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Short Communication

Plant regeneration from transformed calli of the tree species *Casuarina equisetifolia*, Linn.

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C. equisetifolia **is a multipurpose tree, widely used in the afforestation and wasteland development programmes. A successful *in vitro* plant regeneration protocol has been developed for this tree earlier. Further to this, various parameters necessary for the genetic modification using biolistic gene delivery system were standardized. The chimeric calli after 3 months of selection with appropriate antibiotic showed the transgene integration in genomic PCR. The chimeric calli developed shoots when cultured on Murashige and Skoog (MS) medium supplemented with phytohormones Benzyl Amino Purine (BAP) and Indole Acetic Acid (IAA) after it was pre-exposed to an induction medium, (MS with 2,4-Dichlorophenoxy acetic acid (2,4-D) and Kinetin (Kin) at concentrations of 2mg/l and 0.5mg/l respectively) for 30 days. Excised microshoots when treated with Indole Butyric Acid (IBA) followed by Abscisic Acid (ABA) developed roots in one month. Three months old plantlets were transferred to green house in pots containing soil and vermiculite (1:1).**

Key Words: *Casuarina equisetifolia*, biolistic gene delivery, *in vitro* propagation, genomic PCR

Tissue culture reports on tree species are mainly on the commercially important tree species like fruit trees, medicinal trees, trees with industrial importance and trees of aesthetic value (Tabori et al. 2010; Naik and Chand, 2011). In short there are less than 50 tree species with an established protocol for *in vitro* propagation and less than half of this number represents the transgenic trees developed with different traits (McDonnel et al. 2010). Both microprojectile and *Agrobacterium* mediated gene transfer techniques have been used to develop transgenic trees through *in vitro* mediated regeneration system.

The technique of particle bombardment (microprojectile) was developed by John Sanford and co-workers at Cornell University (Sanford et al. 1987). Klein et al. (1987) introduced the idea of using metal microprojectiles as carriers of foreign DNA for plant transformation. Various parameters, instrumentation and culture conditions for biolistic transformation have been reviewed extensively (Christou 1997; Veluthambi et al. 2003). The method essentially involves coating of DNA onto the microprojectiles (gold or tungsten particles) and accelerating them with a very high velocity, given by gunpowder (Vasil et al.

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1993), electric discharge (ACCELTM) (Goldfarb et al. 1991; Ellis et al. 1993) or helium pressure (PDS 1000/He) (Knapp et al. 2001; Tian et al. 2000) into the cells/tissue and selecting the transformed cells/tissues using a selection marker. In this study, the necessary conditions for the microprojectile mediated calli transformation in *C. equisetifolia* have been standardized. The parameters like gap distance, target distance and the helium pressure were studied extensively to find out most suitable combination in transforming the calli.

C. *equisetifolia* is is a non-leguminous nitrogen fixing plant, fix atmospheric nitrogen when nodulated by an ascomycete known as *Frankia* (Diem et al. 1982). Because of its tenacious resistance to salinity and desiccation (NAS, 1980), several third world countries including China, Egypt and India employ these trees in afforestation projects in the arid regions, costal belts and marshy lands. The wood has been exploited extensively for the industrial and household uses. Even though the conditions had been standardized earlier for the plant regeneration (Satheeshkumar et al. 2009), the putative transformed calli were regenerated through a different protocol. PCR based molecular experiments showed the presence of transgene in the putative transformed calli after three months. Unfortunately, the regenerated plants did not show the transgene integration.

