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A Novel Approach of Regeneration from Nodal Explants of Field-grown Litchi (*Litchi chinensis* Sonn.) Fruit Trees

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Abstract: In this investigation, multiple shoot formation using nodal explants of 3-4 year old litchi trees is reported which could pave the way for clonal propagation of litchi from identified elite, adult identified trees in future. Multiple shoot formation was obtained on modified Woody Plant Medium. A continuous proliferating shoot culture was established by repeatedly sub-culturing the original nodal explants on shoot multiplication medium after each harvest of newly formed shoots. The shoots were separated and planted on several media for rooting as this is a perpetual problem with many tree species. This was overcome by giving a pulse treatment to the cut ends of microshoots with IBA (100 mM) in MS liquid medium for 15 min. The shoots were implanted on MS semi solid medium supplemented with IBA (20.6 μ M) and litchi seed powder (1 g L⁻¹) for rooting. This novel approach promoted rooting of shoots *in vitro*. Well established plantlets were successfully transplanted to the nursery after hardening.

Key words: *Litchi chinensis*, axillary shoot proliferation, litchi seed powder, rooting

Introduction

Litchi chinensis Sonn. (litchi) of the family Sapindaceae is an important horticultural crop which bears fruits that rank among the most delicious fruits possessing high nourishment and medicinal value. The aril of the fruit is glutinous, aromatic and contains high amount of vitamin C and phosphorus. The fruit is an excellent thirst quencher reportedly serving as a tonic for brain, heart and liver. Litchi has various names such as litchi, lychee, lici, licy, le-ci, lichee, lichies and leechee (Hayes, 1957). It originated in Southern China and reached India first via Myanmar by the end of the 17th century (Goto, 1960). Natural propagation of litchi occurs by seeds and the reproductive phase normally begins after 8-10 years (Hamilton and Yee, 1970). The seeds lose their viability within 5-6 days (Ray and Sharma, 1985). Propagation via seeds is also undesirable because of the highly heterozygous nature of the plant due to cross pollination (Sarin and Prasad, 2003). The conventional methods of vegetative propagation utilized for litchi are, air layering or marcottage, grafting and budding (Menzel, 1985) which are slow and inefficient (Chapman, 1984; Sarin *et al.*, 2003). In the past several attempts for clonal propagation of litchi were made with marginal success (Kantharajah *et al.*, 1989). Earlier we reported multiple shoot induction and plant regeneration in litchi from the cotyledonary nodes and by *in planta* treatment of the axillary bud regions (Das *et al.*, 1999). In this communication we report a highly reproducible and efficient method of *in vitro* regeneration of elite litchi trees suitable for clonal propagation. The protocol that has been established could prove advantageous to the horticulturists and the industry for developing trees true to the parental type.

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Materials and Methods

Plant Material

Litchi cultivars collected from different geographical regions of India included, Purbi, Deshi, Kasba Illaichi, Late Bedana, Ajhouli, Dehrrase, China and Late-Large-Red (from Bihar Agricultural College, Bhagalpur, Bihar), Muzaffarpur, Kalkattia, Dehradun and Seedless (from Regional Horticulture Division, Gurdaspur, Punjab), Dehradun, Muzaffarpur, Shahi, Rose scented and Early Bedana (from Muzaffarpur, Bihar), Dehradun and Muzaffarpur (collected from Muzaffarnagar, U.P.), Bedana and Kalkattia (from Baidyanath Deoghar, Jharkhand) and Seedless, Dehradun and Dumdum (from Dehradun, Uttaranchal). Grafted saplings of these cultivars established in the nursery served as the source of explants. Nodal cuttings (2-3 cm) from the side branches of the 2-4 year old elite litchi plants were collected and used as explants.

Sterilization of Nodal Cuttings

This was the first crucial step towards clonal propagation. After removing leaves, the nodal cuttings were thoroughly washed first in running tap water followed by 5% solution of detergent, either extran (Merck Ltd.) or savlon (Johnson and Johnson). This was followed by bavistin (B.A.S.F., Ltd.; 1% w/v) treatment for 15-20 min. All subsequent operations were carried out inside a laminar air-flow cabinet. The nodal cuttings were given a quick (30 sec) rinse in 70% ethanol, followed by washing in sterile distilled water. These were further surface-sterilized in 0.5% mercuric chloride solution for 5 min and rinsed thrice with sterile distilled water.

