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BRIEF COMMUNICATION

Plant regeneration in *Robinia pseudoacacia* from cell suspension cultures

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Abstract

A method for plant regeneration in *Robinia pseudoacacia* L. from cell suspension culture was established. Non regenerative friable callus from hypocotyls and cotyledon explants from *in vitro* raised seedling induced on solid Murashige and Skoog (MS) medium supplemented with 0.05 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) was used for initiation of cell suspension cultures on same MS medium but without agar. Single cells were isolated after 3 d and the optimum cell density was 1 - 3 × 10^4 cells per cm^3 of the liquid MS medium. Plating efficiency was 29.6 % and callus formed within 4 weeks was subcultured and transferred to solid MS medium supplemented with 0.6 mg dm^{-3} benzyladenine (BA) along with 0.05 mg dm^{-3} α-naphthalene-1-acetic acid (NAA) for the induction of adventitious bud primordia. The shoots developed were isolated and re-cultured on MS medium containing 0.6 mg dm^{-3} BA. These microshoots after dipping in 1 - 2 cm^3 of 10 mg dm^{-3} indole-3-butyric acid (IBA) for 24 h in dark were cultured on half strength solid MS medium supplemented with 0.05 % charcoal and showed 80 - 82 % rooting within 4 weeks.

Additional key words: benzyladenine, 2,4-dichlorophenoxyacetic acid, indole-3-butyric acid, α-naphthaleneacetic acid, organogenesis.

Cells in suspension can exhibit much higher rates of cell division than do cells in callus culture. Thus, cell suspension offers advantages when rapid cell division or many cell generations are derived, or when a more uniform treatment application is required (Phillips *et al*. 1995). Rapidly growing, fine suspension cultures or friable calluses are generally the most suitable for selection purpose. Where it is possible to regenerate plants from variant cells, selection techniques have potential for the production of crop cultivars with new characteristics such as salt tolerance (Freytag *et al*. 1990), cold tolerance, disease resistance and metal tolerance (Cresswell 1995). The cell suspension is also excellent starting materials for the isolation of protoplasts to be used in a wide range of applications including cell fusion and genetic manipulation (Hall 1991).

Robinia pseudoacacia L., a nitrogen-fixing leguminous tree species has particular advantages such as fast growth and early flowering. For tree improvement from the isolated cells of suspension culture the establishment of regeneration system is required. This species has been regenerated *in vitro* by micropropagation and organogenesis using explants of cambium (Fukuda and Koumoto 1986), axillary bud (Barghchi 1987, Chalupa 1987), leaf (Davis and Keathley 1985), nodal shoot (Han *et al*. 1990), cotyledon (Zou *et al.* 1994), seed (Arrillaga *et al*. 1994) and shoot bud (Kanwar 1996, 2000). However, there is no report on regeneration of plantlets from hypocotyls and cotyledon explants of *Robinia pseudoacacia* by cell culture. In this study we describe the establishment of cell suspension culture from the callus induced from cotyledons and hypocotyls explants and thereafter regeneration of plantlets in this species, using different concentrations and combinations of cytokinin BA and auxin 2,4-D.

The seeds of *Robinia pseudoacacia* L were collected from the university field and surface sterilized by immersing in 0.1 % mercuric chloride for 5 min, then rinsed five times with sterilized distilled water. The sterilized seeds were placed in 100 cm^3 flask containing sterilized sand drenched with sterilized distilled water and were germinated for 15 d at temperature of 25 °C with a 16-h photoperiod.

Explants measuring 4-6 mm excised from hypocotyls and cotyledons from 15-d-old seedlings were placed on solid Murashige and Skoog (1962; MS) basal medium

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Abbreviations: BA - benzyladenine; 2,4-D - 2,4-dichlorophenoxy acetic acid; IBA- indole-3-butric acid; MS - medium of Murashige and Skoog (1962); NAA - α-naphthalene-1-acetic acid.
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supplemented with 0.1 and 0.5 mg dm^{-3} 2, 4-dichlorophenoxyacetic acid (2,4-D). Once the callus was established, it was subcultured on same medium at three weeks intervals. The increase in the fresh mass of the callus was recorded at 7 d intervals.

