International Journal of Agricultural Technology 2018 Vol. 14(6): 911-922
Available online http://www.ijat-aatsea.com
ISSN: 2630-0613 (Print) 2630-0192 (Online)

Efficiency of Antioxidant and Absorbent on Browning and The Optimal Factors of Plant Regeneration from Young Seed of *Gluta usitata* (217 Mae Ka) by Tissue Culture

Rakrawee, R.[1], Kittibanpacha, K.[2], Chareonsap, P. P.[3] and Poeaim, A.[1*]

[1]Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand; [2]Silvicultural Research Group, Forest Research and Development Bureau, Royal Forest Department, Bangkok 10900, Thailand; [3]Plant Genetic Conservation Project, Chitralada Villa, Dusit, Bangkok 10303, Thailand.

Rakrawee, R., Kittibanpacha, K., Chareonsap, P. and Poeaim, A. (2018). Efficiency of antioxidant and absorbent on browning and the optimal factors of plant regeneration from young seed of *Gluta usitata* (217 Mae Ka) by tissue culture. International Journal of Agricultural Technology 14(6):911-922.

Abstract *Gluta usitata* (Anacardiaceae) is a lacquer tree and create lacquer varnish can be utilized in many aspects especially art and culture. Recently, its harvest faced troublesome and yields of varnish tree are decreased. Consequently, the conservation of plant has reduced, and this plant has become extinct. The experiment was chosen to use plant tissue culture technique to preserve and increase this plant. The experiment was compared the effect of Woody Plant Medium (WPM) combined with different concentration of antioxidants were 2, 4, 6, 8 g/L Poly Vinyl Pyrrolidone (PVP), 20, 30, 40, 60 mg/L ascorbic acid or citric acid and absorbents of 1, 2, 3 g/L activated charcoal to reduce browning of phenolic compound in the explants. The results showed that 2 g/L activated charcoal gave the lowest of browning on seed and medium (about 0.9±0.2 points). The addition of 40 mg/L ascorbic acid was generated the highest of shoots (approximately 1.88±0.4 shoots/seed) after culture for 4 weeks. *in vitro* propagation of seed germination on WPM supplemented with 0.5, 1, 1.5, 2 and 3 mg/L 6-Benzyl amino purine (BAP), Thidiazuron (TDZ) and *meta*-Topolin (*m*T) 3% sucrose and 0.2% phytagel was efficiently resulted. Other result revealed that 1.5 mg/L BAP, 2 mg/L TDZ and 2 mg/L *m*T gave the highest seed germination at 1.6±0.2, 1.8±0.2 and 1.6±0.2 shoots/seed, respectively. The best of plant growth regulators for seed germination was 2 mg/L of TDZ after 6 weeks.

Keywords: Absorbent, Antioxidant, Browning, *Gluta usitata* (217 Mae Ka), Plant regeneration

Introduction

Lacquer varnish is the natural lacquer from varnish tree, which is a member of the mango family (Anacardiceae). there are four species including *Rhus verniciflua*, *Rhus succedanea*, *Gluta usitata* and *Gluta laccifera*. Lacquer varnish is very important for art and culture (Tangmitcharoen *et al*., 2015).

* **Corresponding Author**: Poeaim, A.; **Email**: anurug@hotmail.com

However, *Gluta usitata* is the main source of lacquer varnish in Thailand because it can create high quality and quantity of lacquer (Fine Arts Department., 2008).

Gluta usitata, also known as Burmese lacquer (Zipcodezoo, 2014), is a medium to large tree from mixed forest (Wiriyaudom *et al*., 2012). This species is used in Burma and Northern Thailand as a source of lacquer used for producing lacquer varnish or preservative coating and gilding (Tangmitcharoen *et al*., 2015). Recently, its harvest has been troublesome and yield of Burmese lacquer has decrease due to the deforestation and cultivation in National park or Wildlife sanctuary. Moreover, lacquer varnish has caused dermatitis, skin irritation and synthetic lacquer has replaced lacquer varnish. Consequently, the conservation of plant has reduced and this plant is becoming extinction (Tabtong, 2012; Wiriyaudom *et al*., 2012).

