrane preparation following the destruction of cells with liquid nitrogen were homogenized in Tris-HCl buffer, pH 7.8. The homogenate was centrifuged for 5 min at 2000 × g. The supernatant was centrifuged for 35 min at 15000 × g. The isolated enzyme was incubated in medium containing $^{14}$C-glucose for 2 h at 27°C. The reaction was stopped by adding of hot ethanol. Not associated label was removed by washing with 70% ethanol. The radioactivity counted using β-analyzer (LKB, Sweden).

#### *2. Determination of peroxidase activity*

POX activity was determined by diluting of 0.1 ml filtered plant homogenate with 1.9 ml of 0.01 M sodium phosphate buffer (pH 5.8) and 0.05 ml of a 0.02 M guaiacol in 0.01 M sodium phosphate buffer (pH 5.8). Then, 0.05 ml of 0.03% $H_2O_2$ was added. Assays were initiated by adding $H_2O_2$ and the change in optical density at 470 nm was measured for 1 min specific POX activity was calculated by the Boyarkin method (Boyarkin 1951).

#### *3. Determination of cellulase activity*

150 mg of colored no soluble substrate (laminarine) suspended in 0.5 mL of 0.1 M sodium acetate buffer, pH 4.5. 2 mL of crude plant extract was added to substrate solution and incubated for 20 min at 40°C. Obtained solution was filtered and the optical density was determined at 490 nm. Activity units were measured using following formula: 1 EU = 0.1*A, where A - optical density.

### Microscopic assay

The cotton ovule's morphology was assayed using an MBI-6 optical microscope and a Neophot-2 universal optical microscope (Carl Zeiss, Germany).

### Statistical analysis

Data were subjected to ANOVA, (Stat Soft. 2008 ANOVA/ MANOVA) and differences between treatments assessed by a Student's two-sample *t*-test at $P < 0.05$.

## RESULTS AND DISCUSSION

We analyzed the effect of IAA, NAA and $GA_3$ (phytohormones) on protein content and enzyme activity of GS, POX and Ce of 20 dpa integument tissue of naked seed (line 'L-70') cotton, and fiber and integument of linted ('AN-Bayaut-2') cotton. NAA showed no effect on total protein content in 'AN-Bayaut-2', whereas in L-70 it caused an increase of 13% (**Table 1**). $GA_3$ increased the protein content by 35% in 'AN-Bayaut-2' and by 6% in line 'L-70'. We suggested that NAA and $GA_3$ activate genes causing the synthesis of RNA, and lead to synthesis of new kinds of enzymes or their activation. This fact demonstrated increase of protein concentration in all 5-, 10- and 20 dpa integument tissues of seed-bud examples (**Table 1**).

The treatment of flower generative organs with $GA_3$ caused increasing of total protein concentrations in integuments. Accordingly literature data this fact my be explained by stimulation of cell proliferation and protein biosynthesis (Ogawa *et al.* 2003; Sponsel *et al.* 2004) and expression of *de novo* proteins by the action of $GA_3$ (Sun 2004; Thomas *et al.* 2006).

We have investigated the electrophoretic spectrum of total protein isolated from the integuments of control and $GA_3$- and NAA-treated plants. The results were shown in the **Fig. 1**. We have observed expression of some protein bands for the $GA_3$-treatment integuments in comparison to control samples (**Fig. 1**).

Basra *et al*. (1999) showed that plants treated with $GA_3$ before flowering increased the generative period, otherwise the flowering process was slowly inhibited, and the process of fruit ripening was activated after this phytohormone-treatment during the flowering period. The authors concluded that the process of protein biosynthesis is activated and their concentration increases after treatment of the flower with $10^{-6}$ M $GA_3$. The treatment with NAA, before blooming, caused increase in the number of fuzz on the epidermal surface of cells compared to $GA_3$-treatment, according to

