

EFFECT OF DIFFERENT ANTIMICROBIAL AGENTS ON THE FIBER DEVELOPMENT OF *IN VITRO* CULTURED COTTON OVULES

HAFIZA MASOOMA NASEER CHEEMA¹, ASIA KHATOON²,
AFTAB BASHIR^{2*} AND KAUSER A, MALIK³

¹University of Agriculture, Faisalabad.

²National Institute for Biotechnology and Genetic Engineering (NIBGE)
P.O. Box 577, Jhang Road Faisalabad 3800-Pakistan.

³Department of Biological Sciences, F.C. College University, Ferozpur Road, Lahore
*Corresponding author: E-mail: aftabbashir@nibge.org

Abstract

The outer epidermal layer of the cotton ovule may differentiate *in vitro* to form a mass of white fluffy fibers in an appropriate liquid medium. The *in vitro* cultured fibers provide a model for studying not only the cellular physiology and development mechanism but also generate a key resource for promoter evaluation in developing fibers. However, the potential uses of *in vitro* cultured ovules are restricted due to the frequent problem of bacterial and fungal contamination. In this study, the effect of Ampicillin, Gentamicin and Sodium Azide on cotton fiber development was observed at various concentrations (100 µg/mL, 50 µg/mL and 0.5 µg/mL respectively) which were added in the media as contamination controlling agents. The *in vitro* grown ovules were evaluated at 5, 10, 15 and 20 DPA (days post anthesis) for fiber length, percent viable ovules producing fiber, ovules fresh weight and ovules dry weight. It was observed that ovules grown on the media containing either Gentamicin or Sodium Azide were unable to initiate normal number of fiber cells. Addition of Ampicillin to cultures enhanced the fiber length, ovule weight and percentage of viable ovules with fibers. The inhibitory effect of Gentamicin on fiber growth was reversible, when ovules were transferred to Gentamicin -free medium within 3 days after culture initiation. However, Sodium Azide resulted in irreversible fiber development inhibition. These results indicate that Ampicillin was not only efficient to control contamination but also enhanced the fiber and ovule development process.

Introduction

Cotton fiber is an example of the longest cell in plant kingdom. It is a good experimental model for studying plant cell elongation, expansion and cell wall biogenesis (Triplett, 2000; Kim & Triplett, 2001). *In vitro* growth of cotton fibers (Beasley, 1971; Beasley, 1973; Beasley & Ting, 1973) has opened up new horizons in investigating the physiology, biochemistry, transient gene expression and regulation of cotton fiber development (Triplett, 2000). The embryos of interspecific hybrids of *Gossypium* have been rescued using the ovule culture technique (Stewart & Hsu, 1978; Stewart, 1981; Umbeck & Stewart, 1985; Vlachostergios *et al.*, 2007). With the improvement of this technique introgression of different species became possible by recovering the interspecific hybrids of wild and cultivated species (Stewart, 1991, 1992). *Agrobacterium* mediated transformation of cotton ovule cultures has also been reported (Delmer & Holland, 2000). Previously, fertilization was considered essential for inducing *in vitro* fiber growth in the absence of exogenous plant hormones (Beasley, 1971; Beasley and Ting, 1973). Gialvalis and Seagull (2001) reported that unfertilized ovules can also

produce large number of fibers in the absence of hormone in the media. Hormones and fertilization may be essential for fiber growth, but do not play a role in fiber initiation (Gialvalis & Seagull, 2001). Exogenous plant hormones, including indole-3-acetic acid (IAA) and gibberellic acid induce significant increase in fiber production in fertilized ovules (Beasley, 1973; Gialvalis & Seagull, 2001). The use of synthetic auxin (naphthalene-1-acetic acid NAA) promoted fiber elongation and weight as compared to the natural auxin (IAA) by hindering the secondary cell wall cellulose synthesis (Singh *et al.*, 2009). Liquid culture media has been observed to support fiber growth from detached cotton ovules but limited growth on the solid ovule culture medium has been reported (Beasley, 1973). Ovules cultured on solid media lead to decrease in cellulose accumulation in the fiber cell (Triplett & Johnson, 1999). Usually in the liquid culture media, fibers grow only at the surface of the ovule facing the atmosphere (Beasley, 1971) while, the ovular surface submerged in the liquid medium tends to initiate callus formation (Beasley and Ting, 1973). The differentiated fiber development in submerged and air oriented cotton ovule cultures has also been reported by Feng and Brown (2000).