The vegetative propagation of Burmese lacquer can be done by various method. However, these methods caused lacquer varnish getting out from incision and blocking up xylem and phloem flow making the explant die (Keawnapa *et al*., 2015). Furthermore, low seed variability and poor germination frequency restriction the propagation of Burmese lacquer cause seed dormancy to germinate are possibly associated with seed coat because seed cannot absorb water and gaseous exchange for respiration. *In vitro* technique is useful propagation of desirable trees for planting out, research (Sansberror *et al*., 2003) and increases for rapid mass multiplication of plant (Tripathi and Kurami., 2010). Moreover, it also reduces phenolic compound. This method has been reported in some other members of Anacardiceae including *Anacardium occidentale* (Benmahioul *et al*., 2009; Boggetti *et al*., 2001*), Mangifera indica* (Krishna *et al*., 2008), *Pistacia vera* (Onay *et al*., 1996; Tilkat *et al*., 2009; Benmahioul *et al*., 2012) and *Semecarpus anacardium* (Panda and Hazra., 2009). However, there is very few on *in vitro* studies of *Gluta usitata.* In this research describes the efficiently of antioxidants and absorbents to reduce browning and the optimal factors of seed germination of *Gluta usitata* (217 Mea Ka) by tissue culture. The result of this study could be useful to develop an efficient and reproducible method for stimulating *in vitro* multiplication and large-scale propagation of this plant.

Materials and methods

Plant material

Young seeds of *Gluta usitata* (217 Mae Ka) were collected during March from trees growing naturally in Government Organization Ban Huat, Lampang, Thailand. These were surface disinfested in 70% (v/v) ethanol and placed on a

shaker at 230 rpm. Then, dipped the seed into 95%(v/v) ethanol and fire burned 2 times (Pianhanuruk., 2007). After that, cut off the seed coat and cut half of seed for treat on experiments were effect of antioxidants and absorbents and effect of Plant Growth Regulators (PGRs).

Effect of antioxidants and absorbents

The *Gluta usitata* seed treated with WPM medium (Woody Plant Medium, 1981) combined with different concentration of absorbents were 1, 2, 3 g/L Activated charcoal or antioxidants were 2, 4, 6, 8 g/L Polyvinyl Pyrroridone (PVP), 20, 30, 40, 60 mg/L Ascorbic acid or Citric acid. Observe the characteristic and gave the score of browning on seed and culture medium were as follws:- Score 0, no browning on seed and culture medium, Score 1, found that browning on seed exposed into culture medium was little bit, Score 2, found that 50% of browning on seed and distributed in a yellowish brown on culture medium, Score 3, found that browning on seed and culture medium. The growth was recorded by counting the number of shoot, root and measuring the length of shoot using Vernier Caliper after cultured for 4 weeks.

Effect of Plant Growth Regulators (PGRs)

After experiment of antioxidants and absorbents that gave the best of phenolic compounds inhibition for combined in medium to reduce phenolic compound was used. The seeds treated with WPM medium supplemented with 2 g/L activated charcoal and 0.5, 1, 1.5, 2 and 3 mg/L of BAP (6-Benzylaminopurine), TDZ (Thidiazuron), *m*T (*meta*-Topolin) or without Plant Growth Regulators (PGRs). The growth by counting the number of shoot, root and measuring the length of shoot after cultured for 6 weeks wewre obsereved and recorded. The pH of medium was adjusted to 5.8 before sterilization and autoclaved at 121°C for 15 minutes. The culture medium was supplemented with 2% sucrose and 0.26% phytagel and incubated in 16 h photoperiod at 25±2 °C.

Data analysis

All experiments were repeated 2 times with 10 replicates in each repeated. The data were analysed statistically using one-way analysis of variance and significant differences between means were assessed by Duncan's multiple range test at 5% probability. The results are expressed as mean ± standard error (SE).

Results

Poor germination rate, slow growth, browning or blacking of the culture medium and the explant due to leaching of phenolic, which were the problem in plant tissue culture of wood (Panda and Hazra., 2010; Tripathi and Kumari., 2010). In the present study, the experiment was conducted by antioxidants and absorbents to reduce the exudation of phenolic substances content in the plants were also reported in *Anacardium accidentale* (Aliyu., 2005; Sija *et al*., 2015), *Pistacia vera* (Onay *et al*., 2004) and *Sclerocary birrae* (Moyo *et al*., 2012). Furthermore, Plant Growth Regulators (PGRs) is used to increase the growth rate of seed germination and amount of plant.