**Table 1** Enzyme activity of GS, POX, and Ce isolated from 20 dpa integument tissues.

| Samples | Protein content mg/ml (%) | | Enzyme activity | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Integument | Fiber | GS cpm/min/mg of protein (%) | | POX unit/mg of protein (%) | | C unit/mg of protein (%) | |
| | | | Integument | Fiber | Integument | Fiber | Integument | Fiber |
| **Line 'L-70'** | | | | | | | | |
| Control | 5.217 ± 0.2 (100) | - | 1710 ± 80 (100) | - | 7.30 ± 0.4 (100) | - | 7.67 ± 0.4 (100) | - |
| Treatment with IAA | 5.999 ± 0.3 (115) | - | 6120 ± 300 (358) | - | 8.60 ± 0.4 (118) | - | 8.25 ± 0.4 (108) | - |
| Treatment with NAA | 5.913 ± 0.3 (113) | - | 5870 ± 290 (343) | - | 9.20 ± 0.5 (126) | - | 8.46 ± 0.4 (110) | - |
| Treatment with $GA_3$ | 5.530 ± 0.2 (106) | - | 620 ±30 (36) | - | 9.20 ± 0.5 (126) | - | 5.94 ± 0.3 (77) | - |
| **'AN-Bayaut-2'** | | | | | | | | |
| Control | 4.696 ± 0.2 (100) | 1.438 ± 0.07 (100) | 1000 ± 45 (100) | 3460 ±150 (100) | 13.40 ± 0.7 (100) | 0.256 ± 0.01 (100) | 6.39 ± 0.3 (100) | 0 |
| Treatment with IAA | 5.166 ± 0.2 (110) | 1.610 ± 0.08 (112) | 1350 ± 59 (138) | 6890 ± 330 (199) | 13.1 ± 0.6 (98) | 0.287 ± 0.01 (112) | 5.98 ± 0.3 (94) | 0 |
| Treatment with NAA | 4.696 ± 0.2 (100) | 1.508 ± 0.07 (105) | 1200 ±60 (120) | 6440 ± 300 (186) | 12.30 ± 0.6 (92) | 0.304 ± 0.02 (119) | 5.75 ± 0.25 (90) | 0 |
| Treatment with $GA_3$ | 6.348 ± 0.3 (135) | 1.780 ± 0.09 (124) | 10,588 ± 500 (1060) | 13625 ± 600 (394) | 11.10 ± 0.5 (83) | 0.342 ± 0.02 (134) | 5.51 ± 0.25 (86) | 0 |

Values are mean ± SD, experiment repeated at least three times.
Presented averages and standard deviations according to the Student's *t*-test ($P < 0.05$)

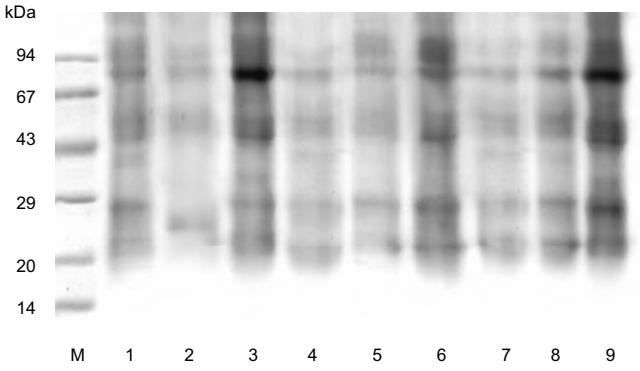


**Fig. 1 Electrophoregramme of total protein from integument of 'AN-Bayaut-2' in gradient PAG (10 to 15%) after 5, 10 and 20 dpa.** M – protein marker-standard; 1 – control; 2 – treatment with NAA; 3 – treatment with $GA_3$ (5 dpa); 4 – control; 5 – treatment with NAA; 6 – treatment with $GA_3$ (10 dpa); 7 – control; 8 – treatment with NAA; 9 – treatment with $GA_3$ (20 dpa).

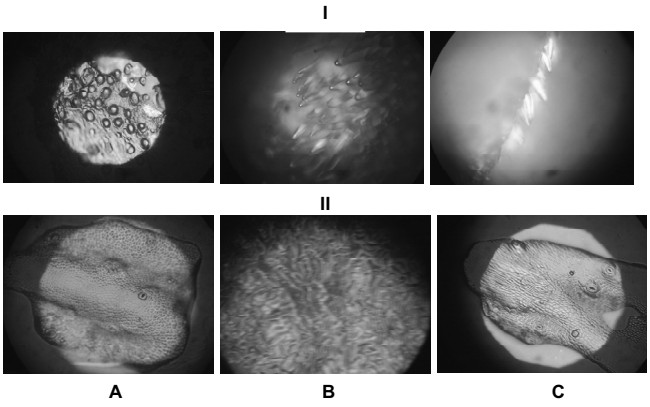


**Fig. 2 Microscopic assay of cotton integuments (I – 'AN-Bayaut-2'; II – 'L-70').** (**A**) Control; (**B**) treatment with NAA ($10^{-6}$ M); (**C**) treatment with $GA_3$ ($10^{-6}$ M).

