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The effect of plant growth regulator and *in vitro* conservation of teak (*Tectona grandis* L.) by tissue culture

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Abstract The plant regeneration of teak (*Tectona grandis* L.) was reported. Result showed that the highest number of shoot induction was 55.49 cm., in length of shoots which tested at 0.25 mg L^{-1} BAP, and averaged 40.29 cm which tested at 0.25 mg L^{-1} *m*T after 4 weeks. The shoots were elongated using shoot tip explants of cryopreservation on teak by encapsulation-dehydration (EnDe), encapsulation-vitrification (EnVi) and vitrification (Vi) techniques. The shoot tips were used as explants, encapsulated apices were precultured on hormone-free MS medium containing 0.3 M sucrose for 2 days, dehydrated, and immersed in liquid nitrogen for 24 h after that thawed in thermal bath at 40 ℃ and washed in unloading solution. Vi and EnVi protocols were used to treat with a loading solution, dehydrated by plant vitrification solution 2 (PVS2). EnDe protocol were encapsulated by desiccated air-dry in laminar air flow and dehydrated with silica gel. Results showed that Vi gave an excellent shoot tip explants for survival and regrowth. Vi were unable to survival. This experiment was preliminary searched to improve and develop the teak for cryopreservation.

Keyword: Cryopreservation, Encapsulation-dehydration, Encapsulation-vitrification, Plant regeneration, Vitrification

Introduction

Teak (*Tectona grandis* L.) is a valuable timber of the family Verbenaceae. It is mostly found in India, Indonesia, Myanmar and Thailand. It is used for ship building, house building, furniture, door frames, bridges and carving. Teak used in this research is Syamindra teak in project at the Royal Chitralada Palace apart from teak conservation project. The reason was chosen Syamindra teak because it is plants in Plant Genetic Conservation Project Under The Royal Initiative. Teak a nearly extinct plant species making it a highly worth conserving plant. Normally, teak tree grown in grown in open environment and often a problem is phytopathy, such as leaf rust, powdery mildew, gall and root rat but also to poor

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dormancy seed which was serious problems for propagation of teak (Akram and Aftab, 2016). According to the problems mentioned above. So there develop protocol for rapid in vitro teak propagation use by tissue culture techniques and study cryopreservation to prevent species extinction of teak. Therefor the researcher has chosen to study the propagation of teak by tissue culture techniques to propagate of teak and it is conservation genetics of teak.

In this research studied plant growth regulators (PGRs) are naturally initiate substances and increase growth and development of a plant such as shoot or roots induction and plant cell division. PGRs that use for this research such as Thidiazuron (TDZ), benzyladenine (BAP) and *meta*-Topolin (*m*T) are a group of auxins and 2,4-Dichlorophenoxyacetic acid (2,4-D), α-Naphthaleneacetic acid (NAA) and Indole-3-butyric acid (IBA) are a group of cytokinin. There have been few studies on the efficient method of micropropagation of teak using nodal explants as an initial plant material and culture on MS medium for shoot induction (Emilio and Juwartina, 2007). Akram and Aftab (2008) studied the effect of TDZ for shoot induction and IBA for root induction using nodal explants of teak.

Cryopreservation is method for long-term storage of plant germplasm in liquid nitrogen (LN, -196 ℃) to avoid intracellular ice formation, which is the cause of fatal cell damage (Lambardi *et al.*, 2000). This is a long-term conservation of germplasm and the cost of maintain is lowest compared to other maintain methods (Engelmann, 2000). It also uses less storage space and sterilization (Musikapala, 2009). Explants commonly used *in vitro* conservation or cryopreservation are somatic embryos, shoot tips and seeds. Cryopreservation methods had chosen uses vitrification (Vi), encapsulation-vitrification (EnVi) and encapsulation-dehydration (EnDe) technique because It is an approved method and has many successful researches. Vitrification is an alternative approach to cryopreservation that enables hydrated living cells to be cooled to cryogenic temperatures in the absence of ice.