Culture of Explants

The nodal cuttings were trimmed at both ends to expose fresh tissue before planting them on the medium. Filter paper bridge technique was followed to provide support to the explants (Das *et al.*, 1999). The liquid Woody Plant Medium (Lloyd and McCown, 1980) for pre-culture of explants was supplemented with adsorbent polyvinyl pyrrolidone (PVP, 0.2%) from Sigma Aldrich and fungicide (bavistin, 30 $\mu\text{L L}^{-1}$). After adjusting the pH to 5.6, an aliquot of 20 mL liquid medium was dispensed into Borosil rimless glass tubes (150×25 mm). The culture tubes were plugged with non-absorbent cotton wrapped in cheese-cloth and autoclaved at 1.06 kg cm^{-2} at 121 °C for 18 min. Cultures were incubated at 25±2 °C under light provided by Phillips cool white fluorescent lamps (40 W) at a photon fluence density of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 16 h per day. After 15 days the explants were transferred to modified liquid Woody Plant Medium supplemented with BAP (11 μM), Kn (2.30 μM), GA_3 (0.60 μM), bavistin (30 $\mu\text{g L}^{-1}$) and PVP (0.2%). Filter paper bridge technique was followed to provide support to the explants. Sucrose was not added to this medium. Different strengths (full, half, one third, one quarter) of Murashige and Skoog semi solid medium (Murashige and Skoog, 1962) supplemented with B5 vitamins (Gamborg and Shyluk, 1981) were tested with different combinations of phytohormones for multiple shoot formation from the nodal cuttings.

Multiple Shoot Formation

After 15 days of culture on modified liquid WPM medium, the explants were transferred to semi solid WPM supplemented with sucrose (3% w/v), deproteinized coconut water (CW, 15% w/v) and casein hydrolysate (CH, 400 mg L^{-1}) from Sigma Aldrich and the same hormone combination as used earlier in liquid WPM medium. Medium was gelled with agar (0.8%). The concentration of PVP and bavistin was reduced to 0.02% and 10 $\mu\text{g L}^{-1}$, respectively. Rapid subculturing (at every 10 days interval) was essential to avoid the local deposition of phenolics. After two subcultures, bavistin and PVP were excluded from the medium.

Multiplication and Elongation of Microshoots

Small (0.5 cm) shoots from 4-5-week-old cultures were excised carefully and transferred to full strength liquid as well as semi solid MS medium supplemented with BAP (6.6 μM), GA₃ (0.15 μM), Silver Nitrate (SN 30 μM) and CH (300 mg L⁻¹) for multiplication and elongation of shootlets. After 15-20 days of subculture, each axillary shoot which had elongated to ca. 7 cm was cut into small segments and transferred to fresh semisolid MS medium of the same composition for further multiplication.

Rooting and Transplantation

The elongated shoots (5-8 cm) were separated from the clump of multiple shoots by giving a gentle cut at the basal region. After giving a pulse treatment with IBA (100 mM) in liquid MS medium for 15 min the shootlets were transferred to different strengths of MS major salts (full, three quarter, half and one quarter) supplemented with 20 μM IBA or NAA along with 1 g L⁻¹ of sterilized litchi seed powder which was prepared from green seeds with soft seed coat. The seeds were collected from immature fruits before ripening, frozen at minus 80°C, pulverized in liquid nitrogen and ground to make a fine powder which was transferred to a glass bottle and autoclaved. Stock of sterilized seed powder was stored at room temperature and used in the medium when required. The rooted plantlets (1-2 inches long) were gently removed from the culture medium and the roots were washed carefully in running tap water to remove agar. The plantlets were transferred to plastic pots (5 cm diameter) containing a mixture of vermiculite and litchi seed powder (3:1) and maintained at 25±2°C; 16 h. photoperiod at 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity and 80% RH. These potted plants were covered with polythene bags to retain high humidity conditions and provided with either half strength MS salt solution devoid of sucrose and myo-inositol or Hoagland's medium twice a week. After 25 days, polythene bags were removed from the plastic pots and the plants were hardened for a week, before transfer to earthen pots (10 cm diameter) containing garden soil mixed with sand and vermiculite (1:1:2) and watered at regular intervals. The plants were transferred to the glass house before transplanting them in the nursery. At least five replicates were maintained for each treatment and the experiments were repeated thrice. Observations on the number of cultures showing bud-break, shoot elongation and rooting were made at regular intervals and contaminated cultures were removed from time to time.

