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In Vitro Plant Regeneration from Cultured Cotyledons of Cotton (*Gossypium herbaceum* L.)

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Abstract

The effect of cytokinins on callus proliferation from cotyledons and plantlet development was studied in cotton. The frequency of callus induction was observed on MS medium enriched with a variety of cytokinins in different concentrations. With the increase of cytokinin concentration, the percentage of callus formation, percentage of shoot developing calli and number of shoots/calli were increased. Among the three different cytokinins studied, BA showed the highest performance. The highest percentage of callus (6.55%) and shoot developing calli (5.87%) was obtained on MS with 1.0 mg/l BA. Highest number of shoots (3.02) per calli was observed on MS media supplemented with 1.0 mg/l Kn. The rooting media composed of MS medium, 0.6% agar, sucrose and fortified with 2.0 mg/l NAA induced root development at the highest percentage (41.23%) with maximum number of roots (3.61) per cutting and length of root (3.62 cm) per culture. The plantlets were acclimatized in natural conditions.

Key words: *In vitro,* Callus, Cotyledons, Cytokinin, Plantlet, Acclimatization.

Introduction

The cotton plant (*Gossypium* sp.) belongs to the family Malvaceae including 35 species only four of which are widely cultivated. The diploids are conventionally divided into six genome groups based on cytological affinities. Cotton is a perennial arborescent tropical plant. This annual plant is generally planted in the late 'Kharif' season (July-August). Cotton (*Gossypium* sp.) is an excellent natural source of textile fiber and is cultivated in many countries. Cotton is one of the important cash crops of Bangladesh. Presently it is grown in an area of about 78,000 acres with a total production of 91,000 bales (Anonymous, 2000). Present production of cotton can hardly fulfill 15-20% of the total requirement for textile mills and handloom industries in the country. Consequently, a substantial amount of foreign exchange is spent every year to meet the demand for lint cotton. So, increased cotton production is the present day need of the country. When compared to other major commercial crops, efficient *in vitro* techniques for regeneration of large numbers of cotton plantlets are limited.

Tissue culture methodology for plant regeneration from cotyledon explant is well established for herbaceous, ornamentals, fruits or vegetable crops. Price and Smith (1979) were the first to report somatic embryogenesis in the cotton, *Gossypium koltzchianum*, although complete plants could not be regenerated. Davidonis and Hamilton (1983) first described plant regeneration from two year old callus of *Gossypium hirsutum* L. cv Coker 310 via somatic embryogenesis. Since then, significant progress has been reported in cotton tissue culture (Zhang and Feng, 1992; Zhang, 1994). Although efficient plantlets regeneration from embryogenic calli through somatic embryogenesis has been improved significantly in recent years, some difficulties still remain. So, an attempt was also made to find out the effect of cytokinins on callus proliferation and plant regeneration of cotton using cotyledonary explant.

Materials and Methods

Cotyledon explants were excised from 90 days old mature seeds of cotton (*Gossypium herbaceum* L.) collected from four different places of Bangladesh. The seeds of were thoroughly washed under running tap water and then treated with 1% savlon and 4-5 drops of Tween 80 for about 15 minutes followed by successive three washing with distilled water to make the material free from savlon. Then the seeds were surface sterilized by immersing the seeds in 0.1% $HgCl_2$ for 7 minutes with gentle shaking. After discarding the $HgCl_2$, the seeds were washed with sterile distilled water at least for 6-7 times till every trace of the sterilent was completely

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removed. Seeds were blotted dry on sterile filter paper under aseptic condition. After removing seed coat the cotyledons were inoculated aseptically for callus initiation in MS Medium (Murashige and Skoog, 1962) variously supplemented with Benzyl adenine (BA), 6-furfuryl aminopurine (Kn) and 2-isopentyl-adenine (2ip.) in various combinations (Table I). All these operation were done inside laminar airflow hood. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 minutes. The cultured Petri dishes were incubated in dark cupboard shelf in culture room. Percentage of explants induced callus was calculated using following formula:

$$\% \text{ of callus formation from explants} = \frac{\text{Number of explants formed callus}}{\text{Total number of explants cultured}} \times 100$$

After 8 weeks, the undifferentiated hard, greenish and small compact calli obtained from cotyledon explants were sub cultured 8-10 times at 6 weeks interval onto MS medium supplemented with same cytokinin formulation of primary medium to induce shoot regeneration. Culture media was maintained in culture shelves at 25±2°C under cool white 2000-3000 lux fluorescent light at 16 hour light and 8 hour dark cycle/day. After sub culturing few of these calli were differentiated to develop adventitious shoot buds (Table I). Percentage of callus explants induced to develop auxiliary shoots was calculated using following formula:

$$\% \text{ of adventitious shoot developing calli} = \frac{\text{Number of shoot developing calli}}{\text{Total number of calli cultured}} \times 100$$

Number of shoots per calli (Table I) was counted after 8 months of culture. Mean number of shoots per calli was calculated using following formula:

$$\bar{x} = \frac{\Sigma Xn}{N}$$

Where, x = Mean number of shoot, Σ = Summation, Xn = Total number of shoot and N = Number of observation.