Effect of antioxidants and absorbents

Gluta usitata possess large amount of phenolic compound *etc*., which was leaching into the culture medium cause browning of media and inhibition of growth of the explant. Antioxidants and absorbents are incorporated in the culture medium to eliminate phenolics released. The four medium additives were 1, 2, 3 g/L Activated charcoal or antioxidants were 2, 4, 6, 8 g/L PVP, 20, 30, 40, 60 mg/L Ascorbic acid or Citric acid used to reduce the phenolics released into the medium, activated charcoal at 2 g/L was significantly more propitious for the growth of seed in culture (Table 1 and figure 1b). The frequency of responding explant after for 4 weeks of culture in WPM medium with activated charcoal at 2 g/L was 0.9 ± 0.10 points of phenolic, this showed that the phenolic content was best reduced and the explant on medium was browning on seed exposed into culture medium was little bit. Citric acid and ascorbic acid at 60 mg/L were 1.1 ± 0.17 and 1.1 ± 0.23 points of phenolic, respectively. It found that browing on seed and medium was little bit (Table 1, figure 1k,1o). In the other hand, PVP at 2 g/L was the most browning on seed and culture medium (2.4 ± 0.16 points of phenolics) which was beginning to turn yellowed after culture for 3-4 days and noticed browning on explant and media more clearly (Table 1 and figure 1d). Observed in all substances used in the experiment after 4 weeks, we found that activated charcoal was browning on seed exposed into culture medium was little bit (Figure 1a-1c) and the higher of percentages of responding seed germination was 75% at 2 mg/L activated charcoal but 3 mg/L activated charcoal had the maximum of number of shoot (1.37 ± 0.49 shoots/seed). WPM medium combined with PVP showed that the seed and medium changed to browning on seed and distributed in a yellowish brown on culture medium (figure 1d-1g). Moreover, the most of seed germination and number of shoot

were 66.66% and $1.21^{abc} \pm 0.49$ shoots/seed at 4 g/L PVP (Table 1) and all concentrations had length of all proximity. WPM medium combined with ascorbic acid or citric acid showed that the explant and medium were 50% of browning on seed and distributed in a yellowish brown on culture medium (figure 1h-1g) but normaly seeds can germinated. It was found that 40 mg/L ascorbic acid had germination rate and number of shoot were 85% and 1.85 ± 0.40 shoots/seed, respectively whereas 20 mg/L citric acid had 77.77% and 0.89 ± 0.20 shoots/seed of seed germination and shoot, respectively (Table 1).

Table 1. Effect of WPM medium supplemented with different concentration of antioxidants and absorbents on seed germination, number of shoot, shoot length and browning on seed and culture medium on seed of *Gluta usitata* after culture for 4 weeks.

Antioxidants/absorbents	Seed germination[1] (%)	Number of shoot mean ± SE	Shoot length mean ± SE in cm	Points of phenolics mean per seed ± SE
Activated charcoal 1 g/L	37.5	$0.37^{a} \pm 0.18$	$3.99^{ab} \pm 0.24$	$1.3^{abc} \pm 0.27$
Activated charcoal 2 g/L	75	$0.87^{bcd} \pm 0.22$	$4.67^{ab} \pm 1.76$	$0.9^{e} \pm 0.10$
Activated charcoal 3 g/L	62.5	$1.37^{ab} \pm 0.49$	$4.55^{ab} \pm 1.37$	$1.4^{cde} \pm 0.22$
PVP 2 g/L	44.44	$0.44^{a} \pm 0.17$	$4.50^{ab} \pm 1.81$	$2.4^{a} \pm 0.16$
PVP 4 g/L	66.66	$1.21^{abc} \pm 0.49$	$4.79^{ab} \pm 1.81$	$2.3^{ab} \pm 0.21$
PVP 6 g/L	50	$0.50^{cd} \pm 0.18$	$7.67^{a} \pm 1.50$	$1.6^{bcde} \pm 0.22$
PVP 8 g/L	62.5	$0.62^{bcd} \pm 0.18$	$5.59^{ab} \pm 1.40$	$1.9^{abc} \pm 0.27$
Citric acid 20 mg/L	77.77	$0.89^{bcd} \pm 0.20$	$2.47^{b} \pm 0.79$	$1.6^{bcde} \pm 0.22$
Citric acid 30 mg/L	12.5	$0.12^{a} \pm 0.12$	$5.34^{ab} \pm 0.00$	$1.8^{abcd} \pm 0.29$
Citric acid 40 mg/L	50	$0.50^{cd} \pm 0.22$	$2.89^{b} \pm 0.94$	$1.2^{cde} \pm 0.24$
Citric acid 60 mg/L	66.66	$0.67^{bcd} \pm 0.16$	$3.76^{ab} \pm 1.21$	$1.1^{de} \pm 0.17$
Ascorbic acid 20 mg/L	57.14	$0.57^{bcd} \pm 0.20$	$3.19^{b} \pm 0.38$	$1.5^{cde} \pm 0.22$
Ascorbic acid 30 mg/L	50	$0.50^{cd} \pm 0.18$	$5.81^{b} \pm 1.53$	$1.4^{cde} \pm 0.30$
Ascorbic acid 40 mg/L	85.71	$1.85^{a} \pm 0.40$	$1.82^{ab} \pm 0.50$	$1.3^{cde} \pm 0.15$
Ascorbic acid 60 mg/L	66.67	$0.17^{a} \pm 0.16$	$3.81^{ab} \pm 0.00$	$1.1^{de} \pm 0.23$

