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DEVELOPMENT OF EFFICIENT MICROPROPAGATION SYSTEM FOR *E. camaldulensis* WITH RESPECT TO AGE OF EXPLANTS

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Efficient regeneration protocol is important for mass propagation, genetic transformation and germplasm alteration of desired plants. Thus, development of an efficient protocol is of the considerable importance for further genetic manipulation. In vitro plant regeneration was studied from two age groups of seedlings (2-11 weeks and 12-21 weeks old) by using their cotyledons, hypocotyls & leaf segments as explants. Shoot organogenesis was evaluated by using different explants on MS medium supplemented with varying concentrations of phtyohormones (1mg/l BAP ; 0.2 mg/l BAP + 1mg/l Zn ; 0.5mg/l BAP + 0.1mg/l NAA; 1.5mg/l BAP + 0.5mg/l NAA). Similarly, rooting of regenerated shoots was optimized on different concentrations of IBA (0.1mg/l;0.5mg/l;1mg/l). Hypocotyls of two to eleven weeks old seedlings as explants showed the best results of direct organogenesis on BAP (0.5mg/l) and NAA (0.1mg/l) in 21 days. However, BAP (0.2 mg/l) and Zn (1mg/l) showed no growth. Indole-3-butyric acid (1 mg/l) induced rooting within a minimum period of 12 days. Overall, rate of shoot and root formation was found to be 50%, respectively. Successfully, the platelets were acclimatized in net house and further transferred to open field conditions.

Keywords: Direct organogenesis, indole-3-butyeric acid, micropropagation, in vitro regeneration

INTRODUCTION

Among the world important hardwood plantation, eucalypts occupied significant position due to their paper pulp, fuel wood and timber. *E. camaldulensis* being widely distributed and drought tolerant species has the ability to adopt changing rainfall as well as temperature conditions. This property has made it useful in economical and industrial prospects (Butcher *et al.,* 2009; Pinto *et al.,* 2013). Medicinal *Eucalyptus* oil is largely employed in the preparation of liniments, inhalants, cough syrups, ointments, toothpaste, and pharmaceutical flavouring. The oil obtained from eucalyptus leaves have found various applications in daily life due to their antiseptic, anti-inflammatory and antipyretic properties (Silva *et al.,* 2003; Akin *et al.,* 2007; Cheng *et al.,* 2009).

Micropropagation of *E. camaldulensis* has been documented previously in vitro via direct organogenesis (Yashoda *et al.,* 1997; Kawaoka *et al.,* 2006; Pinto *et al.,* 2013) and indirect organogenesis (Mullins *et al.,* 1997; Ho *et al.,* 1998; Harcourt *et al.,* 2000; Rahim *et al.,* 2003; Dibax *et al.,* 2005; Dibax *et al.,* 2010; Ahad *et al.,* 2014) and indirect somatic embryogenesis (Prakash and Gurumurthi, 2010). Deepika *et al.* (2011) established in vitro shoot regeneration on MS basal medium supplemented by plant growth hormones (BAP, NAA and TDZ) for various eucalyptus genotypes i.e. *E. grandis*, *E. grandis* × *E. nitens*, *E. grandis* × *E. camaldulensis and E. grandis* × *E. urophylla.* A study on genetic modification of eucalyptus in order to modify the cellulose and hemicellulose biosynthesis to increase the biomass in a short period of time has been reported (Dibax *et al.,* 2010).

In comparison to other woody plants this species is very recalcitrant; the problem with eucalyptus is its lower transformation as well as regeneration ability due to heavy leaching of phenolic compounds (Tournier *et al.,* 2003). During *Agrobacterium* mediated genetic transformation, the transformed tissues usually lose their potential to regenerate and develop plants. Particularly, the failure of well develop rooting systems to initiate roots from transformed shoots show the major obstacle in obtaining whole regenerated transgenic plants. The current study was an attempt to develop a simple, reliable and efficient in vitro regeneration protocol for *E. camaldulensis*. The explants used were cotyledons, hypocotyls and leaf segments. Effect of age of explants on regeneration potency was also monitored.

MATERIALS AND METHODS

***Plant material and sterilization*:** Seeds were collected from Ayub Agricultural Research Institute, Faisalabad, Pakistan. First, they were surface sterilized by submerging in 70% ethanol for 60 second followed by washing with sterile distilled water then immersed in sodium hypochlorite (100%) for 20 minutes. An additional washing with 70% ethanol for 1 minute was also given. Later on, the seeds were rinsed 3-4 times with autoclaved distilled water (Ahad *et al.,* 2014).

