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Multiple Shoot Induction and Plant Regeneration in Litchi (*Litchi chinensis* Sonn.)

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ABSTRACT

Multiple shoot induction in *Litchi chinensis* Sonn. (litchi) has been achieved by two methods: (1) direct germination of litchi seeds in 6-benzylaminopurine (20 mg L^{-1}) supplemented MS liquid medium and supported on a filter-paper bridge and (2) in planta treatment with 6-benzylaminopurine (100 µg on alternate days) of the axillary bud regions of plants germinated and maintained under sterile conditions. While the former method resulted in as many as 27.5±8.6 shoot buds from the cotyledonary node within four weeks, the latter yielded on average approximately 8 shoot buds from each treated node in eight weeks. The cytokinin treatment in planta consisted of placing sterile filter paper moistened with sterile distilled water over the node and adding different concentrations of 6-benzylaminopurine. The shoots elongated and rooted directly in vermiculite after a pulse treatment with IBA (25 mg mL^{-1}) for 15 min. Fungus growth, a serious problem in litchi tissue culture, was controlled using a fungicide, Bavistin, alongwith elimination of organic nutrients from the growth medium.

Key Words: *Litchi chinensis* Sonn.; *In vitro* culture; Multiple shoot induction; Bavistin

Abbreviations: *BAP* (6-Benzylaminopurine), *DMSO* (Dimethyl sulphoxide), *IBA* (Indole-3-butyric acid), *2, 4-D* (2, 4-dichlorophenoxyacetic acid), *MS medium* (Murashige and Skoog (1962) medium), *MS1/2* (half-strength MS medium)

INTRODUCTION

Litchi (*Litchi chinensis* Sonn.) of family Sapindaceae is a tropical tree known for its delicious fruits. It is extensively grown in tropical and subtropical regions of the world, especially in India and China. Since litchi is a cross pollinated plant, it is highly heterozygous, and the progeny is not true to the parental type. Conventional vegetative propagation methods currently being used (air-layering or marcottage) are slow and inefficient (Chapman, 1984). Hence, *in vitro* techniques have potential use in litchi propagation for the large-scale cloning of elite plants. However, litchi has so far proven to be a difficult material for propagation using in vitro culture. Attempts to regenerate plants from explants derived from mature trees have failed to give satisfactory results (Kantharajah *et al.*, 1989). However, the formation of upto 15 adventitious buds was reported when immature litchi embryos were treated with BAP (100 mg L^{-1}) for 3 h followed by culture on hormone free MS medium for 4 weeks (Kantharajah *et al.,* 1992). Direct germination of seeds on cytokinin-supplemented medium has been used for regeneration and propagation of plants from cotyledonary nodes, e.g. for induction of embryogenesis, in *Phaseolus vulgaris* (Malik & Saxena, 1992a) and various other *Phaseolus* spp. (Malik & Saxena, 1992b) as well as in peanut (Saxena *et al.,* 1992).

This method has also been used for multiple shoot induction in pigeon pea (Shiva *et al.,* 1994). We report here that a similar method of culturing of mature seeds on BAP-containing medium can also be used to induce multiple shoot formation in litchi. In addition, we were also able to demonstrate the formation of multiple shoots from other nodal regions of litchi seedlings treated continuously with BAP through moist filter paper. This experiment was designed to induce multiple shoots and rapid plant regeneration through *in vitro* techniques for rapid multiplication of true to type plants of litchi.

MATERIALS AND METHODS

Seeds of litchi (*Litchi chinensis* Sonn.) cv 'Bedana' were obtained from Market of Faisalabad. Mature litchi seeds were first washed in running tap water for 30 min to remove any adherent fruit tissue and dried juice which might serve as a medium for fungal growth. Thoroughly washed seeds were then dipped in autoclaved Bavistin (1%), a fungicide, under sterile conditions for 45–60 min and rinsed three times with sterile water. This was followed by surface sterilization with 1% mercuric chloride for 5 min. All the media used in the investigation were adjusted to 5.8 pH before autoclaving at 1.4 kg cm^{-2} (121°C) for 15 min.

Multiple shoot induction from the cotyledonary node. Mature sterilized seeds of litchi were placed on a filter-paper bridge in culture tubes (25 x 150 mm) containing 20 ml liquid MS supplemented with BAP at various concentrations (5, 10, 15, 20 & 25 mg L^{-1}, respectively). Seeds were placed (1 in each tube) with the embryo portion facing up, and nutrient medium was added until the seed was completely submerged except for the top 1 to 1.5 cm region (in which the embryo is located). The level of the

liquid MS medium was maintained by regular additions of fresh medium every 3 - 4 days (depending on the rate of direct evaporation and transpiration from the seedlings).