[1]/Two repeats and ten explants in each repeat.
[2]/Each value represents the mean ± SE of two repeats per treatment.
[3]/The data were statistically analyzed using Duncan's multiple range test. In the same column, significant differences according to significant difference at the $P \leq 0.05$ level are indicated by different letters.

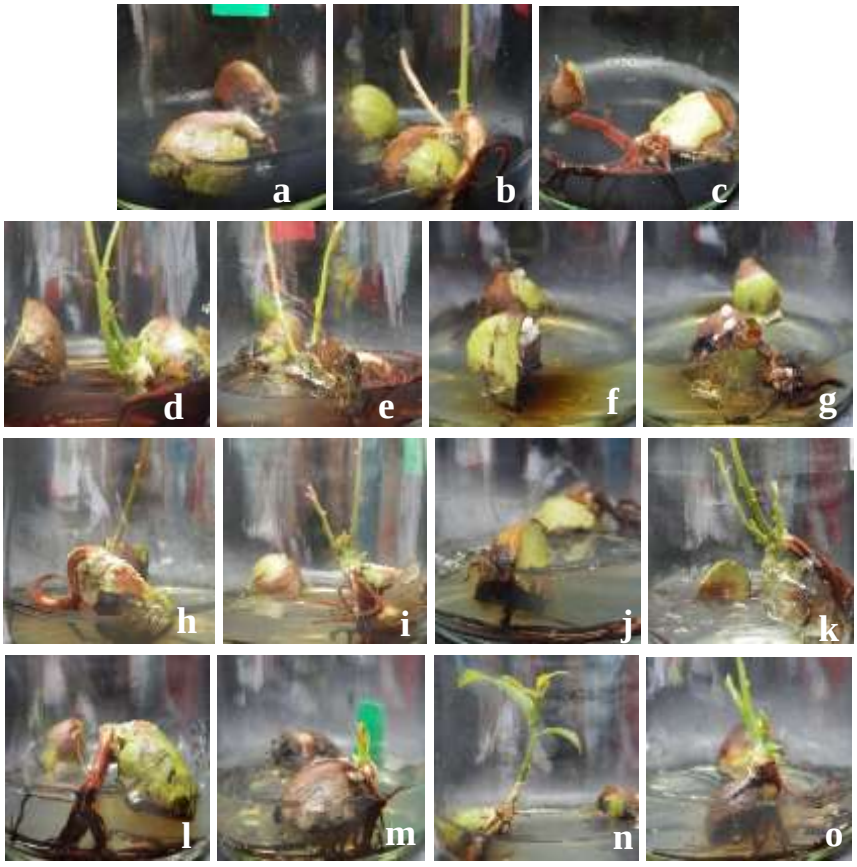

Figure 1. Effect of WPM medium supplemented with different concentration of absorbents were 1, 2, 3 g/L Activated charcoal (1a, 1b, 1c) and antioxidants were 2, 4, 6, 8 g/L PVP (1d, 1g, 1f, 1g), 20, 30, 40, 60 mg/L Ascorbic acid (1h, 1i, 1j, 1k) and 20, 30, 40, 60 mg/L Citric acid (1l, 1m, 1n, 1o), respectively on browning on seed and culture medium on seed of *Gluta usitata* after culture for 4 weeks.

