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The effect of explants plant growth regulators and silver nitrate on *in vitro* callus induction *in Hevea brasiliensis* Muell Arg.

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The effect of explants and silver nitrate on callus induction in *Hevea* was investigated. The explants used for callus induction in this investigation were leaf, petiole, stem leaf, seed integument and root. Callus induction ability was evaluated by using MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l BA gave the best percentage of callus induction in stalk (100%) and seed integument (100%) after 4 weeks of culture. Supplementation with 1 mg/l $AgNO_3$ in callus induction medium resulted in rapid Proliferation of good quality yellow callus after being cultured for 2 weeks. The addition of silver ions which increased growth rate,the highest average callus fresh weight was also obtained at 1 mg/l silver nitrate.

Keywords: *Dicamba, silver nitrate, callus induction, Hevea brasiliensis*

Introduction

Hevea brasiliensis Muell Arg., belonging to the Family Euphorbiaceae, is an economically important perennial tree grown in Thailand and Southeast Asia as the source of natural rubber. *H. brasiliensis* is still propagated by grafting clonal axillary buds onto unselected seedlings as stock plant to maintain intraclonal heterogeneity for both vigour and productivity (Hua*et al*., 2010). Propagation through tissue culture technique has been reported by number of authors interestingly, there have been several reports of *Hevea* callus induction using various explants raised in different culture media and plant growth regulators (PGRs) selected according to the objective of the study. For clonal improvement, most experiments use direct somatic embryogenesis (SE) or SE formation from callus. Successful plant regeneration in rubber tree has been recorded using several explants such as anther (Jayashree*et al*., 1999; Hua*et al.,* 2010), root (Zhou *et al*., 2010) and immature inner integument (Te-chatoand

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Chartikul, 1993; Sushamakumari *et al*., 2000; Montoro *et al*., 2003; Lardet *et al*., 2007).

In order to propagate true-to-type clones, the microcutting technique is always used. This technique begins by culturing axillary buds or cotyledonary nodes and then inducing plantlets from them. However, an efficient protocol for the large scale micropropagation of elite *Hevea* clones has not yet been developed (Nayanakantha and Seneviratne, 2007).

Gaseous components of the tissue culture system, especially ethylene, play an important role in growth and development of plant (Lieberman, 1979; Yang and Hoffman, 1984). Ethylene is recognized as plant PGRs, promoted leaf abscission, fruit ripening and flower senescence. In tissue culture, ethylene can affect callus growth (Kumar *et al*., 2009) its absence also favour to callus formation on anther culture of coffee (Silva *et al*., 2011). Silver nitrate ($AgNO_3$) are reported to potent inhibitor ethylene action (Beyer, 1976). It has also been promoted plantlet regeneration during somatic embryogenesis in a number of crop species, including Brussels sprouts (Williams *et al*., 1990), cassava (Zhang *et al.*, 2001), *Paspalum scrobiculatum* L. (Vikrant and Rashid, 2002), *Ziziphus jujuba* Mill. (Feng *et al.*, 2010), achiote (Parimalan*et al*., 2010) and turnip (Cogbill*et al*., 2010).

In this present study, $AgNO_3$ was applied to culture medium for callus induction and culture of *Hevea brasiliensis*.

Materials and methods

Plant material and culture conditions

Explants derived from seedlings of rubber clone RRIM 600 and native rubber tree grown around Prince of Songkla University, Hat Yai campus, Songkhla province, Thailand, were collected from a young red shoots 1 month after germination and washed in running tap water for 20-30 minutes. The explants were surface sterilized in 70% ethanol for 30 seconds and in 20% sodium hypochlorite for 20 minutes, followed by three rinses with sterilized distilled water. The sterilized explants were separated into leaf, petiole, stem, seed integument and root then cut into 0.5 - 1 cm. length and cultured on Murashige and Skookmedium.The medium's pH was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaved at 1.05 kg/cm^2, at 121ºC for 15 minutes. The cultures were maintained at 28±0.5^o C under fluorescent lamps at 12.5µmol/m^2/s for a 14 hour photoperiod.

Effect of PGRs on callus induction

All types of explants were cultured on MS medium supplemented with 3% sucrose, 2 mg/l 2,4-D, 2 mg/l BA or various concentrations of dicamba (1, 2 and 3 mg/l). under the conditions specified above. The cultures were routinely subcultured at 4 week intervals for 2 months to induce callus. After being cultured for callus formation in term of percentage and was recorded extent of callus formation per explant was recorded.

