Influence of cytokinins, auxins and polyamines on in vitro mass multiplication of cotton (Gossypium hirsutum L. cv. SVPR2)

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Received 23 September 2005; revised 23 March 2006

In the present investigation, the influence of different forms of cytokinins, auxins and polyamines were tested for mass multiplication and regeneration of cotton. Initially, for the identification of effective concentration for multiple shoot induction, various concentrations of BAP, Kin and 2iP along with IAA and NAA were tested. Among tested concentrations, media fortified with MS salts; B5 vitamins; 30 g/l, glucose; 2.0 mg/l, 2iP; 2.0 mg/l, IAA and 0.7 % agar showed best response for multiplication of shoot tip explants (20 shoots per shoot tip explants). In nodal explants, maximum of 18.6 shoots were obtained in the media fortified with MS salts, B5 vitamins, 30 g/l, glucose, 2.0 mg/l, 2iP, 1.0 mg/l, NAA and 0.7 % agar. Effect of different concentrations of polyamines like spermidine and putrescine were also tested along with the above said multiplication media. Among the various treatments, 20 mg/l of putrescine showed best response and the multiple of shoots were increased to 26.5 shoots per shoot tip explants and 24.5 shoots per nodal explants. Elongation of shoots was achieved on multiple shoot induction medium. Significant number of roots were initiated in the medium supplemented with MS salts, vitamin B5 and 1BA (2.0 mg/l). The frequency of root induction was increased by addition of, PVP (10 mg/l) along with root induction medium and after 2 weeks, the roots reached the maximum length of 22 cm. Further, these plantlets were hardened by using sand, soil and vermiculate in 1:1:1 ratio. The hardened plants were transferred to the environmental growth chamber for proper acclimatization. The hardened plants were then transferred to field for boll yielding and they exhibited 100% survival.

Keywords: Acclimatization, Gossypium hirsutum, Hardening, MS medium, Organogenesis, Polyamines.

Cotton (Gossypium hirsutum L.), is a well-known fiber crop and it has been estimated that 180 million people depends on cotton production for textile industry and seed oil. The genus, Gossypium is comprised of 45 diploid and 5 tetraploid species1. The world’s cotton fiber production is obtained from four species, G. arboreum L. (n=13), G. herbaceum L. (n=13), G. barbadense L. (n=26) and G. hirsutum L. (n=26). Among the cotton producing countries, India ranks first in cultivation, with 32% of the world's total area followed by USA (23%) and China (20%)2. Apart from the fiber production, cotton cultivation is also used for isolation of gossypol due to its wide range of biological properties including anti-cancer, antimicrobial, anti-HIV, anti-oxidative and male contraceptive3. The yield of cotton is severely affected by insect pests, fungal, viral diseases and herbs. Cotton bollworm (Helicoverpa zea), pink bollworm (Pectinophora gossypiella), boll weevil (Anthonomus grandis), common bollworm (Heliothis zea) and beet armyworm (Spodoptera exigua) are considered as dangerous insect pests for fibre production. The fungal diseases like Fusarium wilt (Fusarium oxysporum), Verticillium wilt (Verticillium dahliae) and Alternaria leaf spot (Alternaria macrospora) of cotton, causes whole plant wilt and lesions on whole plant. Regarding viral diseases, cotton leaf curl virus causes heavy loss in the yield4. Recent reports proved that in India, nearly 0.2 million hectares of cotton fields are affected by the viral pathogen and cause 30% loss in the yield5,6.

Good quality cotton production with more yields requires controlling of the said biotic stresses. The practice of chemical control methods are expensive, laborious and resource intensive. Some insecticides and fungicides can cause environmental pollution. However, chemical control of pathogens is often difficult, incomplete, costly and transient7. Apart from the insect pests and fungal disease damages,
weeds in cotton field also reduce the percentage of cotton production. Hence, studies on biotic and abiotic stress tolerant plant production through existing or novel methodologies become imperative. In view of this, insect resistant (Bt gene), fungal (Chi II and Glu gene), viral disease tolerant (antisense AVR2 gene) and herbicide resistant (bar gene) cotton cultivars have been produced through genetic transformation studies. For cotton transformation procedures, nowadays micropropagation through shoot tip culture assumes significance in transgenic cotton production via Agrobacterium and biolistic transformation methods, because the shoot tip culture in cotton is thoroughly adapted for biolistic transformation methods, because the shoot tip culture in cotton is thoroughly adapted for Agrobacterium-mediated transformation compared to organogenesis and somatic embryogenesis. In other dicot plants also meristem based methods have been found successful in petunia, pea, sunflower, banana, tobacco and rice. Due to these reasons, the research laboratories and institutions are working to standardize a simple and effective multiplication protocol for cotton regeneration. In view of the importance of the crop, the protocols with even marginal increase in the number of shoots will be important for producing large number of transgenics. Hence, the present work was undertaken to evaluate the effect of various plant growth regulators and polyamines to increase the number of multiple shoots of widely cultivated Indian cotton cultivar SVPR2.