In this research faces limitations and the objective of research was to propagation of teak by tissue culture to propagate rapidly and It is conservation genetics of teak. The aim of cryopreservation study was to germplasm conservation for long-term storage of teak.

Materials and methods

Plant materials

Clean tissue culture of teak (*Tectona grandis* L.) received from Plant Genetic Conservation Project Under The Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG), Bangkok, Thailand (Fig. 1, A).

Culture condition and shoot regeneration

Teaks was cut into shoot tip and node explants with 0.5 - 1 cm in length and cultured on WPM medium containing 30 g L^{-1} sucrose and 2.6 g L^{-1} gellan gum and adjust pH of WPM medium was to 5.6 – 5.8 before autoclaving at 121 ℃ 15 min. WPM medium supplemented with TDZ, BAP and *m*T used 0.25, 0.5, 1 and 2 mg L^{-1} for shoot induction (Fig. 1, B) and incubated in culture room at 25 ± 2 ℃ under 16 h photoperiod (Table 1). The data were recorded for the number of shoots and length of shoots after 4 weeks.

In vitro grown stock plants

Shoots were cultured on WPM supplemented with 0.25 mg L^{-1} BAP and incubated in the culture room at 25 ± 2 ℃ under 16 h photoperiod for stock plants of teak.

Preculture

When shoot elongated, the shoot was cut into shoot tips were precultured on hormone-free MS medium containing 0.3 M sucrose and 8 g L^{-1} agar and adjust pH of MS medium was to 5.6 – 5.8 for 2 days at 25 ± 2 ℃ under 16 h photoperiod in the culture room.

Encapsulation-dehydration (EnDe)

Shoot tips were transferred to Ca-free MS medium containing 0.4 M sucrose and 3% Na-alginate followed by the mixture. A sterile pipette shoot tip was dropped into 0.1 M $CaCl_2$ solution containing 0.4 M sucrose, shaked and left for 30 min to form beads about 5 mm, each bead containing one shoot tip. The beads were placed on the petri dish and dehydrated by air-drying on a laminar air-flow and moisture absorbing silica gel (Fig. 1, C,D) at room temperature for 0, 4, 6 and 16 h to determine the optimal dehydration time (Table 2).

Encapsulation- vitrification (EnVi)

The beads were treated for 0, 20 and 40 min at room temperature with LS composed of MS medium containing 1 M glycerol and 0.8 M sucrose (pH 5.6 - 5.8). After that it was treated for 0, 20 and 40 min at 0 ℃ with PVS2 composed of MS medium containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulphoxide (DMSO) and 0.4 M sucrose (pH 5.6 - 5.8) in cryotubes to determine the optimal vitrification solution time (Fig. 1, E) (Table 3).

Vitrification (Vi)

Shoot tips were treated for 0, 20, 40, 60, 90 and 120 min at room temperature with LS composed of MS medium containing 1 M glycerol and 0.8 M sucrose (pH 5.6 - 5.8). After that were treated for 0, 20, 40, 60 and 90 min at 0 ℃ with PVS2 composed of MS medium containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulphoxide (DMSO) and 0.4 M sucrose (pH 5.6 - 5.8) in cryotubes to determine the optimal vitrification solution time (Table 4).

Long-term storage and thawing

After that, beads or shoot tips were immersed in liquid nitrogen (LN) -196 ℃ for 24 h (Fig. 1, F) after that thawed in the thermal bath at 40 ℃ for 2 min and washed for 20 min with unloading solution (US) composed of MS medium containing 1.2 M sucrose solution (pH 5.6 - 5.8).

Regrowth and data analysis

Beads and shoot tips were cultured on WPM supplemented with 0.25 mg L^{-1} BAP and incubated in the culture room at 25 ± 2 ℃ under 16 h photoperiod for shoot regeneration. The data were recorded for percentages of shoot-tip survival and regrowth after 4 weeks. Data were subjected to analysis of variance (ANOVA) by Duncan's test at $P \leq 0.05$ of statistically significant main effects.