Germination of seeds was obtained on half strength MS basal medium (Murashige and Skoog, 1962) having 3% sucrose and

4% Gelzan (PhytoTechnology Laboratories®). In the current study, seedlings of two age groups (2-11 week and 12-21 week) were used. The first age group was termed as young seedlings and second as old seedlings. For both age groups cotyledons, hypocotyls & leaf segment (0.5-0.8cm) were used as explants.

***Regeneration medium*:** For optimization of regeneration four shoot induction media (SIM1-SIM4) were used (Table 1). Light intensity of 20 µmol m^{-2} s^{-1} was provided by white fluorescent tube lights and the culture room temperature was maintained at 25°C.

Table 1. Combination of different plant regulators used for regeneration in *E. camaldulensis*.

Name of the media	Composition of media in MS medium
SIM1	1.0 mg/1 BAP
SIM2	0.2 mg/1 BAP+1mg/1Zn
SIM3	0.5 mg/1 BAP+0.1mg/1NAA
SIM4	0.5 mg/1 BAP+0.5mg/1NAA

***Rooting medium*:** Elongated shoots were further transferred for rooting in MS medium fortified with IBA (Table 2). Shoots on root induction medium (RIM 1-3) were kept in light at 25°C in tissue culture room for 2 weeks. All media contained 3% sucrose with pH adjusted at 5.8.

Table 2. Rooting media for *E. camaldulensis*.

Name of media	Composition of media in MS medium
RIM1	0.1 mg/1 IBA
RIM2	0.5 mg/1 IBA
RIM3	1.0 mg/1 IBA

***Acclimatization*:** Rooted plantlets with height 6-8 cm were acclimatized in vitro by placing in pots (3x3inch) with a mixture of peat moss, perlite and vermiculite in a ratio of 2:1:1, respectively. Plants were acclimatized under high humidity condition. The humidity was maintained in pots by covering them with polypropylene bags which was gradually decreased by cutting edges of the polypropylene bags until no moisture remained in the bag. Acclimatization was done in green house at 25°C. Hoagland solution (1X, 20 ml) was also given to plants after every 7 day. Acclimatized plants were subjected to hardening in silt and loam soil under net house conditions during spring season.

RESULTS

Response of explants was different on different combination of phytohormones. Direct shoot organogenesis was induced by SIM1 and SIM3 (Fig. 1 a,b,c,d) within 21 days. SIM3 proved to be the best growth hormone combination as it induced more number of bud formation per explant (Table 3). Further, the age of seedlings was monitored to check its effect on regeneration phenomenon. Young as well as old seedlings responded in the same manner on these (SIMI & SIM3) hormonal combinations. Regeneration efficiency of SIM3 was up to 50% (Table 4).

Table 3. Comparison of shoot induction media.

Basal culture medium	PGR combination	Explants inoculated	Explants regenerated	Days
MS	SIM1	12	2	30
	SIM2	12	0	30
	SIM3	12	09	30
	SIM4	12	01	30

Table 4. Regeneration efficiency of SIM3.

No. of experiments	Inoculated explants	Regenerated shoots	% Regeneration
Experiment 1	638	322	50.4
Experiment 2	326	160	49.07
Data generated	Total explants	Regenerated plantlets	Regeneration
Frequency	964	482	50

However, Indirect organogenesis was observed on SIM4 when young seedlings were used as explants, swelling was observed in 19 days while callus induction after 28 days, brown embryonic calli were formed after 36 days of incubation and shoot formation after 45 days of incubation (Fig. 1e & f).

Old seedlings on SIM4, responded in a different manner. Enlargement as well as swelling was seen after 30 days and callus formation initiated in 40 days followed by shoot formation after 60 days of incubation. No callus formation was observed within one month. Whereas, the direct shoot organogenesis was also seen in the some cotyledons and hypocotyls (Fig. 1g). No regeneration was observed on SIM2 even after two months (Table 3). It was concluded that frequency of regeneration decreased with ageing of explants. The results indicated that leaf as explants showed poor regeneration response as compare to hypocotyls irrespective of the age of seedling. Adventitious buds originated as clumps on whole hypocotyls especially at nodal area while in leaf it emerged from petiole region. Most of the leaf portion got necrotic due to heavy release of phenolics when explants were excised to small portions for experimental purposes.