Materials and Methods

Seed germination and selection of explants—The delinted cotton seeds were obtained from Cotton Research Station, Tamil Nadu Agricultural University, Sivpilliputtur, Tamil Nadu, India. The cotton seeds were surface sterilized and germinated in vitro by earlier method. After a particular period of germination, the explants, shoot tips (5-10 days) and nodes (12-16 days), were aseptically removed from the in vitro grown seedlings. About 0.4-0.6 cm long shoot tip and nodal explants were excised for multiple shoot induction.

Tissue culture medium preparation—Basal medium supplemented with MS salts and B5 vitamins was used. The plant growth regulators like BAP, Kin and 2iP were dissolved in 0.1N HCl (2.0 ml) and diluted with distilled water to make 1.0 mg/2.0 ml each and stored in the refrigerator. The polyamines like spermidine and putrescine and the amino acids like pyridoxine-HCl, nicotinic acid, thiamine HCl and mio-inositol were dissolved in distilled water to make 10 mg/10 ml stock. For solidification of the medium, agar (0.7 %) was used throughout the study. All cultures were maintained at 25±2°C with constant light intensity (2500 lux) under 16 h light/day photoperiod. The light source was provided with cool white fluorescent lamps.

Multiple shoot induction and elongation—The above said explants were placed in vertical position in different media for induction, elongation of multiple shoots and rooting of elongated shoots. For high frequency adventitious multiple shoot induction, three important steps were followed. In the initial step, individual treatment of different cytokinins like BAP (1.0-2.0 mg/l), KN (1.5-2.5 mg/l) and 2iP (2.0-3.0 mg/l) were evaluated. After the identification of suitable concentration of cytokinin for multiplication, different auxins (IBA, NAA and IAA) in various concentrations (0.5-5.0) were tested in the second step. In the third step, supplements like polyamines were evaluated. The commonly used polyamines like spermidine (20-40 mg/l) and putrescine (5-40 mg/l) were tested along with identified concentrations of cytokinins and auxins. During multiple shoot induction, influence of each plant growth regulators were noticed. In the present study, elongation of the induced shoots was achieved on the multiple shoot induction medium itself instead of using any other special elongation medium. For multiple shoot induction from the shoot tip and nodal explants, 75 explants were tested with 4 replicates and each experiment was repeated three times.

Root induction, hardening and acclimatization—After complete elongation, shoots (20-25 days old) were transferred to root induction medium fortified with MS basal salts, B5 vitamins and IBA (0.5-5.0 mg/l). During root induction, the impact of different concentrations of PVP was tested to control the secretion of phenolic compounds from the elongated shoots. After rooting, regenerated plants were hardened on plastic pots containing sterile soil, sand and vermiculate in the ratio of 1:1:1. The hardened plants were maintained in environmental growth chamber for proper acclimatization and then the in vitro regenerated plants were successfully transferred to the field. During root induction 95 elongated shoots were tested for each treatment and...
each experiment was repeated 4 times with 5 replications.

Statistical analysis—Mean and standard error were used throughout the study and the values were assessed using a parametric Mood’s median test25. The data were analyzed for variance by Duncan’s Multiple Range Test (DMRT) using the SAS programme (SAS Institute, Cary, N.C.).

Results and Discussion

Adventitious shoot induction and multiplication—The shoot tip explants were collected from 5 to 10 days old in vitro grown seedlings. Among them 7 day old shoot tip explants showed best response for multiple shoot induction because they showed young shoot apices with two to three leaf primordia favouring in vitro induction of multiple shoot apices. More number of leaf primordia and more length of explants affect the regeneration potential. On the other hand, 15 days old nodal explants showed best response for multiple shoot induction compared with younger (5-10 days old) and older (more than 16 days old) explants. Secretion of phenolic compounds was more in old (more than 16 days old) explants with low multiplication potential.

