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The effects of abscisic acid and sorbitol on the slow growth *in vitro* preservation of Mahesak (*Tectona grandis* L.)

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Abstract The conservation of teak (*Tectona grandis* L.) by slow growth was proceeded out to determine appropriate methods *in vitro* preservation using osmotically active compounds and growth retardants. Calli and node explants were cultured on Murashige and Skoog (MS) and Woody Plant Medium (WPM) media supplemented with 1, 2 and 3 mg·L^{-1} abscisic acid (ABA) containing 30 g·L^{-1} sucrose and hormone-free MS and WPM media containing 0.1, 0.2 and 0.3 M sorbitol for reduced the growth rate. Result showed that preservation of calli on MS and WPM media supplemented with 0.2 and 0.3 M sorbitol and node explants were cultured on WPM medium supplemented with 0.2 and 0.3 M sorbitol inhibited the growth and maintained the quality of calli and node explants for 12 weeks. It was found that ABA could not inhibit the growth rate after cultures for 12 weeks. Callus and node explant survival from slow growth preservation were able to regrowth which successfully (100%) tested on WPM medium supplemented with 0.25 mg·L^{-1} 6-benzylaminopurine (BAP) containing 30 g·L^{-1} sucrose after for 2 weeks.

Keywords: Growth retardants, Mahesak (Teak), Preservation, Slow growth, *Tectona grandis* L.

Introduction

Teak (*Tectona grandis* L.) is a tropical forest tree belonging to the family Verbenaceae. It is found in Southeast Asian countries - Indonesia, Laos, Myanmar and Thailand. In Thailand, it is mostly found in Chiang Rai, Chiang Mai, Phayao, Nan, Uttaradit and Nakhon Sawan Provinces. Teak is a valuable timber (Palanisamy *et al*., 2009). The wood is strong and tough and is therefore used for shipbuilding, houses, furniture, bridges and carving (MedThai, 2017). Different parts of teak can be used for medicine, for example, the leaves can be used as a blood tonic, the wood to treat skin conditions and rinds of the fruit are used as headache relief (RSPG, 2014). Generally, teak trees grow in an open environment and often show phytopathy in leaf rust, powdery mildew, gall and root rot (Phongpanich, 2012) and are also affected by insects, e.g. teak beehole borer, teak defoliator, teak canker grub and yellow banded blister beetle (Hutacharern and Eungwijanpanya, 1996). They also show poor dormancy seeds - a serious

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problem for propagation of teak (Akram and Aftab, 2016). Therefore, we study teak propagation by tissue culture techniques, enhance conservation and prevent species extinction. We use teak cv. Mahesak from project at the Royal Chitralada Palace apart from teak conservation project. Mahesak is one of the plants in the Plant Genetic Conservation Project Under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG).

In this research studied the role of abscisic acid (ABA) and sorbitol for slow growth. Slow growth preservation is one of the most common practices for reduced the growth rate of *in vitro* plant cultures, can provide a convenient option for short-term to medium-term germplasm storage (Withers, 1991). A variety of method has been used in combination to reduce the growth rates of *in vitro* plant tissue. Modifications to the culture medium and addition of osmotically active compounds such as sorbitol and using plant growth retardants such as ABA (Jarret and Gawel, 1991) for reduced the growth rate.

The objective of the research was to reduce the growth rate and increases the time between subcultures of teak by slow growth and conservation genetics of teak.

Materials and methods

Plant material

Mahesak (*Tectona grandis* L.) received assistance and cooperation from the RSPG project, Bangkok, Thailand in January 2018. All plants were kept in tissue culture bottle and taken to do experiments at the laboratory, Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

Culture condition

Mahesak explants were cultured on MS (Murashige and Skoog,1962) and WPM (Woody Plant Medium, 1981) medium containing 2.6 g L^{-1} gellan gum (Phyto Technology Laboratories). The medium pH was maintained in the 5.6 - 5.8 range, before autoclaving at 121 ℃ for 15 min and incubated in the culture room at 25 ± 2 ℃ under a 16 h photoperiod.