Materials and Methods

Superior fast growing biotypes of C. *equisetifolia* plantlets obtained from the Department of Forest, Tamilnadu, India were used in the study. Three months old branches detached from the parental tree were dipped in 4000 ppm of rooting hormone, Naphthalene acetic acid (NAA), and planted in small plastic bags containing soil and cow dung in a ratio of 3:1. Rooted plantlets were later transferred to the field and were used as the donor plants for the shoot tip explants. All the plasmids used in the study were maintained in *E.coli* DH5α as glycerol stocks in ultra low freezer (-70°C). Plasmid pUbP5CS is a 7.4 Kb plasmid having *Vigna aconitifolia p5cs* cDNA under Ubiquitin promoter. pHX4 was a 6.4 Kb plasmid containing gene for hygromycin phosphor-transferase (*hph*) from *Streptomyces hygroscopicus* under CaMV 35S promoter. Equipments like Laminar flow hood (Atlantis, New Delhi), PDS- 1000/He Biolistic gene delivery system (Biorad Laboratories, CA, USA), Electrophoresis apparatus (Biotech, R&D Lab, Salem, India) Biotron Growth chamber (Nippon Medical and Chemical Instruments Co. Ltd., Japan), PCR Machine (PTC 150, MJ Research Inc. USA) were used in the study.

Callus induction: Aseptic development of axillary shoots and the phytohormone composition used for callus induction has been published earlier (Satheeshkumar et al. 2008). The cultures were maintained at 25±2°C and 14hr photoperiod. White light was provided by cool fluorescent tubes at a photon flux rate of 400 μmol $m^{-2}s^{-1}$.

Transformation using biolistic gene delivery system: Bombardment experiments were performed using a PDS-1000 He Biolistic gene delivery system (Bio-Rad) with a flight distance of 9cm and He-pressure 1100psi. The calli were cut into small pieces of 2mm diameter and arranged on the center of a petridish containing osmotic media (MS + 2,4-D (2mg/l) + 0.25 M Mannitol + 0.25 M Sorbitol + 0.25% phytagel) and incubated in dark for 4 hours prior to bombardment. Bombardment was performed according to the standard procedure of Sanford *et al.*, 1993. After bombardment, the calli were incubated in dark at 25± 2°C for 24 hours and later transferred to callus maintenance (MS+ BAP (1mg/l)) media.

Selection of transformed calli: Transformed calli after one month of bombardment were selected by culturing on callus maintenance media supplemented with 40mg/l hygromycin. Subculturing was done at an interval of two weeks. After 8 weeks, the survived calli were transferred to regeneration media.

Regeneration of plantlets from putative transformed calli: The hygromycin resistant calli were cultured on MS basal medium supplemented with 2, 4-D (2mg/l) + Kin (0.5mg/l) for four weeks showed shoot induction when subcultured on MS medium supplemented with BAP (1mg/l) and IAA (0.1mgL). Rooting of the microshoots was performed according to the protocol published before (Satheeshkumar et al., 2009).

Molecular analysis of putative transformed calli: Three months old calli after bombardment were selected for the molecular studies to analyze the presence of transgene integration by genomic PCR. DNA extraction for PCR experiments was done by the modified CTAB method (Porebski et al., 1997). PCR was carried out using the specific primers (Forward primer 5'- GAA GAT TGG GAG CTC TGT GC- 3', Reverse primer 5'- ACA AGA AGC TGA GCC GAT GT- 3') designed from the *Vigna* p5cs gene to amplify a sequence of 242bp. PCR products were resolved on a 2% agarose gel and visualized on a UV transilluminator.

Results and Discussion

The transformation experiments were done with two plasmids (details given in materials) pHX4 and pUbP5CS. Plasmid pHX4, give hygromycin resistance to the transformed calli had been used as a selection criterion for transformed cells in this study. Therefore, hygromycin sensitivity assay was performed in order to determine the effective concentration of the antibiotic which completely arrests the growth of untransformed cells. Among the different concentrations of hygromycin (10mg/l, 20mg/l, 30mg/l, 40mg/l and 50mg/l) used, it was observed that, hygromycin at 10 and 20mg/l concentrations had little or no effect on growth and the calli was similar to control. However, at 30mg/l concentration, growth was significantly inhibited. Hygromycin at 40 and 50mg/l concentrations resulted in browning and total inhibition of growth within three weeks (data not given). Therefore, in order to avoid possible escapes during selection, hygromycin at 40mg/l concentration was used for selecting transformed cells in this study.