Cotton ovule culture has numerous applications in the field of plant developmental biology and *in vitro* gene expression studies (Triplett, 2000). The *in vitro* cultured ovules were used as an important tool to study the effect of different temperatures on cellulose biosynthesis (Haigler *et al.*, 1991; Xie *et al.*, 1993), plant-fungal interactions (Mellon, 1986) and examining the structure and biochemistry of naturally pigmented cotton fibers (Ryser *et al.*, 1983). Ovule culture has many advantages over whole plants, especially, the studies involving inhibitors, radio-labeled precursors, or controlled environmental conditions. The comparisons between the fibers produced *in planta* and *in vitro* have been reported (Meinert and Delmer, 1977; Triplett & Timpa, 1995; van't Hof & Saha, 1997; Turley, 1998; van't Hof & Saha, 1998; van't Hof, 1999). Contamination in the culture media during the growth of ovule culture deters the fiber growth and quality that effects reproducibility and sustainability in fiber growth experiments. There is yet no report on the effect of antimicrobial agents on the *in vitro* culturing of cotton ovules. This study was, therefore, undertaken to control the contamination in artificial medium and to address the problem in reproducibility and sustainability in *in vitro* cultured ovule experiments.

Materials and Methods

Cotton plants (*Gossypium hirsutum* L.) cv. CIM-707 were grown under green house conditions. The temperature was maintained at 32°C (± 1 °C). The relative humidity was observed to be 60% throughout the plant growth. A maximum of two insecticide sprays were applied when required. Flowers were tagged on the day of opening, that is given as zero day postanthesis (0 DPA). The ovaries were collected at 0 DPA, soaked in 70 % ethanol for 2-3 minutes, rinsed with water for 1-2 minutes and dissected with flame sterilized blade and forceps to expose ovules. About 25-30 ovules at each development stage were aseptically transferred to 100 mL flasks containing 50 mL liquid ovule culture media (BT media, Beasley and Ting, 1974) and incubated at 28 °C in dark. The pH of the media was adjusted to 5.4 along with the hormonal combination of 5 μ M indole-3-acetic acid and 0.5 μ M gibberellic acid. The Ampicillin, Gentamicin and Sodium Azide were used to test the inhibition of contamination at the concentration of 100 μ g/mL, 50 μ g/mL and 0.5 μ g/mL respectively. The controls included ovules induced in BT media without any antimicrobial agents. The treated and control cultures were initiated on the ovules

collected at 0DPA. Ovule cultures initiated on BT medium at 0 DPA having 50 µg/mL Gentamicin and 0.5 µg/mL Sodium Azide were transferred to culture media without Gentamicin and Sodium Azide on 1, 2, 3, 4 and 5 DPA. All treatments along with control were performed in triplicate. A total of 12 flasks (3 control and 9 from each treatment) were harvested at 5, 10, 15 and 20 DPA each for recording observations on fiber growth and length, fresh ovule weight, dry ovule weight and total viable count of contaminants.

Observed characteristics

I. Percent viable ovules producing fibers: Individual ovules were scored for the presence or absence of visible fibers at 5, 10, 15 and 20DPA, regardless of the length and morphology.

ii. Fiber length: Fiber length was estimated by placing ovules on a watch glass and gently spraying fibers with a spray of distilled water as reported by Schubert *et al.*, (1973). The distance from the chalazal end of the ovule to the tip of the spread fibers was measured manually using a millimeter scale.

iii. Ovules fresh weight: Thirty ovules were taken from each set of cultures induced at 0 DPA, 5DPA, 10 DPA and 15DPA. The liquid media were dried off the ovules by placing them on blotting paper. The ovules were weighed by analytical balance (Ohaus, USA).

iv. Ovules dry weight: Cultured ovules with fiber were dried in oven at 60 °C for 24 hr to evaporate the moisture completely. The cultured ovules taken from three flasks and dried at 5, 10, 15 and 20 DPA were weighed on analytical balance (Ohaus, USA).

v. Contamination: About 1 mL of culture media from flasks harvested at 5, 10, 15 and 20 DPA was diluted 50 times with sterile BT medium. A 100 µL aliquot was spread on solid BT medium. The total viable counts for bacterial colonies were obtained. At 5 DPA four flasks for each treatment were evaluated for TVC (total viable count) and the flask containing contaminants were not included for subsequent evaluation.