Shoot and callus induction

Mahesak shoots were used to prepare shoot tips and node explants, 1 cm long, and cultured on WPM medium (Phyto Technology Laboratories) supplemented with 0.25 mg L^{-1} BAP (Phyto Technology Laboratories) containing 30 g L^{-1} sucrose (Mitr Phol Sugar Corp., Ltd.) for shoot and callus induction.

Slow growth preservation

Calli and shoots obtained from the shoot and callus induction experiments were used for the slow growth experiments. Calli were cut into small pieces, $0.3 \times 0.3 \times 0.3$ cm^3, and shoots were used to prepare node explants 1 cm in length. Calli and node explants were cultured on MS (Phyto Technology Laboratories) and WPM media supplemented with 1, 2 and 3 mg L^{-1} ABA (Sigma Chemical Co.) containing 30 g L^{-1} sucrose. Calli and node explants were cultured on hormone-free MS and WPM media containing 0.1, 0.2 and 0.3 M sorbitol (Sigma Chemical Co.) for slow growth. Cultures were stored in the culture room at 25 ± 2 ℃ under a 16 h photoperiod for 12 weeks after initial culture. The data was recorded for the length of shoot and callus volumes - derived from the maximum sizes of the measure of the callus (width × length × height).

Regrowth

After 12 weeks of culture, calli and node explants were transferred to WPM medium supplemented with 0.25 mg L^{-1} BAP and incubated in the culture room at 25 ± 2 ℃ under a 16 h photoperiod for callus proliferation and shoot regeneration. Percentages of callus and node explant regrowth were recorded after culture for 2 weeks.

Data recording and statistical analysis

Cultures were examined regularly and after 4, 8 and 12 weeks of culturing data were recorded for the length of shoot (cm) and callus volumes (cm^3). Experimental values are given as mean, each value was a mean of 3 explants of three independent experiments. After that, data were analyzed using ANOVA, with Duncan's test at $p \leq 0.05$ for statistically significant effects, using IBM SPSS Statistics 25.

Results

Slow growth treatments

Slow growth preservation from calli and node explants was assessed in 14 treatments with various media (Table 1 and 2). Calli were cultured for 4 weeks for all treatments; the calli were green and survived. After 8 weeks, the green calli turned yellowish brown on MS and WPM media containing 30 g L^{-1} sucrose. After 12 weeks, the calli turned brown on MS and WPM media containing 30 g L^{-1} sucrose and MS and WPM media containing 0.1 M sorbitol. Calli were cultured on MS and WPM media containing 0.2 and 0.3 M sorbitol had lower mean size of callus more than others concentration,

able to inhibit growth and maintain quality callus for up to 12 weeks; calli were yellowish green (Figure 1 and 2F-G). Node explants were cultured for 4 weeks for all treatments; the node explants were green and survived. After 8 weeks, the green node explants turned brown on MS medium containing 0.1 and 0.2 M sorbitol. After 12 weeks, the green node explants turned brown in some parts on MS and WPM media containing 30 g Ł$^{-1}$ sucrose, MS medium containing 0.3 M sorbitol (Figure 3) and WPM medium containing 0.1 M sorbitol. Node explants were cultured on WPM medium containing 0.2 and 0.3 M sorbitol had lower mean length of shoot more than others concentration, able to inhibit growth and maintain quality node explant for up to 12 weeks; node explants were green (Figure 4F-G).

Table 1. Slow growth preservation from callus: different concentrations of ABA and sorbitol in MS and WPM media