Suspension cultures were initiated by transferring 3 - 4 pieces of friable callus (1 g each) after second subculturing in 250 cm^3 flasks containing 70 cm^3 of same liquid MS medium supplemented with 0.5 mg dm^{-3} 2,4-D. These flasks were kept in orbital platform shaker at 120 rpm in dark at temperature of 21 ± 2 °C. Cell suspensions were further maintained by regular transfer of approximately 5 cm^3 of suspension in a flask containing 40 cm^3 of liquid medium. Cell density of suspension culture was adjusted to 1 - 3 × 10^4 cells per cm^3 of liquid medium with the help of haemocytometer. The cell suspension was filtered through autoclaved double-layered muslin cloth into sterile 100 cm^3 graduated cylinder. The contents in graduated cylinder were allowed to settle for approximately 10 min and then the supernatant was poured off. The technique allowed only single cells or maximum of 2 - 3 cells groups to pass through and large cell aggregates were removed.

Molten 10 cm^3 of solid medium containing 0.5 mg dm^{-3} 2,4-D was poured in each of 50 Petri plates, sealed with parafilm and incubated in dark at 25 °C. The growth of single cells was recorded every alternate day for 2 weeks. After 4 weeks, plating efficiency was calculated. Callus formed within 4 weeks was allowed to grow up to 7 weeks.

For shoot induction, calli were transferred to solid MS medium supplemented with 0, 0.2, 0.4, 0.6, 0.8, 1.0 mg dm^{-3} benzyladenine (BA) alone and along with 0.05 mg dm^{-3} α-naphthalene-1-acetic acid (NAA). The shoot primordia regenerated from callus developed into shoots after 3 - 4 weeks. *In vitro* established shoots were cut into small segments (1.0- 1.5 cm) and recultured on the same solid MS medium supplemented with 0.6 mg dm^3 BA alone for multiplication of shoot as reported earlier (Kanwar 1996).

Under aseptic conditions microshoots were cut into approximately 2.0 cm and dipped in 2-3 cm^3 of 2.0 μM IBA for 24 h under dark. Thereafter, microshoots were cultured on growth regulators free solid, half strength MS medium supplemented with 0.05 % charcoal and incubated at 25 ± 2 °C under 16-h photoperiod with irradiance of 20 μmol m^{-2} s^{-1}. After 10 - 15 d, root primordia initiated and developed into well-established rooted plantlets after 3 - 4 weeks. *In vitro* raised plantlets were washed under tap water to remove agar and excessive salts, dipped for 30 min in 0.01 % *Bavistin* and transferred to stratified potting mixture of sand:soil in 3:1 ratio. In the beginning pots were covered to present desiccation, the cover was gradually opened week by week, completely removed after one month. Plants were fully acclimatized to the outer atmospheric conditions.

Non-regenerative and friable callus was formed from cotyledon as well as hypocotyls explants on solid MS medium supplemented with 0.1 or 0.5 mg dm^{-3} 2, 4-D. It was observed that the cotyledon formed callus on 0.5 mg dm^{-3} 2, 4-D was ideal for callus subculturing and cell suspension initiation. The type of explant used for induction of callus and cell suspension cultures mainly depends upon the age of explants (Bonga 1987). Hypocotyl explants were earlier reported to be used for callus induction in *Morus alba* (Ohyama *et al*. 1970), *Leucaena leucocephala* (Nagmani *et al*. 1983), *Robinia pseudoacacia* (Han *et al*. 1990) and *Dalbergia latifolia* Roxb. (Pradhan *et al*. 1998). Cotyledon explants were used for callusing in *Robinia pseudoacacia* (Zou *et al*. 1994). In present investigation, cotyledons were found to be better than hypocotyls explants for induction and maintenance of callus.