In order to standardize the parameters for optimum gene delivery, 60 days old embryogenic callus was used. Co-bombardment was performed with gold particles (1µ) coated with pUbP5CS and pHX4 plasmids. The chamber vacuum pressure, travel distance and gap distance were kept at 28mmHg, 3mm and 9mm respectively. Various combinations of helium pressure (650, 900, 1100 and 1300psi) and target distance (3, 6, 9 and 12cm) were tested to determine the most suitable combination of the two parameters for optimum gene delivery (Fig-1). Depending up on the target tissue/ cell(s), different helium pressures have been used in different tree species like, 400psi (in *Picea glauca*, Li et al. 1994), 900psi (in *P. mariana*, Tian et al. 2000), 1100psi (in *Pinus taeda,* Tang and Tian 2003; Walter et al. 1994) and 1350psi (in *Pinus radiata*, Grace et al. 2005; Bishop- Hurley et al. 2001; *P. taeda*, Tang and Tian 2003). The target distance used for efficient gene delivery in tree species is either, 6cm (in *P. radiata,* Walter et al. 1994; Grace et al. 2005; Bishop-Hurley et al. 2001), 7cm (In *P. pinaster*, Trontin et al. 2002), 9.5cm (in *Picea glauca*, Bommineni et al. 1993) or 12cm (in *P. taeda*, Tang and Tian 2003). However, a target distance of 9cm and helium pressure of 650/900psi was found to

be most effective for gene delivery in this study.

The bombarded calli were then subcultured on callus maintenance medium and allowed to multiply for one month before transferring to the selection medium containing hygromycin (40 mg/l). The amount of selection agent differs according to the plant/tissue used. Tian et al. 2000 and Trontin et al. 2002 used hygromycin at a concentration of 20 mg/l, for selecting transformed cells in *Picea mariana* and *P. abies* respectively. Maximum survival rate of 77.5% and 73.3% were observed for the calli bombarded at 900 or 650 psi helium pressure with a target distance of 9cm (Fig-1).

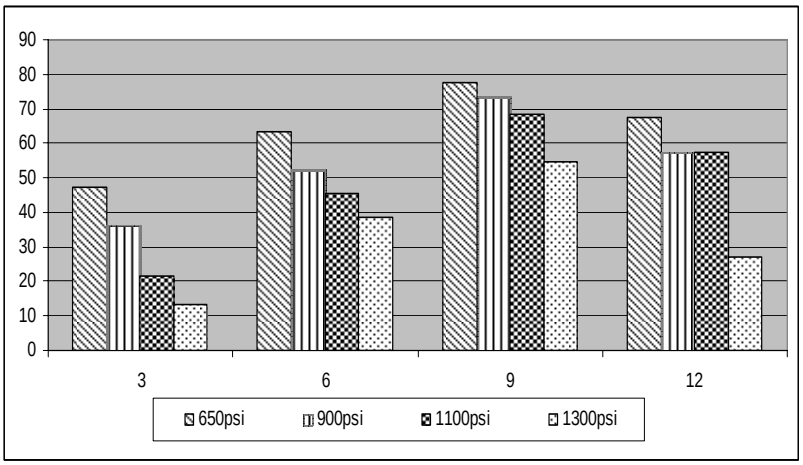

Figure-1 Effect of flight distance and helium pressure on transformation

The calli preparation and bombardment was done as per the protocol given. The chamber vacuum pressure and travel distance were kept at 28mmHg and 3mm respectively. X-axis represents the survival percentage and the Y-axis represents the flight distance in centimeter. Values are the average of three independent experiments with 80-100 replicas.

