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Micro Propagation of *Eucalyptus globules* Through Mature Trees

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Abstract

Eucalyptus globules is an important rapid-growing trees in Eucalypts that is used for agro forestry and tree improvement programs. It has been used as a source of pulp in paper industry, essential oil and honey production. Asexual propagation with conventional methods such as cutting and graft was not possible or obtained success was very low, because of hard proliferation of adventitious roots. This study was focused on micro propagation of *Eucalyptus globules* through mature trees. Apical buds from adult elite trees in forests of northern Iran were collected in different seasons. The method for sterilization and regeneration were optimized by comparing different combination of plant growth regulators. The highest number of induced shoots was obtained in 0.5 mgl^{-1} of BA and 0.5mgl^{-1} of Kin after four weeks. Elongated shoots excised at 1.5 cm in length were rooted in $^1/_4$ strength of macro elements in MS medium with 0.5 mgl^{-1} of IBA plus 0.5 mgl^{-1} of NAA and activated charcoal (2.5 gl^{-1}). These plants were transferred in sterile soil at greenhouse condition and successfully established in the field after gradual acclimation.

Key words: Micro propagation, *Eucalyptus globules,* Mature trees, Bud culture.

Introduction

Eucalypt (*Eucalyptus*) is a rapid-growing tree species, originated from Australia and were represented by about 800 species and hybrids, some of them being commercially important for timber, essential oil and pulp (Gupta and Mascarehans, 1987). Several biotechnological approaches have been developed to circumvent the barriers of Eucalypt breeding due to variability of propagation by seed and lack of extensive genetic selection combined with the long juvenile period to reach the flowering period (Gupta and Mascarehans, 1987; McComb, 1995). Although successful results for *in vitro* cloning have been reported for some species (Bennett *et al.*, 1994), the rooting phase is a critical and limiting step (Hartney, 1980). Micro propagation of *Eucalyptus* may be an important tool for tree improvements and reforestation programs. Plantlets may be used for establishment of cloned seed orchards, testing of cloned candidates and operational planting. Also large number of cloned plantlets may be micro propagated for physiological and silvi cultural studies when genetic uniformity is desired. Foresters have recently appreciated the value of vegetative propagation as a source of uniform trees of known genotype and it can be a proper tool to manipulate the environmental factors and plant material homogeneity (Morabito *et al.,* 1994). In recent years attempts have been made to clone *Eucalyptus* through *in vitro* techniques. In most of these methods, organogenesis was obtained from embryonic of juvenile seedling tissues. Conventional vegetative propagation is not successful for *E. globules* and use of seed propagation is yielding highly variable progeny for continuous pulpwood supplies, then this species is cultivated for woody fibers (Sharma and Ramamurthy, 2000). It is important to have plantations of uniform bole size and quality. Besides eucalyptus wood, essential oil of this is very important in perfume and soap industries and used in some chemical reactions of industries as substrate (Bina and Siddiqni, 1997). Also essential oils of eucalyptus are applied to cure of respiratory and digestive diseases (Mauhachirou *et al.*, 1999). In this paper, we report the

production of plants from nodal stem segments of mature *E. globules* by micro propagation.

Materials and Methods

Abbreviations: BAP: 6- Benzyl amino purine, GA3: Gibberelic acid, IBA: Indol-3-butyric acid, NAA: Naphtyl acetic acid, Kin: 6- furfuryl amino purine, 2iP: -γ,γ,γ –Dimethyl allyl amino purin, MS: Murashige and Skoog (1962), ANOVA: Analysis of variance

Sample preparation

Shoot tips and nodal segments were separated as explants from 30 years old *Eucalyptus globules* trees in forests of northern Iran. For surface sterilization, the explants were immersed in sterilized water containing some drops of soap10 min. Then they were rinsed with distilled water three times and soaked in mercuric chloride solution (0.1%) for different applying times. Then, the shoots were washed repeatedly, cut and cultured under aseptic conditions (Fig. 1).

Micropropagation conditions

Explants were cultured on nutrient MS medium supplemented with a gradient range of hormones (Table 1). Media were solidified with 0.68% w/v agar.