Results and Discussion

Forty Forty percent of the control culture flasks (without antimicrobial agents) develop turbidity at 3 DPA. During 5-20 DPA other symptoms of contamination, like cloudiness of the media and floating fungal spores and bacterial colonies appeared that lead to the retardation of ovules to produce fiber. Considering these symptoms as the critical point we conducted initial experiments to screen for the lowest concentration of Ampicillin, Gentamicin and Sodium Azide that would inhibit contamination and have minimum effect on fiber initiation and development. The rate of contamination appearance was very low during the culturing period by using Ampicillin and Gentamicin at the concentration of 100 µg/mL and 50 µg/mL respectively (Table 2). The Sodium Azide did not let any type of contaminants to appear in the induced ovule cultures but it was inhibitory to fiber development on cultured cotton ovules even when it was added at 0.5 µg/mL concentrations. The control cultures (without any antimicrobial agent) were prone to high rate of contamination. For recording the data of fiber length, only those control cultures were selected which did not show any contamination.

The minimum concentrations of Sodium Azide (0.5µg/ml), Ampicillin (100µg/ml) and Gentamicin (50µg/ml) inhibiting culture contamination were selected to determine their effect on fiber growth. The percentage of ovules producing fibers was highest in the cultures containing Ampicillin, while Sodium Azide had inhibitory effect on ovules producing fibers (Table 1). Sodium Azide had the highest impact on fiber development. It is postulated that the mutagenic nature of Sodium Azide (Turken *et al.*, 2006) may not let free operation of the cellular processes, which in turn may disrupt the transcriptional machinery resulting in the retardation of fiber development. Addition of Ampicillin at 0 DPA to ovule cultures elevated the percentage of fiber producing ovules (Fig. 1 and Fig. 2) and the fiber elongation was markedly improved. However, Gentamicin slowed down the fiber elongation and further development of the fibers as compared to the control (Fig. 1). It indicated that the initiation phase of fiber growth was most sensitive to Sodium Azide, while it responded efficiently to Ampicillin. These results are in agreement with previous reports which emphasized that the first two days of cultivation were critical for fiber initiation (Beasley *et al.*, 1974; Dhindsa *et al.*, 1976; Graves and Stewart, 1988). Previous observations based on uptake of 3H-uridine by ovule cultures and autoradiography of fibers suggested that the majority of RNA synthesis takes place during the first 5 DPA (Berlin, 1986). Sodium Azide seems to arrest the transcriptional activity that could not be reverted even after the ovules were shifted to BT medium lacking Sodium Azide.

Table 1. Effect of Ampicillin, Gentamicin and Sodium Azide on fiber length, ovules fresh weight and ovules dry weight.

Treatments	% Ovules with Fibers ^a	^a Fiber Length (mm)				^b Ovules Fresh Weight (mg)				^b Ovules Dry Weight (mg)					
		5DPA	10DPA	15DPA	20DPA	0DPA	5DPA	10DPA	15DPA	20DPA	0DPA	5DPA	10DPA	15DPA	20DPA
1 Ampicillin	95 %	7	10	14.5	19.5	3	20	83	131	176	1	5.0	11.0	18.0	29.0
2 Gentamicin	40 %	1	2.5	3.0	3	3	6	9	17	29	1	2.0	3.0	7.5	14
3 Sodium Azide	0 %	0	0	0	0	3	3	3	3	3	1	1	1	1	1
4 Control	80 %	7	9	13.5	16.5	3	19	67.5	110.5	168	1	3.5	7.5	16.5	26.5

*The percentage was calculated by dividing the number of ovules producing fiber with total number of ovules cultured on BT media for each treatment.

a: Twenty ovules from each treatment were analyzed for fiber length taken out of three flasks randomly.

b: Thirty ovules from each treatment were taken out of three cultured flasks randomly.

Table 2. Effect of Ampicillin, Gentamicin and Sodium Azide on total viable count of contaminants

	Treatments	Total viable count (cfu/mL)			
		5 DPA	10 DPA	15 DPA	20 DPA
1	Ampicillin	0	1x10 ³	4x10 ³	2.5x10 ⁴
2	Gentamicin	0	0.5x10 ³	1.5x10 ³	0.8x10 ⁴
3	Sodium Azide	0	0	0	0
4	Control	1.3x10 ³	2x10 ⁵	6x10 ⁶	2x10 ⁹

The comparison of fiber length and fiber mass per ovule in the treated and control cultures indicated that Ampicillin had a beneficial effect on the fiber length (Fig. 2). The addition of Gentamicin to cultured ovules shortened the final fiber length (Fig. 1 and Fig. 2). However, the rate of fiber cell elongation increased on Ampicillin addition. It showed that the cell processes necessary to support cell elongation were not interrupted by ampicillin but, somehow, supported ovule cultures and resulted in a higher total biomass (Table 1).