Results and Discussion

Establishment of Nodal Segment Cultures

The nodal segments were cultured in various media (as described in Material and Methods). However, contamination was a major problem during initiation of cultures *in vitro*. The explants were mainly contaminated by endogenous pathogen which had to be isolated and identified for controlling it. The extent of contamination as well as bud break was highly dependent on the season in which the material was collected. Utilizing the elaborate sterilization procedure described earlier, the cultures initiated in the months, February-May showed higher bud break (45%) and less contamination (20%) than those raised later. Since June-August is the period that coincides with rainy season in the North, 90% explants collected in these three months were prone to infection. Therefore, the cultures were routinely raised from February-May. Sterilization steps for the explants which are extremely important for growing disease free material were well standardized in the present investigation.

Seasonal effects on establishment of cultures have earlier been reported for other tree species, viz. neem (Chaturvedi *et al.*, 2004), apple (Hutchinson, 1984), papaya (Litz and Conover, 1981), sweet gum (Sutter and Barker, 1985) and guava (Amin and Jaiswal, 1987). Yu *et al.* (1991) reported that exudation of phenolics and contamination depended on the age of the mother plant from which the explants are taken as well as the growing conditions of the donor plants. They reported a contamination rate of 100% in test material taken after 10 continuous rainy days and 20% after 15 continuous sunny days and found the highest regeneration rate on half strength MS medium fortified with 4.44 μM of BA, 1.142 μM of IAA and 1.734 μM of GA₃. In this investigation, 100%

Table 1: Details of media composition

Media code	Composition
L1	WPM + BAP (11.00 μ M) + Kn (2.3 μ M) + GA ₃ (0.6 μ M) + CH (400 mg L ⁻¹) + CW (15%)
L2	WPM + BAP (6.6 μ M) + GA ₃ (0.15 μ M) + CH (400 mg L ⁻¹)
MS1	MS + BAP (6.6 μ M) + GA ₃ (0.15 μ M) + SN (30 μ M) + CH (300 mg L ⁻¹)
MS2	MS + IBA (20.6 μ M) + litchi seed powder (1 g L ⁻¹)

Table 2: Influence of different media supplemented with BAP (11 μ L) + Kin (0.5 mg L⁻¹) + GA₃ (0.2 mg L⁻¹) + CW (15%) on shoot formation from nodal explants of litchi

Medium type	Shoot No. per responding explant	Percentage shoot formation	Shoot length (cm)	Shoot with basal callus
B5	1.6c	13.5de	1.2c	+
MS	2.0b	33.3b	1.5b	-
MS 1/2	1.2d	19.9cd	1.0d	-
MS 1/3	1.0de	20.2c	1.0d	-
MS 1/4	0.9ef	13.0ef	0.5de	-
MS+ B5	1.4cd	15.0d	1.1cd	-
WPM	3.0a	53.3a	2.5a	+

Total number of explants taken for observation = 100, Mean values within a column having the same alphabet are not statistically significantly different ($p < 0.05$) according to Multiple Duncan Range Test (DMRT), Induction of basal callus shown as + (callus present) and - (callus absent)

contamination was obtained during the rainy season (June-August). Therefore to solve this problem of contamination none of the explants were taken for *in vitro* culture during these months.

Axillary Shoot Proliferation

Axillary shoot proliferation from the nodal explants varied considerably on different strengths of inorganic salts and phytohormone concentration in WPM semi solid medium. Full strength WPM supplemented with various growth regulators (L1 medium, Table 1) resulted in the best response of shoot bud formation in 53.3% of the cultures (Fig. 1A and Table 2). These explants were transferred to liquid (on filter paper bridges) and semi-solid L2 medium (Table 1) after three weeks and after five weeks, respectively. Though the shootlets elongated to ca. 2-3 cm, they appeared yellow and abscission of leaves was observed. Coconut water was excluded from the L2 medium at this stage. Exclusion of CW, incorporation of filter sterilized silver nitrate (30 μ M) in the medium and reducing the concentration of CH to 300 mg L⁻¹ helped in controlling abscission, improved the incidence of bud break and promoted shoot proliferation (Fig. 1B and C).