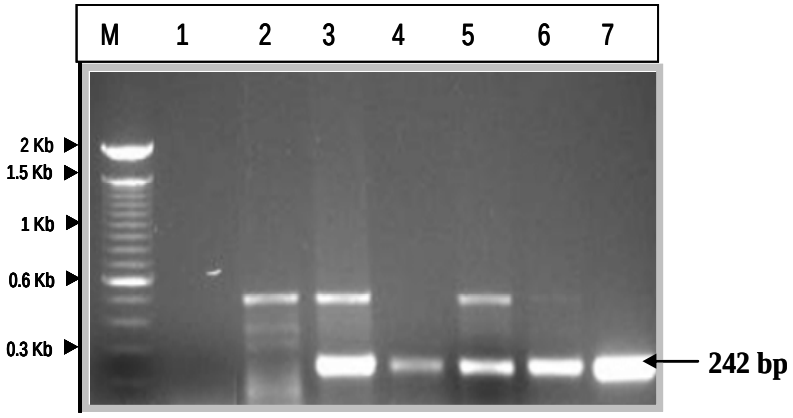

Figure-2 PCR analysis of hygromycin resistant calli

PCR was set with 50ng of genomic DNA from the putative transgenic calli using *Vigna* p5cs specific primers. M-100bp Marker, lane 1- blank, Lane 2 - unbombarded calli, Lane 3-6 - DNA from 4 hygromycin resistant calli, Lane 7- pUBP5CS plasmid

The integration of transgene *p5cs* in the hygromycin resistant calli has been analyzed by PCR with genomic DNA from four randomly selected hygromycin resistant calli using *Vigna p5cs* specific primers. The PCR showed the expected amplicon of 242bp (Fig 2, lane 3-6) corresponding to the positive control (lane 7), indicating the presence of *p5cs* transgene in all the selected calli pieces analyzed, whereas unbombarded calli did not show any amplification (Fig-2, lane 2). It clearly shows that the chimeric calli consists of either pHX4 and/or p5cs gene integrated cells.

The regeneration response of the putative transgenic calli was different from that of untransformed calli. In the revised method, hygromycin resistant calli subcultured on MS basal medium supplemented with 2, 4-D (2mg/l) +Kin (0.5mg/l) showed of shoot bud induction within four weeks (Fig- 3A). When subcultured on MS medium supplemented with BAP (1mg/l) and IAA (0.1mgL), the shoot buds gave rise to shoots (Fig-3B). This observation was similar to one of the previous results published for Douglas-fir, where a cytokine pulse induced shoot induction (Goldfarb et al. 1991). Fully grown shoots were later used in the root induction experiments. Microshoots were rooted as per the protocol of Satheeshkumar et al. 2009 (Fig- 3C). PCR performed with the DNA isolated from four regenerated plants did not show any transgene integration.

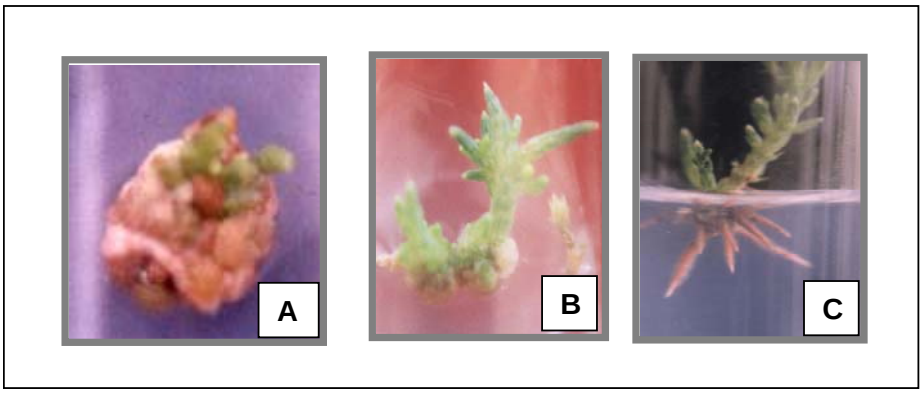

Fig-3 Plant regeneration from hygromycin resistant calli

Hygromycin resistant calli when grown on MS with 2, 4-D (2mg/l) + Kin (0.5mg/l) showed shoot bud induction (A). Shoot proliferation in MS with BAP (1mg/l) + IAA (0.1mg/l) (B) and root induction of *in vitro* regenerated shoots (C).

Conclusions

The conditions standardized for *C. equisetifolia* transformation was found to be efficient to transfer the co-bombarded plasmids to the genome of *C. equisetifolia*. The regeneration conditions had to be revised compared to the control calli, owing to the fact that the post-bombardment selection changes the normal physiology of the calli. The chimeric calli showed the transgene integration, but the regenerated plants were devoid of any transgene integrated to its chromosome.

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