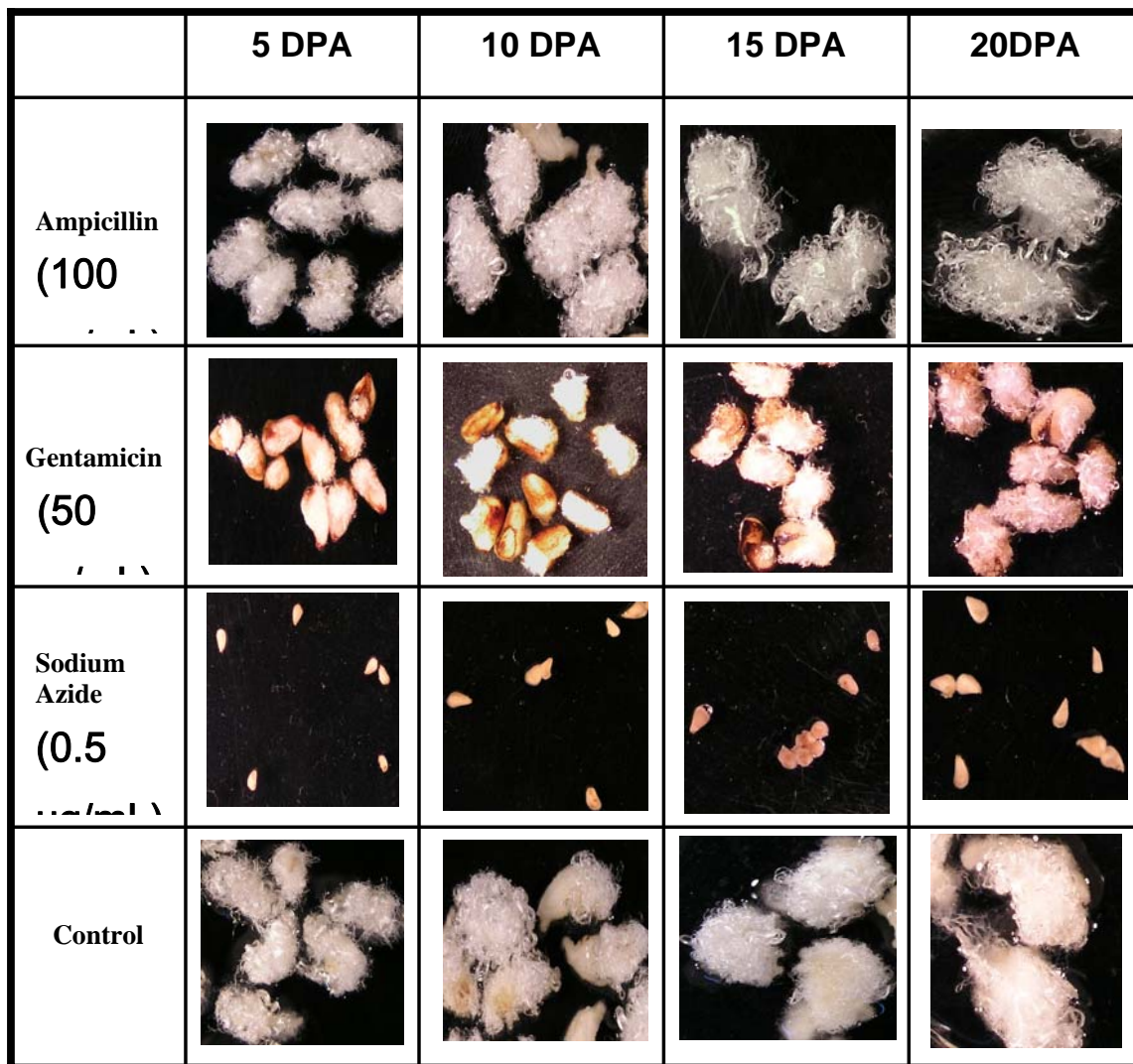


Fig. 1. Photo-gallery of the cotton ovules grown *in vitro* on a media containing Ampicillin, Gentamicin and Sodium Azide along with the control at 5DPA, 10 DPA, 15 DPA and 20 DPA.

Ovules fresh and dry weight determined between 0 and 20 DPA in ovule cultures was not affected by Ampicillin (Table. 1). Gain in ovule mass during development coordinated well with the increase in cell number and volume on Ampicillin application. The Gentamicin treatment resulted in the loss of ovular mass in coordination with the reduced fiber length when compared to the control (Table 1). The negative impact of Gentamicin on ovular fresh and dry weight can be directly related to the reduced fiber length. This observation is in agreement with the idea that fiber elongation is a complex interplay of temporal gene regulation and turgor pressure raised by the basal

cells of the ovular epidermal layer (Ruan, 2005). The slow rate of ovular cell's multiplication and expansion was considered to be due to reduced fiber initiation and elongation.