Influence of BAP, Kin and 2iP on multiple shoot proliferation—Five days after inoculation, the effect of growth regulators on growth response of explants was observed. Multiple shoot formation was observed at all the concentrations of BAP, Kin and 2iP tested for both the explants. From the different concentrations evaluated, individual treatment of 2.5 mg/l of 2iP showed best response for multiple shoot induction from shoot tip explants and produced 14.6 shoots per explant (Fig. 1a). In the case of BAP treatment, 1.5 mg/l of BAP produced 7.2 shoots per explants (Fig. 1b). Like BAP treatments, Kin treatments also produced low number of multiple shoot when compared with 2iP. Among the different concentrations tested 2.0 mg/l of Kin showed best response and produced 8.9 shoots per shoot tip explants (Fig. 1c). In the case of nodal explants, 2.5 mg/l of 2iP showed best response and produced 12.0 multiple shoots. Individual treatment of BAP and Kin showed moderate response and they produced 6.5 and 8.0 shoots per nodal explants, respectively.

Usually in cotton, individual concentration of BAP or Kin was used for the induction of multiple shoots. In previous studies, BAP and Kin mediated plant multiplication and regeneration have been noticed26. BAP has been found to be ideal hormone for direct shoot multiplication of cotton by using shoot tip, node and meristamatic explants27. In previous reports, they have regenerated maximum of 8-10 shoots per nodal and shoot tip explant and they have not observed any significant difference between the number of shoots regenerated from the shoot tip and nodal explants28. In other dicot plants also BAP or Kin supplementation has been used for micropropagation. High frequency of multiple shoot induction has been observed in Syzygium alternifolium by using BAP, Kin and 2iP29. In their studies, maximum of 27.6 shoots are regenerated from the embryonal axis meristamatic explants. Like their results, the superiority of cytokinin has been noticed in several plant species during multiple shoot induction from shoot tip and nodal explants26, 30-32. In some cases, superiority of
Kin has been noticed for the high frequency direct multiplication from the different types of explants (shoot tip, node and meristematic tissue explants) and cytokinins tested (BAP, 2iP and TDZ)\textsuperscript{33}. In few plants, like \textit{Vicia faba} and \textit{Phaseolus vulgaris}, combinations of two cytokinins are used for effective direct multiple shoot proliferation from shoot tip and nodal explants\textsuperscript{34,35}. But in the present study, supplementation of 2iP showed better results for multiplication of shoot tip and nodal explants. This was probably due to variation in endogenous growth regulator contents and physiological status of the plant species and genotype\textsuperscript{36}. Like our results, 2iP mediated high frequency of multiple shoots from the shoot tip and nodal explants. This supplementation of 2iP showed better results for multiple shoot induction from shoot tip explants (Table 1). Maximum of 20.2 shoots were regenerated at these concentrations of auxins with 97% response. In the case of NAA treatment, significant increase was not observed for multiple shoot induction when compared with control. To our knowledge, no reports have been published for multiple shoot induction from shoot tip explants by using 2iP and IAA. But in other dicot plants, combined effect of cytokinins with auxins has been reported\textsuperscript{38}.

In the case of nodal explants, medium fortified with MS salts, B5 vitamins, 30 g/l, glucose; 2.5 mg/l, 2iP and 1.0 mg/l, NAA showed best response. At this concentration, maximum shoots (18.6/explant) were successfully regenerated with 97.8% response without basal callus formation. Addition of IAA produced low number of multiple shoots as compared to NAA concentrations of auxins with 97% response. In the case of NAA treatment, significant increase was not observed for multiple shoot induction when compared with control. To our knowledge, no reports have been published for multiple shoot induction from shoot tip explants by using 2iP and IAA. But in other dicot plants, combined effect of cytokinins with auxins has been reported\textsuperscript{38}.

### Table 1 — Influence of IAA and NAA in the multiplication of shoot tip and nodal explants cultured on medium supplemented with MS salts, B5 vitamins, and 2iP (2.5 mg/l)