Results

Shoot regeneration

Result indicated that shoot regeneration from node explants of teak on various PGRs after 4 weeks of initial culture. TDZ, BAP and *m*T as well as BAP at 0.25 mg L^{-1} (Fig. 2, G) showed the best result when compared to other for the highest number of shoots (10) and length of shoots (55.49 cm), followed by 10, 40.29 cm at 0.25 mg L^{-1} *m*T (Fig. 2, H) and 10, 35.09 cm at 0.5 mg L^{-1} TDZ, respectively (Fig. 2 I) of the number of shoots and length of shoots respectively. Hormone-free WPM medium (Fig. 2, J) was the best growth at 0.25 mg L^{-1} *m*T for length of shoots but lower in number of shoots (Table 1).

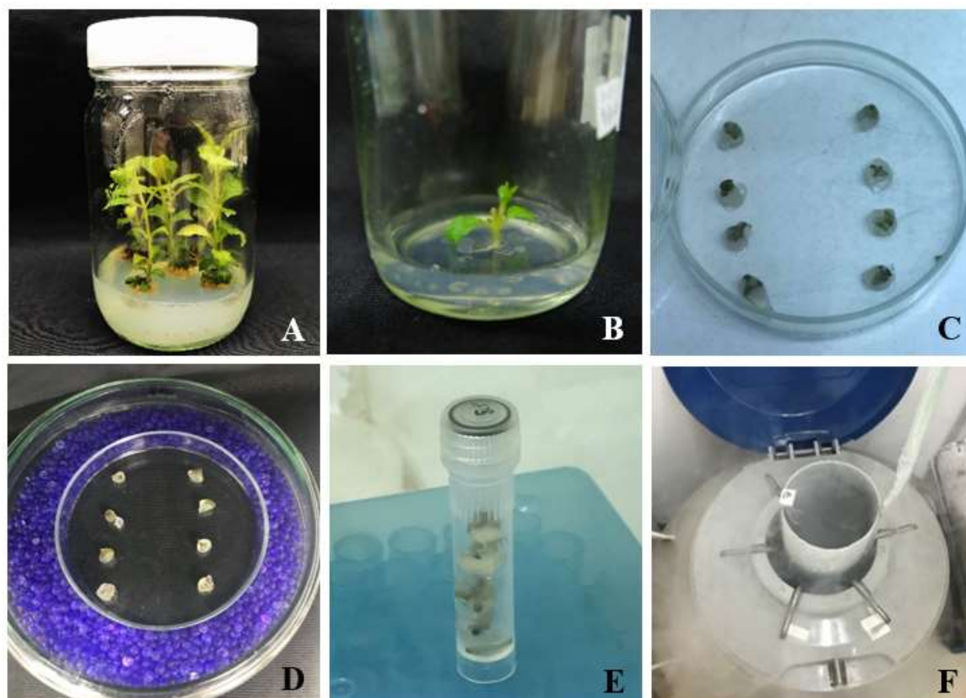

Figure 1. Stock clean tissue of teak (A). Cultured on WPM medium from node explants (B). Encapsulation- dehydrated by air-drying on a laminar air-flow (C). Encapsulation- moisture absorbing silica gel (D). Encapsulation- vitrification in cryotubes (E). Shoot tips were immersed in liquid nitrogen (LN) -196 ℃ for 24 h (F)

Encapsulation-dehydration (EnDe)

The rate of survival was highest of 100%, and 80% percent regrowth at D1 of control (LN-) after 4 weeks (Fig. 3K) but, (LN+) after 3 days of culture shoot tip green turned brown at D1, D2 and D5. LN+ were unable to survival (Table 2, Fig. 3L).

Encapsulation- vitrification (EnVi)

The highest (LN-) survival rate and regrowth rate were 100 % and 90 %, respectively at T1 after 4 weeks (Fig. 3 M). Nevertheless, the lowest survival rate (LN+) was cultured after 2 days, the shoot tip become green turned brown at T1 and T4 (Fig. 3N), but T6, T8 and T9 were turned from green to brown after 4 days. It showed more survive time than soaking in PVS2(Table 3).