Effect of Plant Growth Regulators (PGRs)

After experimenting, gave the best phenolic compounds inhibition. It was used as a ground for seed germination. The proliferation potential of seed germination was assessed on WPM medium combined with 2 g/L activated charcoal and various cytokinins were 0.5, 1, 1.5, 2 and 3 mg/L of BAP, TDZ, *m*T or without PGRs, the results are summarized in Table 2. WPM medium supplemented with TDZ and *m*T were more effective in seed germination than BAP. The result showed that, the maximum percentages of seed germination was 2 mg/L *m*T (95%) compared with all medium used in the experiment after

culture for 2 weeks. Furthermore, the highest length of shoot was 1.67 ± 0.22 shoots/seed at 2 mg/L *m*T (Table 2, figure 2n) and all concentration of *m*T had microshoot at the base of the trunk (figure 2k-2o). However, all concentrations of TDZ can be induced seedling germination was similar and the most of shoot and multiple shoots (figure 2f-2j). Especially, the highest of shoots were 3.00 ± 0.86 shoots/seed at 1.5 mg/L TDZ but 1 mg/L TDZ had the most percentages of seed germination was 82% (Table 2, figure 2h). Moreover, the seeds were cultured in WPM medium with TDZ had callus with green colour and hard, which were found in incision on seed. WPM medium combined with different concentrations of BAP found that 78.26% of seed germination at 1.5 and 2 mg/L BAP but the maximum of shoot was 1.60 ± 0.22 shoots/seed at 1.5 mg/L BAP (Table 2, figure 2c) and on base of shoot had microshoot (figure 1a-1e) whereas WPM medium without PGRs can be induced seed germination than 0.5 and 1 mg/L BAP. There were significant differences in the experiments showed that there are many concentrations at the most of shoot length at 1 mg/L of *m*T, 0.5 mg/L of BAP and 0.5, 1 mg/L of TDZ were 6.88^{a} ± 0.91, 6.82^{ab} ± 1.78, 6.45^{ab} ± 0.54 and 6.47^{ab} ± 1.04 cm (Table 2), respectively. It was also found that all the concentrations used in the experiments induced root emergence (Figure 2).

Table 2. Influence of WPM medium supplemented with different concentration of BAP, TDZ or *m*T on seed germination, number of shoot, shoot length and root germination on seed of *Gluta usitata* after culture for 6 weeks

PGRs (mg/L)	Seed germination (%)	Number of shoot mean ± SE	Shoot length mean ± SE in cm	Root germination (Yes/No)
0	73.60	1.16^{cde} ± 0.2	5.30^{ab} ± 8.95	0.71^{a} ± 0.14
BAP 0.5	66.66	0.83^{cde} ± 0.16	6.82^{ab} ± 1.78	1.00^{a} ± 0.17
BAP 1	63.60	0.87^{e} ± 0.20	6.64^{ab} ± 0.66	1.00^{a} ± 0.17
BAP 1.5	78.26	1.60^{bcde} ± 0.22	5.37^{ab} ± 0.72	0.91^{a} ± 0.12
BAP 2	78.26	1.00^{cde} ± 0.16	6.03^{ab} ± 1.46	1.13^{a} ± 0.21
BAP 3	71.4	0.75^{de} ± 0.16	5.65^{ab} ± 1.05	1.42^{a} ± 0.20
TDZ 0.5	80.95	1.75^{bcd} ± 0.16	6.45^{ab} ± 0.54	1.19^{a} ± 0.19
TDZ 1	82.00	1.71^{bcd} ± 0.28	6.47^{ab} ± 1.04	1.00^{a} ± 0.20
TDZ 1.5	80.00	3.00^{a} ± 0.86	5.58^{ab} ± 0.65	1.10^{a} ± 0.17
TDZ 2	80.00	2.40^{ab} ± 0.54	5.60^{ab} ± 0.87	0.82^{a} ± 0.08
TDZ 3	73.07	1.87^{bc} ± 0.12	3.74^{b} ± 0.37	1.00^{a} ± 0.19
*m*T 0.5	88.00	1.10^{cde} ± 0.23	4.85^{ab} ± 0.62	1.60^{a} ± 0.27
*m*T 1	75.00	1.38^{bcde} ± 0.18	6.88^{a} ± 0.91	1.40^{a} ± 0.38
*m*T 1.5	80.00	1.30^{cde} ± 0.30	4.58^{ab} ± 0.44	1.56^{a} ± 0.37
*m*T 2	95.00	1.67^{bcd} ± 0.22	5.92^{ab} ± 0.69	1.38^{a} ± 0.28
*m*T 3	76.19	1.20^{cde} ± 0.20	6.23^{ab} ± 0.91	1.42^{a} ± 0.34