<table>
<thead>
<tr>
<th>Concentration of auxins (mg/l)</th>
<th>Number of shoots / explant</th>
<th>Percentage of response</th>
<th>Basal callus formation</th>
<th>Number of shoots / explant</th>
<th>Percentage of response</th>
<th>Basal callus formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.5 ± 0.61 f</td>
<td>85.4 ± 2.5</td>
<td>-</td>
<td>12.0 ± 0.45 f</td>
<td>90.2 ± 1.8</td>
<td>-</td>
</tr>
<tr>
<td>IAA</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>0.5</td>
<td>18.5 ± 0.45 d</td>
<td>92.4 ± 3.4</td>
<td>-</td>
<td>13.4 ± 0.65 e</td>
<td>93.5 ± 1.9</td>
<td>+</td>
</tr>
<tr>
<td>1.0</td>
<td>18.9 ± 0.52 c</td>
<td>94.6 ± 2.9</td>
<td>-</td>
<td>13.8 ± 0.66 de</td>
<td>95.3 ± 2.4</td>
<td>+</td>
</tr>
<tr>
<td>1.5</td>
<td>19.2 ± 0.65 bc</td>
<td>96.0 ± 1.5</td>
<td>-</td>
<td>15.0 ± 0.85 ed</td>
<td>96.8 ± 2.2</td>
<td>++</td>
</tr>
<tr>
<td>2.0</td>
<td>20.2 ± 0.85 a</td>
<td>97.0 ± 1.9</td>
<td>-</td>
<td>17.5 ± 0.55 a</td>
<td>98.2 ± 1.8</td>
<td>++</td>
</tr>
<tr>
<td>2.5</td>
<td>19.5 ± 1.22 b</td>
<td>94.6 ± 2.1</td>
<td>-</td>
<td>16.1 ± 0.68 b</td>
<td>97.1 ± 1.9</td>
<td>++</td>
</tr>
<tr>
<td>3.0</td>
<td>18.7 ± 0.75 cd</td>
<td>91.5 ± 2.2</td>
<td>-</td>
<td>15.2 ± 0.54 c</td>
<td>95.8 ± 1.6</td>
<td>++</td>
</tr>
<tr>
<td>3.5</td>
<td>18.0 ± 0.89 e</td>
<td>88.4 ± 2.4</td>
<td>-</td>
<td>14.1 ± 0.85 d</td>
<td>92.1 ± 1.7</td>
<td>-</td>
</tr>
<tr>
<td>NAA</td>
<td></td>
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<td></td>
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<tr>
<td>0.25</td>
<td>14.8 ± 1.24 d</td>
<td>74.5 ± 3.1</td>
<td>+</td>
<td>13.9 ± 0.84 e</td>
<td>92.5 ± 1.5</td>
<td>-</td>
</tr>
<tr>
<td>0.50</td>
<td>15.1 ± 0.85 c</td>
<td>78.4 ± 1.5</td>
<td>+</td>
<td>14.8 ± 0.65 d</td>
<td>93.9 ± 2.1</td>
<td>-</td>
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<tr>
<td>0.75</td>
<td>15.6 ± 0.64 b</td>
<td>79.2 ± 0.9</td>
<td>+</td>
<td>15.9 ± 0.56 c</td>
<td>95.7 ± 2.2</td>
<td>-</td>
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<tr>
<td>1.00</td>
<td>16.2 ± 0.95 a</td>
<td>83.5 ± 2.4</td>
<td>+</td>
<td>18.6 ± 0.68 a</td>
<td>97.8 ± 2.3</td>
<td>-</td>
</tr>
<tr>
<td>1.25</td>
<td>15.0 ± 0.55 cd</td>
<td>82.8 ± 2.6</td>
<td>++</td>
<td>16.2 ± 0.35 b</td>
<td>95.2 ± 1.5</td>
<td>-</td>
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<tr>
<td>1.50</td>
<td>14.5 ± 0.45 de</td>
<td>79.2 ± 2.5</td>
<td>++</td>
<td>15.0 ± 0.95 cd</td>
<td>93.0 ± 1.8</td>
<td>-</td>
</tr>
<tr>
<td>1.75</td>
<td>13.8 ± 0.56 e</td>
<td>73.2 ± 2.0</td>
<td>++</td>
<td>13.7 ± 0.91 ef</td>
<td>90.5 ± 1.9</td>
<td>-</td>
</tr>
</tbody>
</table>

Number of explants tested – 75.
Means within a column followed by the same letters are not significant at $P=0.05$ according to DMRT.
multiplication of cotton nodal explants\textsuperscript{14}. In their studies they have obtained low number of multiple shoots (10 shoots/explant) when compared with present results. In other dicot plants also, combination of cytokinins (BAP) with auxins (IAA) has been used for efficient multiple shoot proliferation from the shoot tip and nodal explants\textsuperscript{39}. In their studies, they obtained around 15 shoots directly from the explants.

In some cases, combinations of Kin with IBA have been used for the enhanced proliferation of multiple shoots in micropropagation studies\textsuperscript{40}. These results proved that combination of cytokinins with any auxins showed superior results for direct multiple shoot induction. Hence, the process of multiplication depends on types of auxins and cytokinins. In our study also, variation in the use of cytokinins and auxins for multiplication of shoot tip and nodal explants was observed.