Treatments	Mean callus volume[1,2] (cm³)			Color of 12 weeks
	4 weeks	8 weeks	12 weeks	
MS+30 g Ł$^{-1}$ sucrose	$0.05^{e} \pm 0.01$	$0.06^{e} \pm 0.01$	$0.06^{d} \pm 0.01$	brown
MS+1 mg Ł$^{-1}$ ABA +30 g Ł$^{-1}$ sucrose	$3.30^{a} \pm 0.78$	$5.30^{a} \pm 0.96$	$5.93^{a} \pm 0.89$	green
MS+2 mg Ł$^{-1}$ ABA +30 g Ł$^{-1}$ sucrose	$1.65^{bc} \pm 0.63$	$3.43^{bc} \pm 0.55$	$3.72^{bc} \pm 0.59$	green
MS+3 mg Ł$^{-1}$ ABA +30 g Ł$^{-1}$ sucrose	$1.12^{cde} \pm 0.59$	$1.78^{cd} \pm 0.85$	$2.18^{c} \pm 1.07$	green
MS+0.1 M sorbitol	$0.15^{de} \pm 0.01$	$0.15^{e} \pm 0.01$	$0.15^{d} \pm 0.01$	brown
MS+0.2 M sorbitol	$0.12^{de} \pm 0.02$	$0.12^{e} \pm 0.02$	$0.12^{d} \pm 0.02$	yellowish
MS+0.3 M sorbitol	$0.11^{de} \pm 0.01$	$0.11^{e} \pm 0.01$	$0.11^{d} \pm 0.01$	yellowish
WPM+30 g Ł$^{-1}$ sucrose	$0.12^{de} \pm 0.00$	$0.15^{e} \pm 0.03$	$0.15^{d} \pm 0.03$	brown
WPM+1 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose	$2.57^{ab} \pm 0.30$	$3.99^{ab} \pm 0.45$	$4.22^{b} \pm 0.43$	yellowish
WPM+2 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose	$1.72^{bc} \pm 0.75$	$2.22^{c} \pm 0.99$	$2.39^{c} \pm 1.08$	green
WPM+3 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose	$1.50^{bcd} \pm 0.68$	$1.97^{c} \pm 0.92$	$2.08^{c} \pm 0.96$	yellowish
WPM+0.1 M sorbitol	$0.10^{de} \pm 0.02$	$0.10^{e} \pm 0.02$	$0.10^{d} \pm 0.07$	brown
WPM+0.2 M sorbitol	$0.13^{de} \pm 0.01$	$0.13^{e} \pm 0.01$	$0.13^{d} \pm 0.02$	yellowish
WPM+0.3 M sorbitol	$0.07^{e} \pm 0.01$	$0.07^{e} \pm 0.01$	$0.07^{d} \pm 0.02$	yellowish

[1]/Each value was a mean of 3 explants of three independent experiments;
[2]/Each value represents the mean ± SD; means identified with different letters were significantly different by Duncan's test ($p \leq 0.05$).

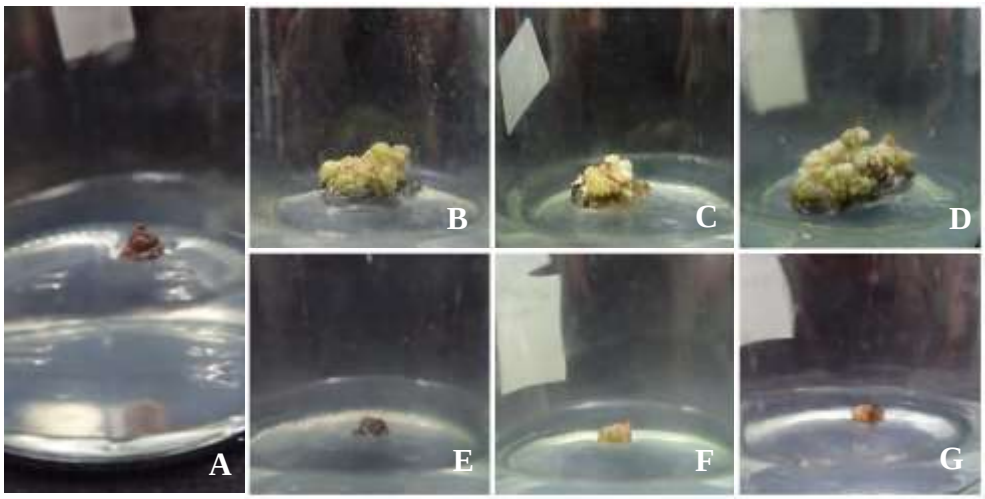

Figure 1. Effect of various ABA and sorbitol on MS medium for slow growth preservation from callus after cultures for 12 weeks: (A) MS+30 g Ł$^{-1}$ sucrose; (B) MS+1 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (C) MS+2 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (D) MS+3 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (E) MS+0.1 M sorbitol; (F) MS+0.2 M sorbitol; (G) MS+0.3 M sorbitol