Effect of silver nitrate on callus induction

Leaf and petiole explants cultured on the MS medium add to 3% sucrose, 2 mg/l 2,4-D, 2 mg/l BA and two different concentrations of silver nitrate (0, 1 mg/l) which was lower than previous experiments. The cultures were maintained under the same conditions as described above. After 2 and 4 weeks of being cultured, callus induction percentage was recorded.

Effect of silver nitrate on callus culture

Four-week-old callus derived from culturing on the MS medium supplemented with 3% sucrose, 2 mg/l 2,4-D and 2 mg/l BA were transferred to proliferation medium which was solidified MS mediumsupplemented with various concentrations of silver nitrate (0 and 1 mg/l). After 4 weeks of culture, the proliferation rate of callus were recorded.

Statistical analysis

Each experiment consisted of treatment replication. What design is perform Mean values were analyzed using a one-way analysis of variance (ANOVA). Significant differences among treatments were detected using Duncan's multiple range tests (DMRT) at the 0.05 level of probability.

Results

Effect of PGRs on callus induction

MS medium supplemented with 2,4-D and BA at the same concentrations of 2 mg/l promoted the formation of callus from all types of explants of rubber seedlings. Among those explants seed integument and stem gave the highest percentage callus formation at 100, followed by petiole, leaf significant different ($p<0.05$) to that obtained from root (Table 1). Replacement 2,4-D and

BA with dicamba at all concentrations could not induce callus from all explants except leaf explants.

Callus produced was differed from explants to explants and plant growth regulators. Characteristic of callus obtained from the present study was classified into 3 types. Callus was initiated from leaf, petiole, stem, seed integument and root. Experimental results show that callus formed at different type among five kinds of explants in the MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l BA, leaf and root promoted type I callus, petiole and stem promoted type II callus, seed integument promoted type III callus but Dicamba promoted types II of leaf explant (Table 1). The type III callus gave higher active growth then type I and type II. The effects of plant growth regulators on type callus from explants, resulting in 3 type of callus formation; type I, compact, hard and white gray Show fig 1B; type II, compact, hard, wool swollen and white gray Show fig 1C; type III, granular, friable and yellow Show fig 1A. Dicamba in induce medium resulting type II callus.

Effect of silver nitrate on proliferation of callus

In order to proliferate callus from leaf and petiole, the effect of silver nitrate was investigated and the result show that callus looked better than that of controlled treatment. In the absent of silver nitrate, callus swollen without cell division (Fig. 1). The stimulatory effect of silver nitrate on callus proliferation was 1 mg/l. Different concentration of silver nitrate had influences on the type of callus (Table 2).

Effect of silver nitrate on callus growing

Silver nitrate play important role in enhancing callus growth. Concentration of the chemical at 0 to 1 mg/l containing in the MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l BA increased the growth rate of callus leading to the highest average fresh weight (Table 3).

Table 1. Effect of PGRs on callus formation from culturing various explants raised on MS medium for 4 weeks

Concentration of substance			Explants	Callus induction (mean %)	Type of callus
2,4-D (mg/l)	BA (mg/l)	di (mg/l)			
2.0	2.0	-	leaf	91.67[a]	I
			Petiole	94.44[a]	II
			stem	100[a]	II
			Seed integument	100[a]	III
			root	66.67[b]	I
-	-	2.0	leaf	80.00[ab]	II
			Petiole	0	
			stem	0	
			Seed integument	0	
			root	0	
-	-	2.5	leaf	80.00[ab]	II
			Petiole	0	
			stem	0	
			Seed integument	0	
			root	0	
-	-	3.0	leaf	66.67[b]	II
			Petiole	0	
			stem	0	
			Seed integument	0	
			root	0	
		C.V. (%)		12.67	

Mean values followed by the same letter(s) within a column are significantly different ($p < 0.05$)

Table 2. Effect of silver nitrate on callus formation containing MS medium together with 2 mg/l 2,4-D and 2 mg/l BA for 4 weeks

Silver nitrate (mg/l)	Explants	Callus induction (mean %)		Type of callus
		2 weeks	4 weeks	
0	leaf	0	92.88[a]	I
	Petiole	0	93.75[a]	II
1.00	leaf	20.00[a]	90.00[a]	III
	Petiole	27.78[a]	94.44[a]	III
C.V.(%)		23.25	11.33	