Effect of spermidine and putrescine on multiplication—The use of polyamines in tissue culture studies has increased in recent period, particularly in somatic embryogenesis for enhanced plant regeneration. In the present investigation, different concentrations of spermidine and putrescine were tried for high frequency multiple shoot induction of cotton shoot tip and nodal explants. In the multiple shoot induction medium, supplementation of 20 mg/l of putrescine showed best response for the enhanced production of multiple shoots. At this concentration, maximum of 26.5 shoots per shoot tip explants (Fig. 2a) and 24.6 shoots per nodal explants were formed (Fig. 2b). At all the concentrations of spermidine and putrescine tested increased multiple shoot formation was noticed as compared to controls. In the case of spermidine, maximum of 24.2 shoots per shoot tip explants (control-20.2 shoots / shoot tip explants) and 23.4 shoots per nodal explants (control-18.6 shoots/nodal explants) were observed. The callus formation occurred at the base when the medium was supplemented with increasing concentrations of spermidine and in the case of putrescine treatment at all the concentrations, callus formation was completely absent. The induced multiple shoots were subcultured on the same medium for elongation. After 3 weeks, the induced shoots reached maximum of 3.5 cm (Fig. 2 c, d). Polyamines mediates more number of direct shoot proliferation and elongation have been reported earlier in several dicot plants\textsuperscript{41,42}. To our knowledge no report has been published so far for polyamines mediated direct multiple shoot induction in cotton. Enhanced effect of polyamines on multiple shoot bud induction per explant could be ascribed to their stimulatory effect on cell division and/or to their inhibitory effect on production of ethylene\textsuperscript{37,38}. Usually, the polyamines have been used for the induction of callus and somatic embryos\textsuperscript{43}. In cotton also, high frequency somatic embryo induction is achieved by putrescine treatment\textsuperscript{44}. In our studies, addition of putrescine showed enhanced direct multiple shoot induction from shoot tip and nodal explants.

Root induction and hardening—The elongated shoots (2.0 to 2.5 cm in length) obtained from both the explants were successfully rooted on the medium fortified with MS salts, B5 vitamins and IBA. Root induction was achieved within 10 days of culture. IBA alone is effective for induction of roots. Usually root induction in cotton is difficult and genotype dependent compared to other dicot plants. In such cases, rootone has been used to induce the roots from elongated shoots in sterilized soil\textsuperscript{11}. In some cultivars of cotton (Anjali-LRK516), root induction has been obtained on the medium supplemented with low concentration of NAA. In the present study, 2.0 mg/l of IBA was effective for root induction. At this concentration, maximum of 4.9 roots were initiated from the elongated shoots without intervening callus formation and the initiated roots reached maximum length of 20.4 cm within three weeks of culture (Table 2).

During root induction the impact of different concentrations of PVP were tested to control the phenolic oxidation process, because the secretion of phenolic compounds from the elongated shoots reduced the root induction frequency. Among various concentrations tested, 20 mg/l of PVP showed significant results. The percentage of root induction was increased by addition of PVP in root induction medium. After PVP fortification, about 6.6 roots per shoot were initiated with 22.8 cm length of the root (Table 3, Fig. 2 e). The increased root length of regenerated plants showed increase in the survival percentage of hardened and field grown plants. For hardening process sand, soil and vermiculated soil were used (1:1:1 ratio). The hardened plants were maintained in the environmental growth chamber for acclimatization (Fig. 2f). After proper acclimatization the hardened plants were transferred to field for boll yielding and bolls were produced after 25 days of proper irrigation.
cotton, in our study, superior results were observed for high frequency of multiple shoot induction with 100% survival rate of regenerated plants.

Direct regeneration (without intervening callus) of functional plantlet in tissue culture is a pre-requisite for any successful in vitro multiplication and transformation programme as the regeneration through callus is known to induce somaclonal variations. Hence, the studies on the production of simple direct regeneration protocols are needed for all the economically important crops. Compared with

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Fig. 2—Different developmental stages of micropropagation of cotton variety SVPR 2 through shoot tip and nodal explants. (a)- Induced multiple shoots from shoot tip explants after 1 week of culture. Bar = 1 cm; (b)- Cluster of shoots obtained from nodal explants after 1 week of culture. Bar = 0.8 cm; (c & d)- Elongated multiple shoots in clusters after 4 weeks of culture. Bar = 3.5 cm; (e)- Rooted elongated shoots after a week of culture. Bar = 5 cm and (f)-Hardened plants grown in environmental chamber. Bar = 7 cm
earlier reports, the protocol described in the present study could lead to active growth and multiplication of cotton plants, because delay in multiple shoot induction from the explants, suppressed root induction from the elongated shoots and phenolic excretion from the explants were controlled. Hence, we conclude that the protocol described in the present study may be useful for other cultivars of cotton regeneration, multiplication and transformation.

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