Multiple shoot induction in plant. Four to five weeks old seedlings with a fully expanded first pair of primary leaves that had been germinated and grown on vermiculite in culture bottles (175 mL capacity with Magenta B-caps) were used. Moist, autoclaved filter papers (15 x 5 mm) were placed on the leaf axils (nodal region) of the seedlings to ensure direct contact of the hormone to the axillary meristem, and 100 µL solutions of various concentrations of BAP (0.25, 0.5, 0.75 and 1 mg mL^{-1}) were added to the filter paper. This treatment was continued for 8 weeks with 100 µL of the respective concentration of BAP being supplemented to the filter paper on alternate days. All cultures were incubated at 25° ± 2°C under light provided by two Phillips cool-white fluorescent lamps (40 W) at a photon fluence density of 50 µmol m^{-2} s^{-1} for 16 h per day.

Induction of roots. Individual shoots were excised from the node. The proximal end (lower portion) of the shoot was dipped in an auxin solution for 15 min, transferred to autoclaved vermiculite in 7.5 cm plastic pots and watered with Hoagland's medium (Hoagland & Arnon, 1950). Different auxins (NAA, 2, 4-D & IBA) at various concentrations (1, 5 & 25 mg mL^{-1}) were tested for their efficacy in root induction. The pots were covered with transparent plastic bags and kept under low illumination (1 Phillips cool white fluorescent lamp, 40 W) for 2–3 weeks, with daily watering with Hoagland's nutrient solution, after which the plastic bags were removed over a period of 1 week and the plantlets were transferred to soil. All experiments were repeated at least twice with similar results.

Data presented in the tables are treatment means obtained from a single experiment. The data were subjected to the F-test to detect differences between treatments, and Tukey's multiple comparison test was used to separate treatment means.

RESULTS AND DISCUSSION

Multiple shoot induction from the cotyledonary node. Litchi seeds germinated within the first 5 days of culture on MS medium supplemented with BAP. The seedlings grew normally until after 10 days when both primary root and shoot growth were inhibited. By day 14 of the culture, the axillary shoots at the cotyledonary nodes started elongating. Multiple shoots were visible after approximately 3 weeks of culture with the formation of new shoot buds adjacent to the elongated axillary buds. The maximum number of shoot initials was observed in 4 to 5 weeks old cultures. Though new shoot initials continued to form even after 5 weeks, the older buds started browning and withered away. Almost all the germinated seeds produced multiple shoots and as many as 27.5 ± 8.7 shoot buds were obtained after 4 weeks of culture in MS medium supplemented with 20 mg L^{-1} BAP (Table I). In control plants germinated on MS basal medium and only the elongation of the 2 pre-existing axillary buds was recorded.

Agar versus filter paper. The nature of the support provided to the seeds was found to be a critical factor for the success of this method of multiple shoot induction. Litchi seeds cultured on BAP-supplemented MS semi-solid medium turned black and their germination was completely inhibited. Though the cause for this inhibition of germination on semi-solid medium needs to be investigated, it is speculated that the excess phenolics released by litchi may not diffuse rapidly on agar-solidified medium and that the resulting local concentration of phenolics around the seeds might be auto-toxic. The germination method using litchi seeds on filter-paper bridges proved to be a better one, probably because the rapid diffusion of phenolics in liquid medium prevented toxic levels from being reached. Fresh MS liquid medium supplemented with BAP was added every 3–4 days to compensate the loss of liquid through transpiration and evaporation. This was essential for the continuous contact of the embryonic region of the seed (before germination) and cotyledonary nodal region of the seedlings (after germination) with the medium, which was critical for obtaining multiple shoots. These results are similar to the ones observed in *Vigna mungo* L. (Das *et al.,* 1998) and *Cajanus cajan* L. (Shiva *et al*., 1994) where multiple shoot initiation from the nodal region was possible only through the direct contact of the nodal region with the medium. Perhaps this is due to the inability of other plant parts (root or lower regions of the seed) to take up cytokinin or because of the poor translocation of cytokinin to the cotyledonary node from other parts.