optical microscopic of control and phytohormone-treated ovules. The treatment of unfertilized seed-buds revealed the reverse effect: $GA_3$ increased a fuzz number compared to NAA. Therefore, an exogenous phytohormone effect depends on period of influence on cotton on the dpa. Studying 'AN-Bayaut-2' cotton ovule structure on the epidermis surface showed "blubber" cells especially near chalazal holes (**Fig. 2, I**). The process of fuzz appearance and their elongation increased after $GA_3$-treatment in first day flowering ($10^{-6}$ M) (Turley *et al.* 2002).

The surface of 'L-70' cotton ovules remained largely unchanged after treatment with NAA and $GA_3$. However, there was an increase in the number of ovule cells which lead to distortion of lank cell surface, to appearance of folds, and to longitudinal deepening (Bowman *et al.* 2000). However appearance of fuzz was not observed (**Fig. 2, II**).

The investigation of the influence of phytohormones to the activity of GS, POX and Ce which considered as a fiber development associated enzymes revealed that a treatment of naked seed ovules with NAA increased activity of GS by 243%, POX by 29%, and Ce by 10% (**Table 1**). The activation of the analyzed enzymes in 20 dpa integument tissue of L-70 under NAA was associated with an increase in protein quantity. Protein synthesis and the increase of enzyme activity are closely interconnected (Sakalo *et al*. 2004). According to our results (**Table 1**), the increase of enzyme activity correlates with increase of protein content under NAA-treatment in 'L-70'. NAA also increased the activity of GS in linted 'AN-Bayaut-2' by 20%, while POX and Ce activities were reduced by 8 and 10%, respectively. Furthermore, treatment of ovules with $GA_3$ reduced GS activity in integument tissue of 'L-70' by 64%; Ce by 23%, and POX acclivity increased by 29%. The activity of GS increased (by 960%) in integument of AN-Bayaut-2, while activities of POX and Ce were reduced by ~15% (**Table 1**).

The specificity of plant reaction to different phytohormones is controlled by genetic background of the cultivar (Chow *et al.* 2006). The cause of physiological activity has been explained by two hypotheses: 1) exposure of plants to exogenous gibberellin causes the plant to convert gibberellin to the active form; and 2) plant cells have different specific receptors to gibberellins, and gibberellins activity depends on the degree of their affinity of the molecular structure to the specific receptor. In our case, the various effects of phytohormones may be caused by the presence of different receptors in line 'L-70' and cv. 'AN-Bayaut-2' (Gubler *et al.* 2002; Chow *et al* 2006). Several means of phytohormone inactivation exist in plant cells such as binding of proteins, glycolysis, POX and polyphenoloxidase oxidation. In our previous study (Akhunov *et al*. 2001), we showed the presence of proteins which inhibited GS activity from integument tissue of naked seed of line 'L-70'. In the current investigation, we studied the effect of protein fractions of phytohormone-treated integument tissue on the GS

**Table 2** Effect of proteins isolated from 20-days integument of naked seed on GS activity.

| Sample | Inhibition (%) |
|---|---|
| **Line 'L-70'. Control integument** | |
| Initial material | 84 |
| 1st fraction | 77 |
| 2 fraction | 14 |
| **Integument treated with IAA** | |
| Initial material | Stimulation 47 |
| 1st fraction | Stimulation 168 |
| 2 fraction | 39 |
| **Integument treated with NAA** | |
| Initial material | Stimulation 40 |
| 1st fraction | Stimulation 170 |
| 2 fraction | 40 |
| **Integument treated with $GA_3$** | |
| Initial material | 36 |
| 1st fraction | 96 |
| 2 fraction | 20 |

Values are mean ± SD, experiment repeated at least three times.
Presented averages and standard deviations according to Student's *t*-test ($P < 0.05$)