Modified MS (9) medium with 3% sucrose and 100 mgl^{-1} ascorbic acid were used as basal medium (were autoclaved at 120°C temperature under 1.06 kg/cm^2 pressure for 20 min). Then, they were incubated in standard culture room conditions (16 h light, light intensity of 63 PPFD, 22-25 °C). Explants at these treatments were embedded into the medium (5 explants/plate × 6 plate). After 30 days, the growth parameters including shoot length (cm) and shoot number and shoots greenness was recorded. ANOVA was performed using shoot number, shoot length growth, leaf color and rooting percentage. Duncan's multiple range tests was used to compare treatments with in clones when a significant difference was found between treatments. The results of statistical analysis were considered significant when they were outside 95% confidence intervals.

Six growth regulator treatments in MS medium with ½ nitrate supplemented with activated charcoal ($2.5gl^{-1}$) and IBA, NAA, IAA in 0, 0.5 and 1 mgl^{-1}, respectively were used for rooting induction (Table 2). Shoots were selected at 15-20 mm pieces. Explants at these treatments were embedded into the medium (4 explants/plate × 3 plate). After 2 months, root number and existence or lack of root hairs was recorded. *In vitro* derived plantlets were transferred to 4:1:4 peat: per lit: vermiculite pots with polyethylene caps in greenhouse condition.

Results

In the sterilization phase, the best treatment in each season was presented in table 3. In comparison of these results, the best treatment (about Viability percent of explants) in the year was soaking of buds in $HgCl_2$ solution (0.1 %) for 10 min in spring. Shoots measuring 10-15 mm were formed in 5-6 weeks. The MS medium was achieved for proliferation of some of the *Eucalyptus* species. Shoot proliferation was not good on Kinetin and 2iP media, while shortened inter node and growth inhibition was observed. Analysis of variance records showed that effect of growth regulators were significant on shoot length and greenish rate (Table 4). Shoots sub cultured in MS medium supplemented with 0.5 mgl^{-1} BA plus 0.5 mgl^{-1} Kin were the best treatment for proliferation of shoots (Fig. 1B, Table 5). Up to 20- 40 shoots could be obtained from one nodal culture.

The use of two different multiplication media improved shoot condition and subsequent rooting of *E. globules* even if it does not always increase shoot multiplication. One fourth of macro elements of MS medium and 0.5 mgl^{-1} of IBA plus 0.5 mgl^{-1} of NAA was also found to be best in rooting (Fig. 1C, Table 6). The percentage of roots was 50. Tissue cultured plants were transferred in soil successfully (Fig. 1D).

Table 1. Shooting different treatments of *E. globules*.

Hormone (mgl^{-1}) / Treatment	BA	GA	Kin	2iP
T1	0.5	0.5	0.5	-
T2	-	0.5	-	0.5
T3	0.5	0.5		0.5
T4	0.5	0.5	-	-
T5	-	0.5	0.5	0.5

-: Non- growth regulator

Table 2. Rooting different treatments of *E. globules*.

	IBA		NAA	
Hormone (mgl^{-1}) / Treatment	**0.5**	**1**	**0.5**	**1**
T6	+			
T7		+		
T8			+	
T9				+
T10	+		+	

+: Existence of growth regulator; -: Non- Growth regulator

Table 3. Sterilization treatments of *E. globules* buds.

Season	The best treatment	Viability%	Infection%	Necrosis%
Spring	$HgCl_2$ 0.1 % 10 min	76	24	0
Summer	$HgCl_2$ 0.1 % 12 min	73	18	9
Autumn	$HgCl_2$ 0.1 % 15 min	26	60	14
Winter	$HgCl_2$ 0.1 % 20 min	12	62	26

Table 4. Analysis of variance of different cytokines effects on growth factors.

Source of Variation	DF	Mean Square	*F*-value	*p*
Effect of Growth Regulators on Shoot Length	4	1.676	22.88	0.000*
Effect of Growth Regulators on Greenish Rate	4	4.98	7.13	0.006**
Experiment Error (Shoot Length)	10	0.07	-	ns
Experiment Error (Greenness Rate)	10	0.69	-	ns

**: significant different (p<0.01), *: significant different (p<0.05), ns: non- significant

Table 5. Effect of hormone treatments on shoot length and leaf greenish means in Duncan grouping

Treatments	Hormones (mgl^{-1})	Mean of Shoot length	Mean of Leaf greenish
T1	BA 0.5+ K0.5+G0.5	2.5 a	4 a
T2	G0.5+2iP 0.5	1 c	2.5 c
T3	2iP 0.5+ BA0.5+G0.5	1 c	2.5 c
T4	BA0.5+G0.5	0.5 d	2d
T5	2iP 0.5+ K0.5+G0.5	2 b	3b