Elongation and Multiplication of Shootlets

Shootlets (0.5 cm long) were excised carefully from the explants and transferred to MS1 medium (Table 1). Each shootlet (Fig. 2A) produced a clump of shootlets from the base after 6 weeks (Fig. 2 B-C). Thus, three to four-fold shoot multiplication could be achieved on MS1 medium. Yu *et al.* (1991) also obtained the highest regeneration as well as multiplication rate only when the shootlets were excised from the mother explants. The medium composition used by them was, however, different [half strength MS supplemented with BA (4.4 μ M) + IAA (1.14 μ M) + GA₃ (0.2 μ M)].

Initiation and *in vitro* propagation of mature trees in general is difficult due to various problems which include mainly recalcitrance of the tissue, contamination and field establishment. The method of multiple shoot induction reported by Kantharajah *et al.* (1989) involved culturing immature embryos, which are difficult to obtain and tedious to culture because of the labour and time involved in isolating the immature embryos. Das *et al.* (1999) reported multiple shoot formation by direct germination of litchi seeds in MS liquid medium supplemented with 6-benzylaminopurine (20 mg L⁻¹) and supported on a filter-paper bridge. In the same investigation they also reported *in planta* treatment of the axillary bud regions of plants germinated and maintained under sterile conditions with 6-benzylaminopurine (100 mg L⁻¹) on alternate days for multiple shoot induction. Both methods of multiple shoot induction were effective for the five genotypes of litchi tested (cv. Deshi, Kasba, Bedana, Purbi and Shahi). Chandra and Padaria (1999) cultured shoot buds of

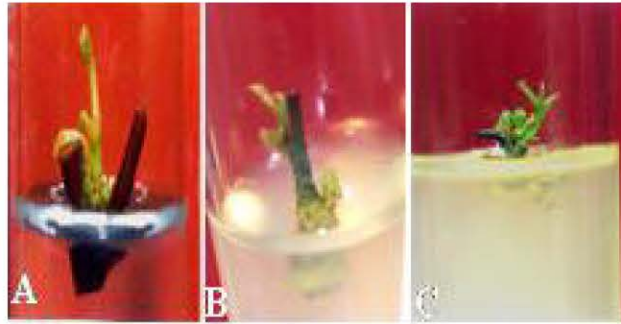


Fig. 1: (A-C) Shootlet formation from the nodal segments of litchi on L1 medium (WPM + BAP (11 μM) + Kin (2.3 μM) + GA₃ (0.6 μM) + CW (15%)]

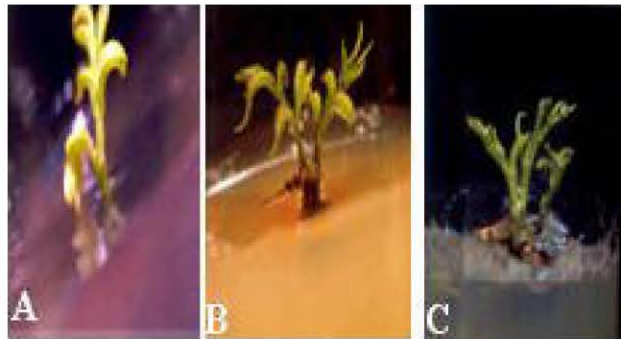


Fig. 2: (A-C) Multiplication and elongation of shootlets on MS1 medium [MS + BAP (6.6 μM) + GA₃ (0.15 μM) + SN (30 μM) + CH (300 mg L^{-1})]

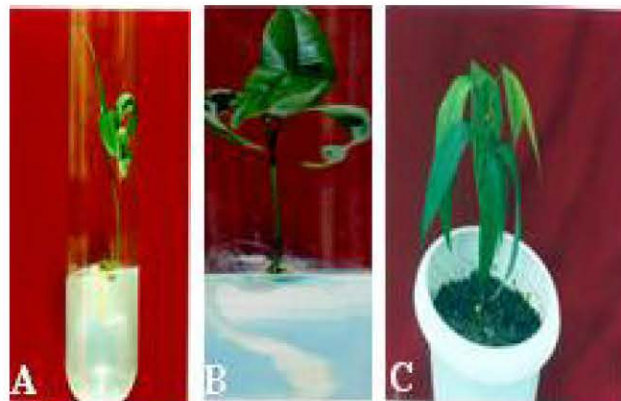


Fig. 3: (A-B) Root induction at the base of shootlets of litchi on MS2 semisolid medium [MS medium supplemented with IBA (20.6 μM) + litchi seed powder (1g L^{-1})] (C) Hardened regenerated plants in pots containing a mixture of soil: sand: vermiculite (1:1:2)