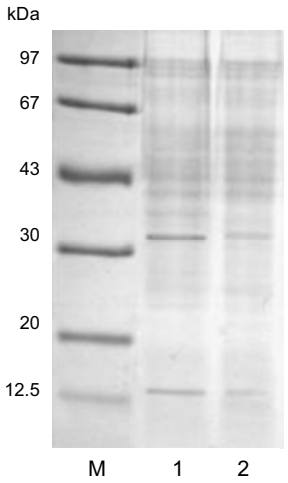


**Fig. 3 Electrophoresis of proteins from 20 dpa integument tissue of line 'L-70'.** M – protein marker-standard; 1 – proteins from $GA_3$ ($10^{-6}$M)-treated samples; 2 – extract of control samples.

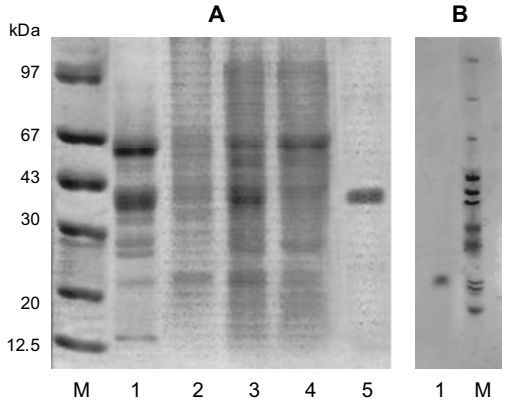


**Fig. 4 Electrophoresis of fractions obtained after chromatography on DEAE-TSK.** (**A**) M – protein marker-standard; 1 – fraction 1; 2 – fraction 2; 3 – fraction 3; 4 – fraction 4; 5 – fraction 5. Isoelectrofocusing in 7% PAG at presence of ampholins in diapason pH 3.5-10. (**B**) M – mixture of pI markers; 1 – protein – inhibitor.

activity. Proteins from integument of naked seed line 'L-70' suppressed GS activity both in control and in experimental variants (**Table 2**). The first fraction from $GA_3$-treated integument tissue, obtained after gel filtration on TSK-gel column, suppressed GS activity by 96%. In this case the gene-inhibitor is in the dominant state (Musaev *et al.* 1988) and, possibly, during $GA_3$ treatment an activation of inhibitor gene occurred. The increase of GS to 170% was observed under the effect of proteins from integument. The activation of GS (or intensification of synthesis this enzyme) stimulated under the action of this phytohormone.

Electrophoretic determination of protein components from ovule integument tissue of naked seed 'L-70' line revealed that in the control and $GA_3$-treated plants, there are two proteins detected with molecular masses 37 and 13 kDa, respectively, in addition to a large number minor protein components (**Fig. 3**). Proteins bands of samples treated with $GA_3$ are expressed more clearly than control.

These results suggest that proteins which possess inhibitory effect to GS activity are present in total protein extract of $GA_3$-treated integument tissue of 'L-70' line. To purity this protein fraction we used size-exclusion chromatography on TSK-HW-55F. The separation of total protein on TSK-HW-55F showed the presence of three fractions. The first fraction decreased GS activity by 63%, while the second and the third fractions had lower inhibitory effect (20 and 25%, respectively) as compared with control. The protein fraction composition was investigated by electrophoresis (**Fig. 4A**). This fraction (fraction 5) was homogenous with a molecular mass of ~ 37 kDa. The pI is 4.2 (**Fig. 4B**).

## CONCLUSION

Thus, phytohormones IAA, NAA and $GA_3$ have different effects on protein biosynthesis and alter the activity of glucansynthase, peroxidase and cellulase enzymes active during fiber formation. The study showed an increase in the number and length of fiber in linted cotton under the action of phytohormones. The changes in structure of ovule surface of naked seed were marked, but appearance of fiber was not observed. This suggests that naked seeding of 'L-70' cotton line is genetically determined. Furthermore, the content of protein-inhibitor with Mw 37 kDa increased in integument tissue of naked seed cotton under the effect of $GA_3$. The research of modulators of cotton fiber development will allow understanding the typical mechanisms of development of fiber with different quality. The knowledge about these properties is demonstrates the importance of parental selection, and control of growth processes.

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