Table 6. Effect of hormone treatments on root induction Means

Treatment	Hormones (mgl^{-1})	Percent of root induction	Percent of Necrosis
T1	IBA 0.5	12	10
T2	IBA 1	10	50
T3	NAA 0.5	28	20
T4	NAA 1	25	35
T5	NAA, IBA 0.5	50	10

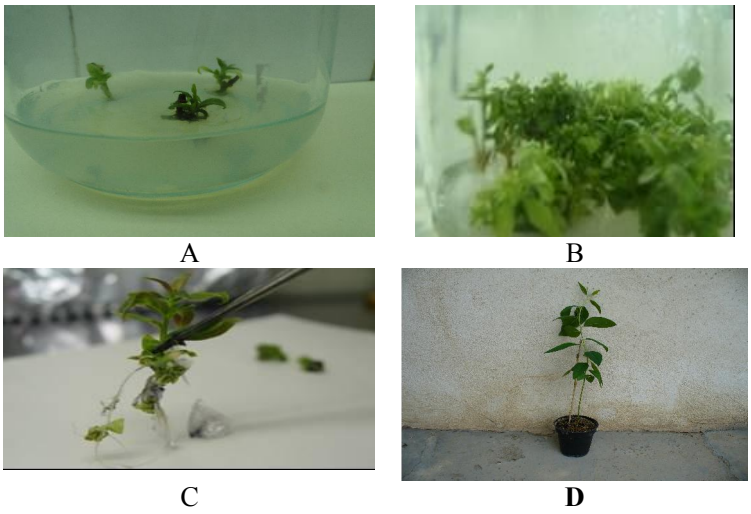

Fig. 1. The different stages of *Eucalyptus globules* micro propagation. A: Culture of buds, B: Proliferation of shoots, C: Rooting of shoots, D: *In vitro* plant.

Discussion

Novel advances in micro propagation of forest trees have opened great opportunities for mass propagation of selected valuable genotypes (Assareh, 1998). Selection of explants, composition of nutrient media, concentration of hormones and methods used for micro propagation had significant effects on shoot multiplication and rooting rates of *E. globules* explants. In the sterilization phase, the best treatment was immersion of buds in $HgCl_2$ solution (0.1 %), formerly used by Gupta *et al*. (1981) on *E. citriodora*.

The best of shoots proliferation were done in MS medium containing BA and Kinetin. Lakishmisita (1993) on micro propagation of *E. tereticornis* was achieved similar results. Bennet (1994) showed that in the medium culture of *E. globules* with Kinetin, the shoots were longer in contrast to other treatments. Mascarehans (1982) reported that the composition of BA and IBA produced the regeneration of *E. globules* shoots.

Our results indicate that the species can be rooted by addition of IBA on the induction medium. Auxines are involved in the process of adventitious root formation (Glocke *et al.,* 2006). In some other woody plants, IBA is commonly used to promote root initiation (Onay, 2003; Bhatt and Dhar, 2004). In our study, the absence of IBA in the rooting medium did not lead to root formation. Several authors have reported auxin as the only required hormone during the initiation phase, and inhibitory for root out growth (Shwab and Martins-Loucao, 1988; Elhamdouni *et al.*, 2000). This inhibition of rooting is often accompanied with callus formation. The presence of callus on the shoots increased time for rooting as well as the number of roots formed. Studies on *in vitro* rooting of explants of *Eucalyptus* (Fazal *et al.,* 2003) also showed increased IBA concentrations resulting in increased callus formation. The rooting response was correlated with the production of particular flavonoid (Curir *et al.*, 1990). Endogenous cytokines appear to have a role in adventitious root production (Bollmark *et al.*, 1988). It is possible that BAP is more effective than Kin in switching on endogenous cytokinin production. However, shoots induced from juvenile material were more

amenable to rooting than those from mature trees. Shoots were sub cultured to MS medium contained activated charcoal (2.5 gl^{-1}) and GA (1 mgl^{-1}) without auxines, was suitable for elongation prior of rooting. Charcoal is indispensable to induce good development of roots, clamping the reaction of tannins In fact, the tannin constitutes a physiological inhibitor (Souayah, 2002; Custodio *et al.* 2005). Curir *et al*. (1990) demonstrated the importance of multiplication medium on rooting in *E. gunnii Hook* and the presence of GA_3 in the shoot multiplication medium suppressed rooting.

This paper is demonstrated the production of plants from nodal stem segments of mature *E. globules.* The cloned propagated plants, will be used for tree improvement and agro forestry programs

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