Multiple shoot induction in plant. Seedlings treated with BAP at the leaf axils shed their leaves after 7–10 days from the start of the treatment, and further elongation of the main shoot was inhibited. Multiple shoot formation was observed after 7–8 weeks of BAP supplementation in planta as stated in Materials and methods. The maximum number of shoots (8 shoots) was observed when a BAP stock solution of 1 mg ml^{-1} was applied at alternate days onto the filter paper (Table II).

Induction of roots. Individual shoots were separated from

Table I. Effect of different concentrations of BAP on the number of multiple shoots formed from the cotyledonary node of *Litchi chinensis* (cv. 'Bedana') after 4 weeks of culture. (Values are means of three replicates with 20 seeds in each replication)

BAP (mg L^{-1})	Percentage of germinated seedlings responding	Mean no. of shoots per cotyledonary node[a]
10	20	8.0 ± 0.8[a]
20	100	27.5 ± 8.7[b]
50	10	3.0 ± 1.4[c]
100	0	0[d]

[a] Means followed by the same letter do not differ significantly (P = 0.05) as indicated by one-way ANOVA followed by Tukey's multiple comparison test.

each other and rooted by giving a short pulse (15 min) of auxin treatment. For the induction of roots, 25 mg mL^{-1} IBA was found to be the best concentration resulting in approximately 80% of the treated shoots forming roots after 4 weeks (Table III). This method combines the rooting and the hardening steps, thus reducing the time required for clonal multiplication. The rooted plantlets appeared to be phenotypically normal. The absence of somaclonal variation could be because of the lack of callus phase in the process of regeneration. The entire process of regeneration starting from a seed upto the stage of establishment of regenerants in soil took approximately 2 months, and 22 plants could be recovered from each cultured seed. On the other hand, in planta cytokinin treatment resulted in the recovery of 6.8 plantlets per axillary node in approximately 4 months.

Use of fungicide. A high level of fungal contamination is one of the major difficulties in the *in vitro* culture of litchi. Surface sterilization of seeds with 0.1% mercuric chloride for even upto 15 min was ineffective in controlling seed-borne fungus, which seems to be endophytic in origin. Shields *et al.* (1984) evaluated various fungicides in plant tissue culture for their effect in reducing fungal overgrowth. However, the use of fungicides is not a common practice in plant tissue culture. In the present investigation, Bavistin, a systemic fungicide of the benzimidazole group, was successfully used to control the fungal contamination problem in litchi culture. Bavistin was not only necessary for sterilizing the seeds, but also its continuous presence in the medium was essential in controlling fungus harbored. Root formation in an excised shoot after a 15 min. pulse treatment with IBA was followed by direct implantation in vermiculite and watering with Hoagland's solution (after 30 days of auxin treatment). In addition, organic nutrients were excluded from the culture medium to minimize contamination. The in planta method in which BAP was applied by means of filter paper at the nodal region of plants growing in the open was hampered because of the rapid drying out of the filter paper. The enclosed nature of the culture bottles used for growing seedlings ensured a high humidity, which consequently prevented the filter paper from drying out. Perhaps this method of clonal propagation could be applied to field-grown plants as well by ensuring sufficient moisture on the filter paper used for BAP treatment.

Table II. Effect of different concentrations of BAP treatment in planta for 8 weeks on the number of multiple shoots in *Litchi chinensis* (cv. 'Bedana'). (Values are means of three replicates with 20 nodes in each replication)

BAP (mg L^{-1})	Percentage of treated nodes responding	Means no. of shoots per node
0.25	0	0^a
0.5	0	0^a
0.75	50	3 ± 0.8^b
1	100	8.5 ± 1.3^c

[a] Means with the same letter do not differ significantly (P = 0.05) as indicated by one-way ANOVA followed by Tukey's multiple comparison test

Table III. Effect of pulse treatment with different concentrations of auxin on root formation from *Litchi chinensis* shoots (cv. 'Bedana'), after 4 weeks of culture. (Values are means of three replicates with 20 shoots in each replication)

Auxin (mg ml^{-1})	Percentage of shoots responding	Mean no. of roots per shoot [a] ± SD
NAA (1)	0	0^a
NAA (5)	0	0^a
NAA (25)	45	1.2 ± 0.8^b
2,4 D (1)	0	0^a
2,4 D (5)	0	0^a
2,4 D (25)	0	0^a
IBA (1)	0	0^a
IBA (5)	0	0^a
IBA (25)	80	1.8 ± 0.8^b

[a] Means with the same letter do not differ significantly (P = 0.05) as indicated by one-way ANOVA followed by Tukey's multiple comparison test.

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