Regenerated shoots (2.5-3cm) obtained from SIM3 were shifted to rooting media. At concentration of 1 and 0.1mg/l IBA within 12 days, root induction was observed (Fig. 1h). Delayed root formation (after 27 days) was seen on MS medium supplemented with 0.5mg/l IBA (Table 5). On each plantlet an average of 4-6 roots were formed. Root growth was normal but after four weeks of culture, browning was observed. Root regeneration was observed from 25 to 50% in inoculated shoots. In micropropagation, hardening of tissue culture derived plantlets is a critical step. But in present study,

Figure1. Photographic presentation of in vitro shoot and root induction in *E. camaldulensis*: a,b. Organogenesis on SIM3 (0.5mg/l BAP+ 0.1mg/l NAA) by direct regeneration; c,d. regeneration on SIM1; e,f. shoot regeneration on SIM4 (1.5mg/l BAP+0.5mg/l NAA) via indirect organogenesis; g. Shoot and root formation in old seedlings; h. Root induction on RIM3 (1mg/l IBA); i,j,k. acclimatized *E. camaldulensis* plants.

transfer of plantlets in pots having silty and loamy soil in net house gave 100% survival rate and thus plants were successfully acclimatized (Fig. 1 i,j,k).

Table 5. Root formation of *E. camaldulensis*.

PGR (IBA mg/l)	Shoot inoculated	Shoots formed roots	Culture time	Media efficiency
0.1	4	1	12	25%
0.5	4	1	27	25%
1	6	3	12	50%

DISCUSSION

Eucalyptus species are the mostly propagated as a raw material for production of cellulose. Developing a regeneration protocol for this species would be an important step towards the improvement of its properties for the utilization in pulp and paper industries. Several reports regarding transformation and micropropagation methods in various eucalyptus genotypes are available (Kawaoka *et al.*, 2006; Deepika *et al.*, 2011; Ahad *et al.*, 2014).

Sterilization is an important step in order to get in vitro seedlings as explant material. During present work, in process of sterilization, the most common contamination was fungal spores and its hypae. Use of NaClO proved effective in controlling contamination. These results were supported by (Dibax *et al*., 2010) and (Nugent *et al*., 2001) who reported different concentrations of NaClO helpful for sterilization of eucalyptus. According to Tournier *et al.* (2003) in comparison to other woody plants, the problem with eucalyptus species is their lower transformation and regeneration rate due to leaching of phenolic compounds. Thus, evaluation of efficient shoot inducing medium, best target material for the differentiation and age of explant with respect to transformation is of vital importance. On SIM3 and SIM4 (Table 1) young seedlings were proved the best target tissue for regeneration; it showed survival up to 60-70%. This regeneration response decreased to 10-20% in case of old seedlings. González *et al*. (2002) recommended 2 and 15 days old cotyledons and hypocotyls after germination for eucalyptus genetic transformation and concluded that actively dividing cells and tissues were more efficient as compared to older explants. Work done on *E. gunnii* leaves showed that regeneration was initiated from meristems located in the leaf base and connected to the vascular bundles (Herve *et al.*, 2001) However, Bandyopadhyay and Hamill (2000) documented more regeneration capabilities in hypocotyl derived calli in *E. nitens* and *E. globules.*

The current study has same findings in regenerative response of cotyledons and hypocotyls. A combination of auxins (NAA) and cytokinins (BAP) in lower concentration favored direct organogenesis from hypocotyls and cotyledons of *Eucalyptus tereticornis* (Girijashankar, 2011; Prakash and Gurumurthi, 2009). From early times, Indole butyric acid (IBA) is widely used auxin to stimulate the rooting phenomenon in plant cuttings (Blazich, 1988; Hartmann and Kester, 1975; Wiesman *et al*., 1988). In present research 1mg/l IBA showed root formation in 12 days (Table 5). Current results are in consistent with published literature. The documented data also showed, 1mg/l IBA as successful in inducing roots in various genotypes of eucalyptus (Ho *et al.*, 1998; Prakash and Gurumurthi, 2009). However, another report by Kawaoka *et al.* (2006), showed 0.5mg/l IBA effective in root formation in the case *E. camaldulensis.*

***Conclusion*:** The protocol established in this study provides a simple and efficient system for introduction of desirable characters in *Eucalyptus camaldulensis*. This shows potential for in vitro mass propagation and the development of germplasm under different climatic conditions. It also suggests that in vitro young seedlings (2-12 week age) are good target tissues for producing transgenic plants via micropropagation.

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