Vitrification (Vi)

The maximum (LN-) percentage survival rate and percentage regrowth were 100 % and 100 %, respectively at T1, T2, T3, T7, T10 and T15 after 4 weeks (Fig. 3 O,=P). However, LN+ culture after 1 day, shoot tip turned from green to brown at T1, T2 and T7(Fig. 3Q). The highest survival rate was T22, T23 and T24 that shoot tip turned from green to brown after 7 days of culture (Table 4, Fig. 3R).

Table 1. Effect of various PGRs in WPM medium for shoot formation of teak after 4 weeks

WPM + PGRs (mg L^{-1})			Number of shoots[/1]	Length of shoots[/1,2] (cm)
TDZ	BAP	*m*T		
0	0	0	8	38.64[bc] ±3.83
0.25			8	10.84[g] ±1.26
0.5			10	35.09[bcd] ±0.86
1			9	30.15[def] ±1.39
2			8	24.80[f] ±2.27
	0.25		10	55.49[a] ±1.59
	0.5		9	31.04[def] ±1.55
	1		10	28.74[def] ±0.86
	2		9	26.14[ef] ±2.15
		0.25	10	40.29[b] ±1.25
		0.5	9	33.01[cde] ±2.95
		1	9	34.59[bcd] ±3.49
		2	8	31.28[def] ±2.45

[1]/Each value is a mean of 10 explants of three independent experiments.
[2]/Each value represents the Mean ± SE; mean values followed by different letters are significantly different according to Duncan's test at $P < 0.05$.

Discussion

The optimum response of shoot induction and length of shoots was from shoot tips and node explants of teak on WPM medium supplemented with 0.25 mg L^{-1} BAP was in the same manner with Tiwari *et al.* (2002) who reported that node explants cultured on MS medium supplemented with 22.2 µM BAP gave maximum culture establishment (76.8%).

Widiyanto *et al.* (2005) reported that the best medium for shoot formation was MS medium with 10 µM BAP.

Table 2. Effects of dehydration method with survival rate and regrowth of shoot tip in beads of teak by encapsulation-dehydration (EnDe)

	Dehydration method		**LN-[1]**		**LN+[2]**	
	silica gel (h)	laminar air-flow (h)	Survival rate (%)	Regrowth (%)	Survival rate (%)	Regrowth (%)
D1	0	0	100	80	0	0
D2	4	-	50	20	0	0
D3	6	-	0	0	0	0
D4	16	-	0	0	0	0
D5	-	4	50	20	0	0
D6	-	6	0	0	0	0
D7	-	16	0	0	0	0

[1]/ LN- = without liquid nitrogen
[2]/ LN+ = included with liquid nitrogen
Note: Data were collected after 4 weeks of initial culture.

Table 3. Effects of time duration of treated LS and PVS2 with survival rate and regrowth of shoot tip in beads of teak by encapsulation- vitrification (EnVi)

	Treatment		**LN-[1]**		**LN+[2]**	
	LS (mins)	PVS2 (mins)	Survival rate (%)	Regrowth (%)	Survival rate (%)	Regrowth (%)
T1	0	0	100	90	0	0
T2	0	20	70	50	0	0
T3	0	40	50	30	0	0
T4	20	0	90	60	0	0
T5	20	20	40	20	0	0
T6	20	40	20	10	0	0
T7	40	0	80	50	0	0
T8	40	20	10	0	0	0
T9	40	40	10	0	0	0

[1]/ LN- = without liquid nitrogen
[2]/ LN+ = included with liquid nitrogen
Note: Data were collected after 4 weeks of initial culture.