To obtain a fine suspension culture, it is of prime importance to initiate suspension cultures from a friable callus source. Therefore, white and friable callus obtained from cotyledon explants were used for the experiments. As the friability of the cells increases, it becomes much easier to achieve a full separation of the cells. It was also reported that the degree of the friability of callus tissue increased when maintained on a semisolid medium for two to three passages (Bhojwani and Razdan 1990). During the incubation period, the biomass of suspension cultures increases due to cell division and cell enlargement, this continued for a limited period. It was necessary to transfer them to a fresh medium of same composition.

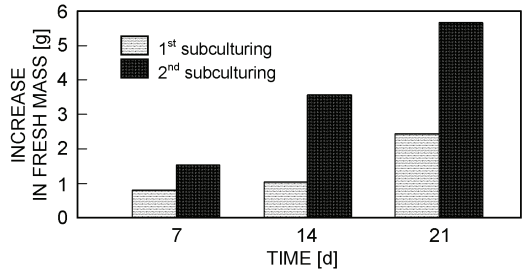

Fig. 1. Increase in fresh mass of cells after first and second subculturing on MS medium supplemented with 0.5 mg dm^{-3} 2,4-D. Means of 3 repetitions.

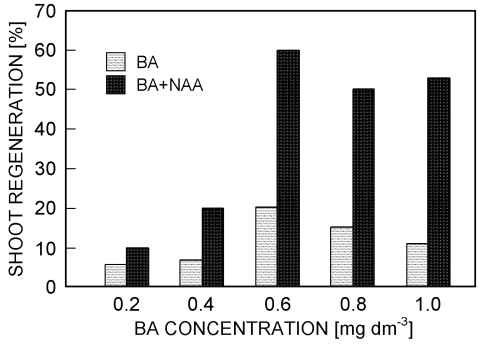

Fig. 2. Effect of different concentrations of BA alone or with 0.5 mg dm^{-3} NAA on induction of shoots from callus. Means of 3 repetitions.

Callus subcultured on MS medium containing 0.5 mg dm^{-3} 2,4-D showed much faster increase in fresh mass after first as well as second subculturing (Fig. 1). The best results were obtained at 14 and 21 d of culturing during second subculturing. The concentration of 0.5 mg dm^{-3} 2, 4-D was found to be the best for isolation and separation of cells in liquid MS medium.

The cell density increased by their subculture onto fresh liquid medium on 4^{th} day and thus maximum cell density was found on 7^{th} day which was 4.83×10^4 cells dm^{-3}. It was observed that cells were subsequently isolated after keeping them in shaker for 3 d. The youngest cells were nonvacuolated round, small and some were elongated or granular (Fig. 3*A*). After 10 d of incubation of Petri plates in dark 4 - 8 cell colonies emerged (Fig. 3*B*) and after 21 - 28 d white spheroid