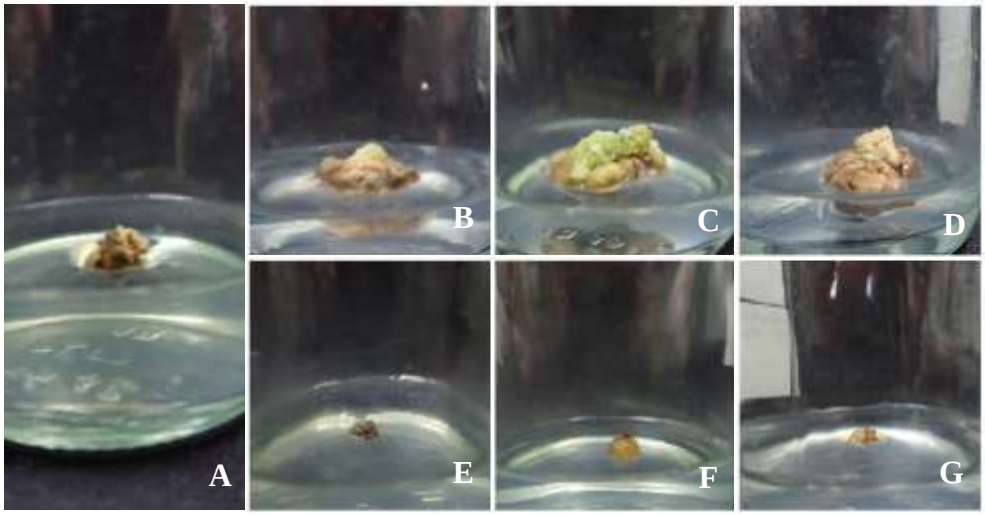

Figure 2. Effect of various ABA and sorbitol on WPM medium for slow growth preservation from callus after cultures for 12 weeks: (A) WPM+30 g Ł$^{-1}$ sucrose; (B) WPM+1 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (C) WPM+2 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (D) WPM +3 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (E) WPM+0.1 M sorbitol; (F) WPM+0.2 M sorbitol; (G) WPM+0.3 M sorbitol

Table 2. Slow growth preservation from node explant: different concentrations of ABA and sorbitol in MS and WPM media

Treatments	Mean length of shoot[1,2] (cm)			Color of 12 weeks
	4 weeks	8 weeks	12 weeks	
MS+30 g Ł$^{-1}$ sucrose	1.12^{ab} ±0.28	1.53^{a} ±0.39	1.53^{ab} ±0.39	brownish
MS+1 mg Ł$^{-1}$ ABA +30 g Ł$^{-1}$ sucrose	1.08^{ab} ±0.38	1.31^{ab} ±0.36	1.45^{abc} ±0.40	green
MS+2 mg Ł$^{-1}$ ABA +30 g Ł$^{-1}$ sucrose	0.63^{bcd} ±0.08	0.76^{bcd} ±0.12	0.86^{bcde} ±0.16	green
MS+3 mg Ł$^{-1}$ ABA +30 g Ł$^{-1}$ sucrose	0.75^{bcd} ±0.28	1.06^{abc} ±0.43	0.90^{bcd} ±0.42	green
MS+0.1 M sorbitol	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	brown
MS+0.2 M sorbitol	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	brown
MS+0.3 M sorbitol	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	brown
WPM+30 g Ł$^{-1}$ sucrose	1.29^{a} ±0.11	1.57^{a} ±0.10	1.61^{ab} ±0.12	brown
WPM+1 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose	0.96^{abc} ±0.10	1.24^{ab} ±0.09	1.92^{a} ±0.58	green
WPM+2 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose	0.52^{cde} ±0.08	0.82^{bcd} ±0.18	1.12^{abcd} ±0.26	green
WPM+3 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose	0.42^{de} ±0.06	0.52^{cde} ±0.05	0.64^{cde} ±0.07	green
WPM+0.1 M sorbitol	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	brown
WPM+0.2 M sorbitol	0.13^{e} ±0.06	0.25^{de} ±0.12	0.29^{de} ±0.14	green
WPM+0.3 M sorbitol	0.13^{e} ±0.08	0.28^{de} ±0.14	0.31^{de} ±0.15	green