Table 3: Rooting response of shoots on full MS, 1/2 MS and 1/4 MS basal media or basal media (semi solid and liquid) supplemented with IBA and litchi seed powder after five weeks of transfer

Treatment	% Shoots forming roots	% of surviving after transplantation
Full strength MS	0	0
1/2	0	0
1/4MS	0	0
Full MS + IBA (20.6 $\mu\text{M L}^{-1}$) + seed powder (1000.0 mg L^{-1}) a	70 \pm 1.0	60 \pm 0.5
1/2 + IBA (20.6 $\mu\text{M L}^{-1}$) + seed powder (1 g L^{-1}) a	60 \pm 1.0	50 \pm 0.5
1/4 + IBA (20.6 $\mu\text{M L}^{-1}$) + seed powder (1 g L^{-1}) a	45 \pm 1.0	25 \pm 0.5
Full MS + IBA (41.2 $\mu\text{M L}^{-1}$) + seed powder (1 g L^{-1})	50 \pm 1.0	20 \pm 0.5
1/2 + IBA (41.2 $\mu\text{M L}^{-1}$) + seed powder (1 g L^{-1})	75 \pm 1.0	10 \pm 0.5
1/4 + IBA (41.2 $\mu\text{M L}^{-1}$) + seed powder (1 g L^{-1})	60 \pm 1.0	05 \pm 0.5

Values represent mean \pm SD of the number of shoot. Each treatment consisted of 5 shoots

litchi seedlings cv Seedless on MS medium supplemented with 0.2 mg L^{-1} BA + 0.1 mg L^{-1} IAA + 0.5 mg L^{-1} GA₃ and obtained shoot differentiation and growth. In the present investigation an alternative method of direct regeneration of litchi plantlets from nodal explants taken from 3-4 year old plants in the field has been demonstrated for the first time.

Rooting of the Shoots

For rooting, MS and WPM media were tested at full, half, one third and quarter strength respectively, of the major inorganic salts. All the media, liquid as well as semi solid were supplemented with IBA (20 μM) and 1 g L^{-1} litchi seed powder. Before transfer of the shoots into the rooting medium a short pulse treatment with IBA (100 mM) for 15 min was essential. After the pulse treatment of basal part, the shoots were transferred to MS2 semi solid and liquid medium, respectively (Table 1). Both the media proved very effective as 70% of the shoots responded to form roots (Table 3). Rooting was observed directly from the base of the shoots on MS2 semi solid medium (Fig. 3A-B) after 4 weeks and maximum response was observed after 5-6 weeks. Liquid MS2 medium was equally good as the semi solid MS2 medium.

Generally an auxin is essential for rooting either at a high concentration for a short duration pulse or in optimum concentration in the medium. For shoots obtained from cotyledonary nodes, a pulse treatment with IBA (25 mg L^{-1}) for 15 min followed by implantation on vermiculite resulted in establishment of regenerated plantlets (Das *et al.*, 1999). Kantharajah *et al.* (1992) observed adventitious rooting in 65% of shoot cultures on MS medium supplemented with NAA (0.5 mg L^{-1}) and activated charcoal (ca. 1%). Yu (1991) obtained plants with roots, stem and leaves when the regenerated shoots were grafted on stocks cultured in tubes with a seedling age of 15-20 days. However, in the present study, rooting of the shoots proved particularly difficult as various media and grafting strategies were tested. Finally 70% of the shoots could be rooted on MS medium supplemented with IBA (20 μM) + litchi seed powder (1 g L^{-1}) after a pulse treatment of IBA (100 mM) for 15 min. The alternate approach used in this investigation for root regeneration involved the use of litchi seed powder, which may serve as the natural source of rooting hormones. The use of litchi seed powder to promote establishment of roots is novel and could be used in a commercial venture for clonal propagation from adult trees.

Hardening and Transplantation

Following the protocol described under Materials and Methods, the plants were hardened and successfully transferred to the nursery (Fig. 3C).

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