Inhibition of fiber cell growth by Gentamicin was reversible for up to 3 DPA and irreversible by Sodium Azide, when the ovules cultured with these treatments were shifted to the media free of these antimicrobial agents. After 20 DPA on Gentamicin -free BT media, the appearance of cultures pretreated with Gentamicin for up to 3 DPA was nearly indistinguishable from control cultures. However, pre-exposure to Gentamicin for 5 days significantly altered the ovular capability to revert back and produce fibers equivalent to the controls. These results suggest that there is a window of opportunity in the first 3 DPA for fiber cell initials to develop into elongated fiber cells.

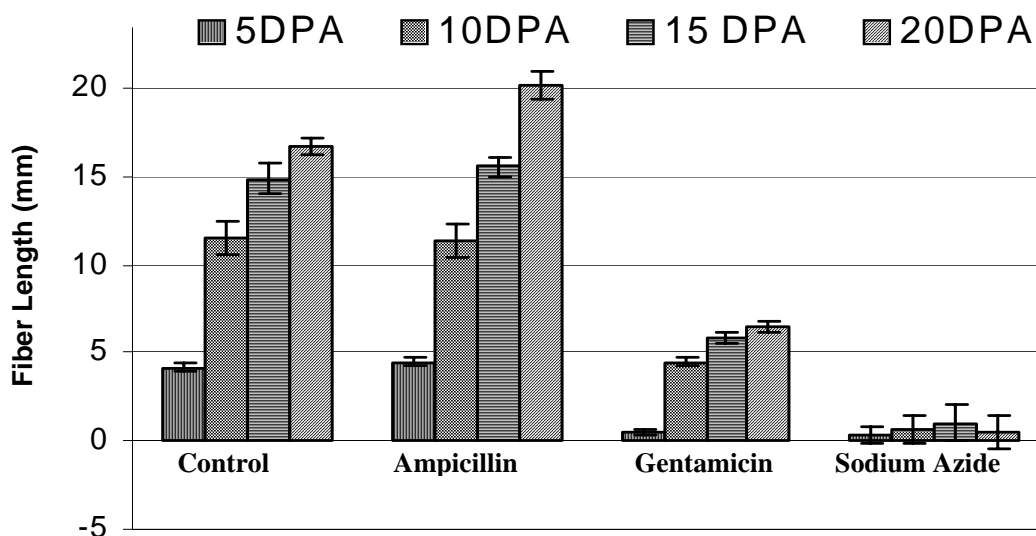


Fig. 2. Effect of Ampicillin, Gentamicin and Sodium Azide at the concentration of 100 $\mu\text{g}/\text{mL}$, 50 $\mu\text{g}/\text{mL}$ and 0.5 $\mu\text{g}/\text{mL}$ respectively on fiber length of *In vitro* grown ovules at 5 DPA, 10 DPA, 15 DPA and 20 DPA.

The results indicated that the ovular cells did not differentiate between the 3 DPA period, while a prolonged exposure resulted in loss of their capacity to differentiate. It showed that the genes which are required for the differentiation of fiber initials can not be switched on after 3 DPA. Similar results suggesting the importance of the ovule development period (4-6 DPA) was observed on treatment with exogenous abscisic acid (Dhindsa *et al.*, 1976) or bromodeoxyuridine (Dhindsa, 1978). Similarly, the treatment of ovules at pre-anthesis in BT medium without IAA and GA3 for several days indicated their loss of capacity to support fiber development (Graves and Stewart, 1988). It showed that any factor delaying expression of the ovule's normal developmental program would result in reduced fiber production.

Collectively, these experiments showed that Ampicillin, Gentamicin and Sodium Azide inhibited the microbial contamination successfully. Ampicillin enhanced the fiber and ovule growth resulting in increased fiber length and ovular mass. On the other hand, the ovules may be held up to 3 days in media containing Gentamicin with little or no effect on subsequent fiber development. But, Sodium Azide had an irreversible effect on ovular and fiber development. The studies provide information on the beneficial impact

of Ampicillin in ovule culture. Microbial contamination has been very problematic in the *in vitro* ovule culture studies. The use of Ampicillin at a concentration of 100 µg/ml can overcome the contamination problem and would be very beneficial in the fiber analysis programs focused on modifying fiber traits.

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