For rooting of shoots, the well grown healthy shoots were separated and transferred to MS medium supplemented with different concentrations of IBA, NAA and IAA (Table II).

After 10-12 weeks, the plantlets were 3-5 cm height and developed a good root system and they were ready for transplanting into beds. Before transfer, the closures of the plantlet containing flasks were removed and kept in growth room for 3-5 days for acclimatization. In field, care was taken to avoid damage to the roots and to ensure good contact between roots and soil. The plants were kept in an environment with high relative humidity for the first few days following transplantation by covering the beds with polyethene sheets and regular spraying of water.

Results and Discussion

The case of cotyledon has served advantages in *in vitro* (Gogala and Camloh, 1988). Microbial contamination of such explant has never been a serious problem. Cotyledon explants of cotton (*G. herbaceum* L.) were cultured onto MS medium supplemented with different concentrations of BA, Kn and 2ip. After inoculation the size of cultured cotyledon explants were enlarged and the colour changed from white to green within eight weeks. Irrespective of growth regulator formulations a few percent cotyledon explants of cotton swelled up with one or two weeks of incubation and became dark green. Most of the calli differentiated within 3 weeks of culture. The calli were greenish, compact, hard and they grew slowly. Callus proliferation was noticed from the basal part of the cotyledons. Localized callus proliferation was also noticed from cut surface of the cotyledon. Only a few percent of the explants in all treatments induced to develop callus (Plate A). Mean data regarding to the percentage of cotyledon explants induced to develop callus explants in BA, Kn and 2ip are given in Table I. Among the cytokinins supplemented media, the highest percentage (6.55%) of cotyledon explants were induced to develop callus in MS + 1.0 mg/l BA. On the other hand, the percentage of callus inducing explants was the lowest (4.22%) in MS + 0.1 2ip supplemented media (Table I). The present study indicates that the percentage of callus forming explants has risen with the increase of cytokinin concentration.

For shoot regeneration the well grown callus were separated and sub cultured in MS medium containing the same concentration of cytokinins (Table I). Organogenic differentiation was noticed from cotyledon derived callus after 8-10 subsequent subculture at 6 weeks interval. Organogenic adventitious shoot bud development was also reported by Srivastava *et. al.* (1989) and Islam *et. al.* (1995) from neem cotyledon; Kathal *et. al.* (1986) and Niedz *et. al.* (1989) in melon. Adventitious bud differentiation was observed form calli showing embryoid like structures which were later differen-

tiated into tube like protrusions and eventually developed into buds. Among the cytokinins, MS + 1.0 mg/l BA showed the highest (5.87 %) and MS + 0.1 mg/l 2ip showed the lowest (3.97 %) performance (Table I).

Table I: Effect of different concentrations of cytokinins in MS medium on percentage of callus formation, percentage of shoot developing calli and number of shoots/calli

Cytokinins (mg/l)	% of callus formation (M±SE)	% of shoot developing calli (M±SE)	Number of shoots/calli (M±SE)
MS+0.1 BA	6.12±0.40	4.72±0.56	2.5±0.42
MS+0.5 BA	6.32±0.38	5.19±0.58	2.65±0.96
MS+1.0 BA	6.55±0.31	5.87±0.41	2.8±0.65
MS+0.1 Kn	5.55±0.58	5.20±0.85	2.72±0.75
MS+0.5 Kn	5.67±0.57	5.35±0.62	2.9±0.15
MS+1.0 Kn	5.87±0.56	5.58±0.61	3.02±0.43
MS+0.1 2ip	4.22±0.93	3.97±0.90	2.4±0.58
MS+0.5 2ip	4.37±0.78	4.15±0.89	2.52±0.52
MS+1.0 2ip	4.52±0.76	4.26±0.89	2.7±0.69

Each value represents an average of 10 replicates and each experiment was repeated at least thrice

Adventitious buds subsequently grew to shoots (Plate B) within 8-10 sub culture. In general, the results of shoot formation were slow in all media. However, the number of shoots/calli was highest (3.02) in MS + 1.0 mg/l Kn and lowest (2.4) in MS + 0.1 mg/l 2ip. It is observed from the present study that, with the increase of cytokinin concentration, the number of shoot per culture also increased. For shoot elongation the adventitious shoots were further sub cultured in the same medium.