Note: results were only recorded for new part proliferation;
[1]/Each value was a mean of 3 explants of three independent experiments;
[2]/Each value represents the mean ± SD; means identified with different letters were significantly different by Duncan's test ($p \leq 0.05$).

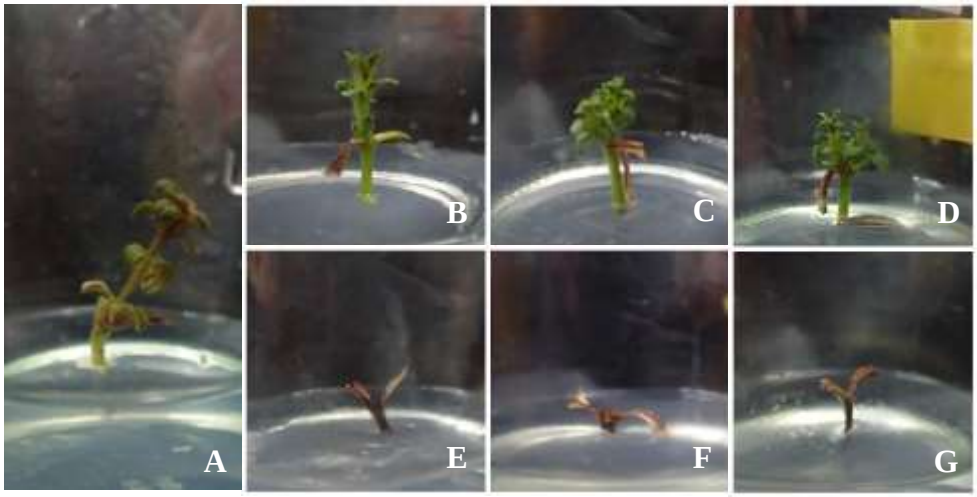

Figure 3. Effect of various ABA and sorbitol on MS medium for slow growth preservation from node explant after cultures for 12 weeks: (A) MS+30 g Ł$^{-1}$ sucrose; (B) MS+1 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (C) MS+2 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (D) MS+3 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (E) MS+0.1 M sorbitol; (F) MS+0.2 M sorbitol; (G) MS+0.3 M sorbitol

Figure 4. Effect of various ABA and sorbitol on WPM medium for slow growth preservation from node explant after cultures for 12 weeks: (A) WPM +30 g Ł$^{-1}$ sucrose; (B) WPM+1 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (C) WPM+2 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (D) WPM+3 mg Ł$^{-1}$ ABA+30 g Ł$^{-1}$ sucrose; (E) WPM +0.1 M sorbitol; (F) WPM +0.2 M sorbitol; (G) WPM +0.3 M sorbitol

Regrowth

Callus and node explants from slow growth experiment were transferred to WPM medium supplemented with 0.25 mg Ł$^{-1}$ BAP for regrowth, 100% regrowth was observed (Figure 5).