Table 4. Effects of time duration of treated LS and PVS2 with survival rate and regrowth of shoot tip of teak by vitrification (Vi)

	Treatment		**LN-[1]**		**LN+[2]**	
	LS (mins)	PVS2 (mins)	Survival rate (%)	Regrowth (%)	Survival rate (%)	Regrowth (%)
T1	0	0	100	100	0	0
T2	0	20	100	100	0	0
T3	0	40	100	100	0	0
T4	20	0	100	90	0	0
T5	20	20	100	80	0	0
T6	20	40	100	90	0	0
T7	40	0	100	100	0	0
T8	40	20	100	80	0	0
T9	40	40	100	70	0	0
T10	60	0	100	100	0	0
T11	60	20	80	60	0	0
T12	60	40	80	50	0	0
T13	60	60	70	40	0	0
T14	60	90	70	50	0	0
T15	90	0	100	100	0	0
T16	90	20	80	50	0	0
T17	90	40	70	40	0	0
T18	90	60	70	40	0	0
T19	90	90	60	30	0	0
T20	120	0	90	60	0	0
T21	120	20	60	30	0	0
T22	120	40	60	50	0	0
T23	120	60	50	20	0	0
T24	120	90	40	10	0	0

[1]/ LN- = without liquid nitrogen
[2]/ LN+ = included with liquid nitrogen
Note: Data were collected after 4 weeks of initial culture.

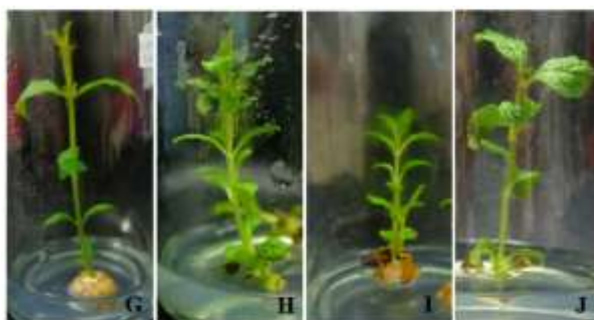

Figure 2. Effect of various PGRs in WPM medium for shoot regeneration from node explants after 4 weeks. (G) WPM supplemented with 0.25 mg L^{-1} BAP; (H) WPM supplemented with 0.25 mg L^{-1} *m*T; (I) WPM supplemented with 0.5 mg L^{-1} TDZ; (J) Hormone-free WPM medium

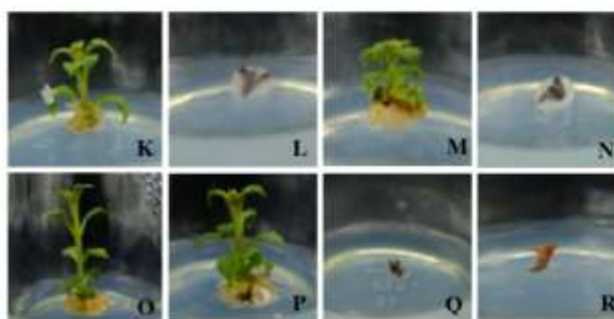

Figure 3. Effect of cryopreservation on WPM supplemented with 0.25 mg L^{-1} BAP. (K) EnDe (LN-) at D1 after 4 weeks of culture; (L) EnDe (LN+) at D1 after 3 days of culture; (M) EnVi (LN-) at T1 after 4 weeks of culture; (N) EnVi (LN+) at T1 after 2 days of culture; (O) Vi (LN-) at T1 after 4 weeks of culture; (P) Vi (LN-) at T7 after 4 weeks of culture; (Q) Vi (LN+) at T1 after 1 day of culture; (R) Vi (LN+) at T24 after 7 days of culture

Cryopreservation techniques support in the long-term storage and conservation of tropical hardwood tree species throughout the world. Cryopreservation, the low temperature (-196 ℃) storage of cells, tissue, embryos, or seeds, is a system that can be considered as a secure maintaining to living collections (Benson, 2008). Cryopreservation has generally three standard protocols in plant cryopreservation that is controlled-rate cooling, vitrification, and encapsulation-dehydration (Reed, 2008). In this research cryopreservation study was to germplasm conservation in liquid nitrogen by three methods; vitrification, encapsulation-vitrification and encapsulation-dehydration. The results showed that vitrification, shoot tip turned from green to brown slower than other method. Similar result is reported in *Rubus* vitrification which was successful with the highest mean regrowth of 71% (Gupta and Reed, 2006). In summary, the shoot tips and node explants resulted in superior starting

materials for highly shoot regeneration with BAP. Cryopreservation in the development stage, vitrification is tended to success in the future.

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