Mean values followed by the same letter(s) within a column are not significantly different ($p < 0.05$)

Table 3. Effect of silver nitrate containing MS medium together with 2 mg/l 2,4-D and 2 mg/l BA for 4 weeks on callus proliferation in term of callus fresh weight

Silver nitrate (mg/l)	Callus (fresh weight;mg±SD)
0	0.3443 ± 0.0904^{b}
0.10	0.3537 ± 0.1877^{b}
0.25	0.4142 ± 0.1602^{ab}
0.50	0.4240 ± 0.1751^{ab}
1.00	0.6127 ± 0.2791^{a}
C.V.(%)	43.85

Mean values followed by the same letter(s) within a column are significantly different ($p < 0.05$)

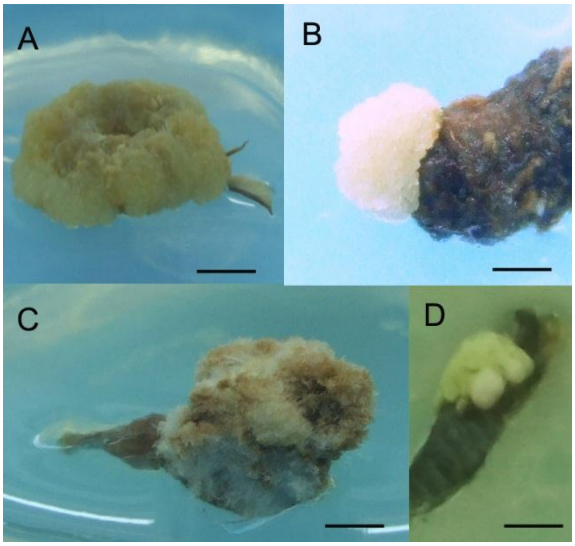

Fig. 1. Callus induced from rubber tree explants (A) Type III callus from leaf stalk in MS medium with silver nitrate at 1 mg/l (B) Type I callus from leaf stalk (C) Type II callus from leaf (D) Type I callus from leaf (Scale bars= 2 mm)

Discussion

Callus formation *H. brasiliensis* from different explants was affected by PGRs. 2,4-D gave an efficiency on callus induction in rubber tree, which has been reported by many researches (Jayasree *et al*., 1999; Kouassi *et al*., 2008; Sobha *et al*., 2003; Zhou *et al*., 2012). Different PGRs and explants have contrast on callus induction, high concentration and combination PGRs were used to investigate the influences on three type of callus formation in rubber tree (Zhou *et al*., 2010). As three kinds of explants make difference callus size and type but the best callus formation was obtained with low levels of PGRs in *Catalpa bungei* (Juan *et al*., 2010).

Browning is problem in *H. brasiliensis*, ethylene enhanced the activities of peroxidases and bound polyphenol oxidase, associated with the metabolism of phenolic products and tissue browning (Houst*iet al*., 1992). Action of silver nitrate in plant tissue culture is assumed to be associated with the physiological effects of ethylene, Inhibition of ethylene action by silver ions (Beyer, 1976). The positive effect of silver nitrateon plant tissue culture had been observed in many plants, whereas some cases have demonstrated the even negative effect (Zhang *et al*., 2001; Vikrant and Rashid, 2002; Feng*et al*., 2010; Parimalan*et al*., 2010; Cogbill*et al*., 2010). In the present study, the addition of silver nitrate in the induction medium at concentrations ranging from 1 to 3 mg/l was successful in callus formation in all the explants tested, Similar results were also reported in coffea (Silva *et al*., 2011) wheat (Wu *et al*., 2006) canola (Ali *et al*., 2007) maize (Rakshit *et al*., 2010) zinnia (Anantasaran and Kanchanapoom, 2008). The type III callus was induced leaf and petiole by using sliver nitrate, It gave highest grow rate. In addition, silver nitrate provides silver ions which Inhibit enzyme related in ethylene production, thus increased growth rate of callus (Fei *et al*., 2000), and increased frequency of embryogenesis as well (Zhang *et al*., 2001; Huang and Wei, 2004). Thus, the present study suggests that it is possible to improve the frequency of callus induction and its maintenance in *H. brasiliensis*.

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