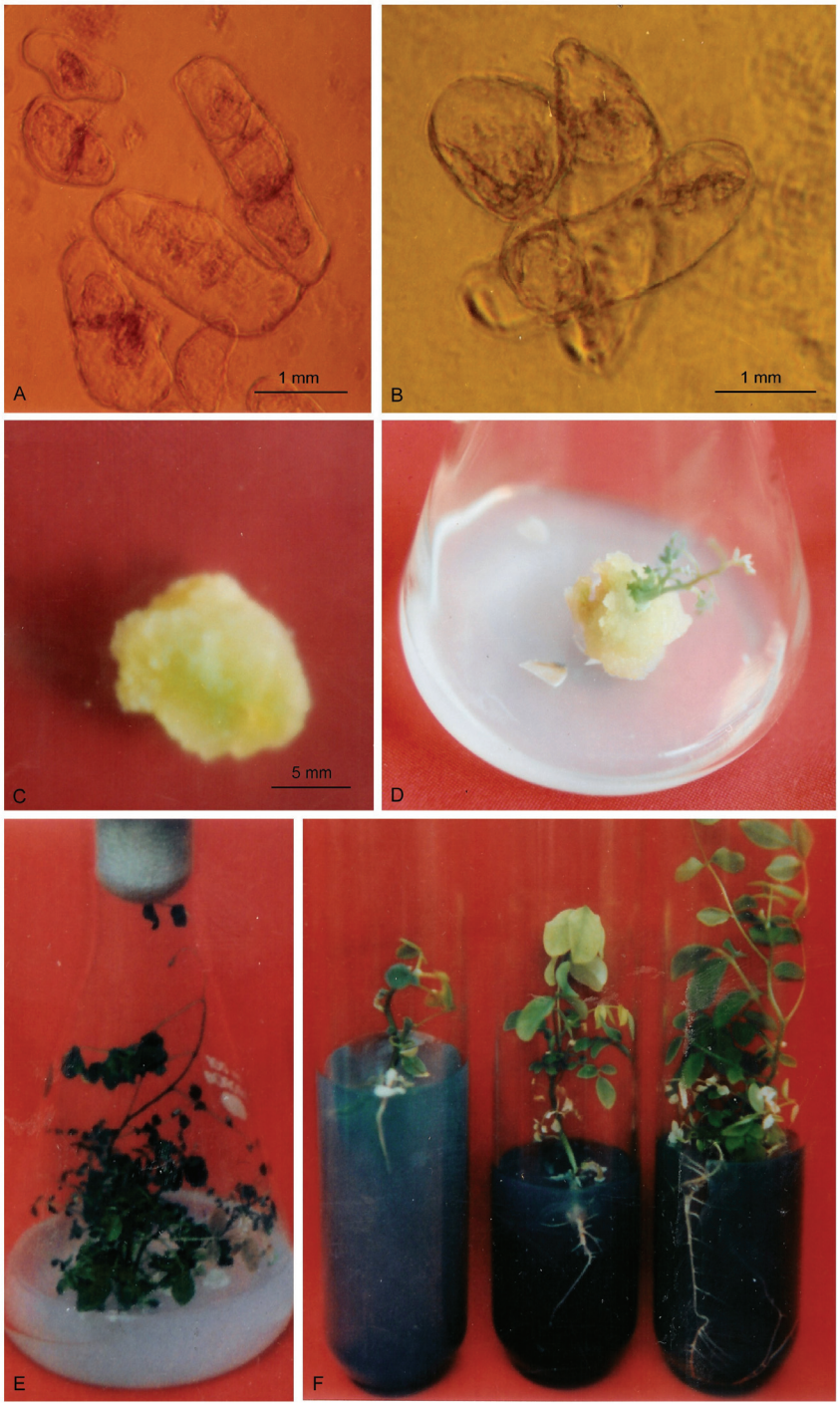

Fig. 3. Plant regeneration from cell culture : *A* - isolated single cells from callus, *B* - first cell divisions, *C* - induced callus, *D* - shoot regeneration from callus, *E* - shoot multiplication, *F* - *in vitro* root induction.

microcolonies formed. Plated single cells showed poor cell division, however cell aggregates of 2 - 5 cells showed enhanced division leading to microcallus formation (Fig. 3*C*). This may be attributed to the reason that single cells may be damaged during isolation or were unable to adapt to the phase change to liquid agar medium where the nutrients are less readily available and inhibitory metabolites build up around the cells (Bergman 1960).

In the present investigation, shoot initiation in callus derived from suspension culture of *Robinia pseudoacacia* obtained in MS medium supplemented with 0.05 mg dm^{-3} NAA and 0.6 mg dm^{-3} BA (Fig. 3*D*). Shoots were remultiplied on MS medium supplemented with 0.6 mg dm^{-3} BA (Fig. 3*E*). Similarly, Pradhan *et al.* (1998) observed shoot bud differentiation on calli derived from cell suspension cultures of *Dalbergia latifolia* on MS medium supplemented with 2.7 μM NAA and 13.3 μM BA. Results presented in Fig 2 shows that the best treatment was found to be 0.6 mg dm^{-3} BA along with 0.05 mg dm^{-3} NAA to induced 60 % shoot regeneration from callus derived cell cultures. In the present studies 85 - 90 % rooting on micropropagated shoots of *Robinia pseudoacacia* was observed within 15 d (Fig. 3*F*). Well-established root system was observed within 9 month and could be acclimatized in pots containing soil and sand to produce plantlets.

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