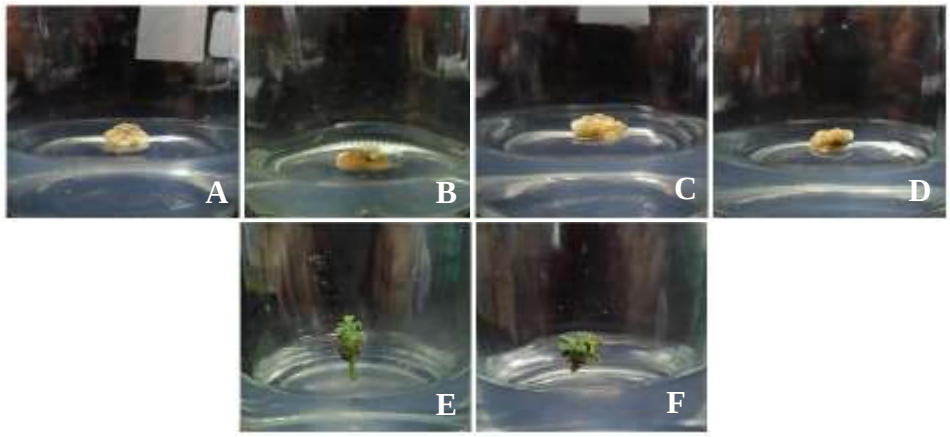

Figure 5. Effect of regrowth after cultures for 2 weeks on WPM medium supplemented with 0.25 mg Ł$^{-1}$ BAP transferred from the best treatments of slow growth preservation: (A) callus on MS+0.2 M sorbitol (B) callus on MS+0.3 M sorbitol (C) callus on WPM+0.2 M sorbitol (D) callus on WPM+0.3 M sorbitol (E) node explant on WPM +0.2 M sorbitol (F) node explant on WPM+0.3 M sorbitol

Discussion

The efficacy of slow growth preservation used growth retardants such as ABA and osmotically active compounds such as sorbitol and sucrose for reduced the growth rate of callus and node explant. The optimum results for calli were achieved with MS and WPM media supplemented with 0.2 and 0.3 M sorbitol. For node explants, we were achieved with WPM medium supplemented with 0.2 and 0.3 M sorbitol in culture room at 25 ± 2 ℃ under 16 h photoperiod for 12 weeks. Moges *et al*. (2003) reported that slow growth *in vitro* preservation of African violet microshoots cultured on hormone-free MS medium containing 0.16 M mannitol and sorbitol under 16 h photoperiod; they were able to decrease shoot growth and maintain quality explants for up to 12 weeks. Sharaf *et al.* (2012) reported slow growth *in vitro* preservation of shih (*Artemisia herba-alba*) microshoots cultured on medium containing 0.1, 0.2 and 0.3 M sucrose, mannitol and sorbitol in 16 h photoperiods at 25 ℃ and were able to inhibit growth and maintain quality explants for up to 12 weeks. The explants were able to slow growth preservation longer by increase the concentration of osmotically active compounds. Gopal *et al*. (2002) reported the maximum of potato microplant survival (55.5-77.8%) after 12 months of preservation was on MS medium containing 20 g L^{-1} sucrose supplemented with 40 g L^{-1} sorbitol. In the same with Gopal and Chauhan (2010) reported that the maximum of potato microplant survival rate (58%) were achieved with MS medium containing 20 g L^{-1} sucrose plus 40 g L^{-1} sorbitol after 18 months without subcultures.

In the study of slow growth preservation, ABA could not inhibit the growth rate. In contrast of Saab *et al*. (1990) mentioned that ABA accumulation has roles in the maintenance of root elongation and inhibition of shoot elongation. In many researchers reported that ABA was able to inhibit growth and maintain quality explants such as Sharaf *et al*. (2012) observed that slow growth *in vitro* preservation of wild shih (*Artemisia herba-alba*) microshoots cultured on medium supplemented with 0.5 mg L^{-1} ABA, able to reduced growth and maintained achieve survival and complete regrowth for up to 12 weeks. Jarret and Gawel (1991) reported that 10 mg L^{-1} ABA were inhibited axillary shoot development and did not affect viability of cv. Jewel explants after 365 days of culture.

Slow growth preservation can be increase period times between each subculturing. But, preservation has large problematic of *in vitro* micropropagation. There was also continued concern about the level of somaclonal variation under slow growth conditions (Jarret and Gawel, 1991). However, preservation of Cassava under slow growth conditions for up to 10 years did not affect genetically stable (Angel *et al.*, 1996). In summary, sorbitol was excellent osmotically active compounds for slow growth preservation at 0.2

and 0.3 M sorbitol. Calli and node explants were able to regrowth successfully on WPM medium supplemented with 0.25 mg L^{-1} BAP.

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