Table II: Effect of different concentrations of IBA, NAA and IAA in MS medium on percentage of root forming explants, number of roots/shoot and length of roots /shoot

Growth regulators (mg/l)	% of root formation (M±SE)	number of roots/shoot (M±SE)	length of roots /shoot (M±SE)
MS+0.5 IBA	31.11±0.69	1.45±0.49	1.5±0.86
MS+1.0 IBA	32.52±0.53	1.90±0.64	1.7±0.59
MS+ 2.0 IBA	32.91±0.56	1.97±0.89	1.7±0.53
MS+0.5 NAA	38.75±0.39	2.87±0.31	3.30±0.88
MS+1.0 NAA	38.28±0.51	3.19±0.59	3.37±0.85
MS+ 2.0 NAA	41.23± 0.36	3.61±0.68	3.62±0.64
MS + 0.5 IAA	32.81±0.64	1.59±0.58	0.57±0.21
MS + 1.0 IAA	33.97±0.94	1.64±0.99	0.88±0.32
MS + 2.0 IAA	35.50±0.49	1.89±0.79	1.12±0.66

Each value represents an average of 10 replicates and each experiment was repeated at least thrice

Individual shoots from *in vitro* grown shoot clumps (1 cm or more) were excised and after trimming off basal leaves they were transferred to rooting media composed of MS basal salt having 0.6% agar, sucrose and fortified with different concentrations of IBA, NAA and IAA.

In most of the cases, root initiation started within 3-4 weeks after shoot inoculation in culture media (Plate C). The shoot, which failed to form roots within this period, were unable to develop root even after 8 weeks of culture. Among the 9 rooting media 2.0 mg/l each of IBA, NAA and IAA induced root development at the highest percentage. These media showed maximum number of roots per cutting (1.97, 3.61 and 1.89 respectively) and length of root per culture (1.7, 3.62 and 1.12 respectively) (Table II). Out of three auxins tested, performance of NAA was better than that of IBA and IAA. It was observed that high concentrations of IBA, NAA and IAA improved rooting frequency but it also induced callus development at the base of the microcuttings. These findings showed similarities with the results of Joarder *et. al.* (1993).

Rooted plantlets were successfully transferred to pots and acclimatized *ex vitro* condition (Plate D). The plantlets thrived well in the soil with vigorous growth. Increased vigor of tissue culture derived plant under natural condition has also been reported by many authors (Zimmermann, 1986; Swartzetai *et. al,* 1981).

The present study includes a standard protocol development for regular plant regeneration through callus phase from cotyledons. It has been reported that juvenile tissues such as, embryo and cotyledons are good source of explant for callus induction and subsequent plant regeneration either through organogenesis or embryogenesis (Arnold and Erikson, 1978; Hammerswechlag *et. al.,* 1985; Espinasse *et. al.,* 1989; Miller and Chandler, 1990; Karim, 1996; Abu Backer and

Plate A: Callus induction on cotton cotyledon in MS + 1.0 mg/l BA after 8 weeks of culture.

Plate B: Adventitous shoot development from cotyledonary callus in MS + 1.0 mg/l BA after 8 months of culture.

Plate C: Rooted shoots showing roots in MS +2.0 mg/l NAA after 12 weeks of culture.

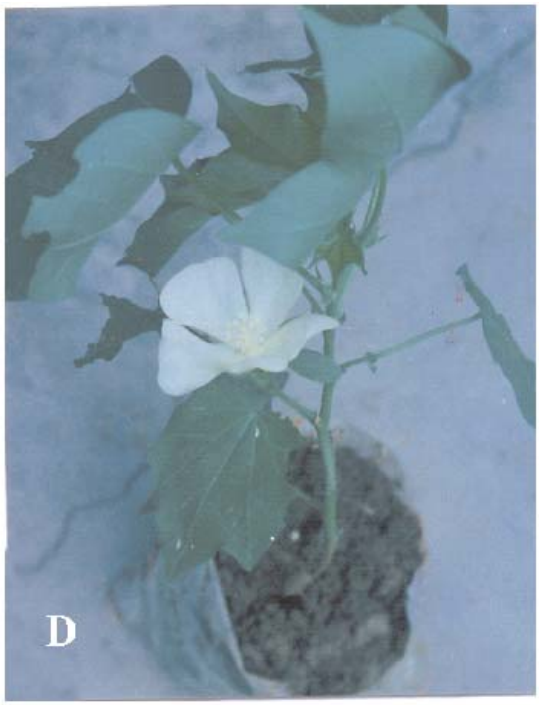

Plate D: *In vitro* grown plantlets in poly bag and acclimatized under *ex vitro* condition.

Alagumanian, 1999). Nevertheless, mature tissues are generally less responsive when compared with immature tissues (Sommer and Caldras, 1981; Lazzeri *et. al.,* 1985).

Conclusion

The result of the present study reveals that *in vitro* plant regeneration from unorganized callus tissue using cotyledon explants of cotton although take prolong time (6-10 times subculture at 4-6 week intervals) and regeneration frequency is very low but it could be possible to regeneration in *in vitro* condition. Moreover, these plantlets could be successfully transferred to the natural environments.

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