[1]/Two repeats and ten explants in each repeat.
[2]/Each value represents the mean ± SE of two repeats per treatment.
[3]/The data were statistically analyzed using Duncan's multiple range test. In the same column, significant differences according to significant difference at the $P \leq 0.05$ level are indicated by different letters.

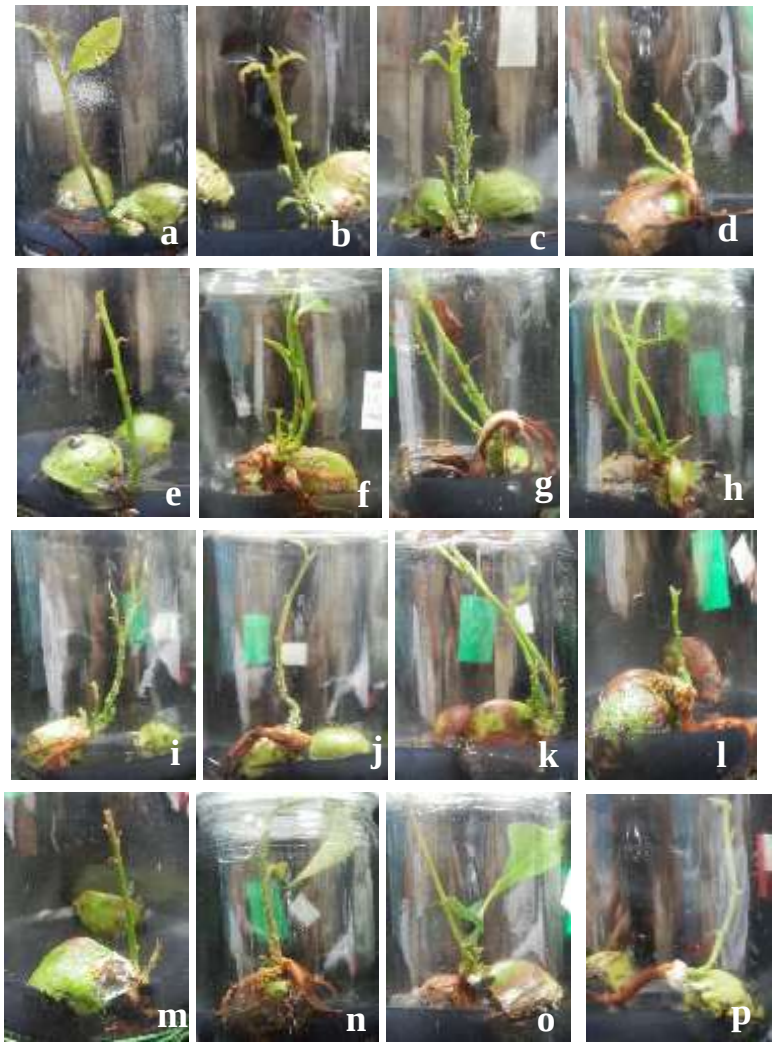

Figure 2. Influence of WPM medium supplemented with BAP were 0.5, 1, 1.5, 2 and 3 mg/L (2a, 2b, 2c, 2d, 2e), TDZ were 0.5, 1, 1.5, 2 and 3 mg/L (2f, 2g, 2h, 2i, 2j) and *m*T were 0.5, 1, 1.5, 2 and 3 mg/L (2k, 2l, 2m, 2n, 2o) and without PGRs (2p), respectively on seed of *Gluta usitata* after culture for 6 weeks

Discussion

A hard seed coat and presence of phenolics in seed influenced poor germination by creating a barrier that interferes with the absorption of water

and for gaseous exchange for respiration (Panda and Hazra., 2009). In this experiment cut the bark of the seeds of *Gluta usitata*. By burning the fire before cut, the seed coat can be easily removed, and the contamination rate reduced as well as in *Tectona grandis* Linn. F. (Pianhanuruk., 2007). The experimental results of effect of antioxidants and absorbents found that activated charcoal at 2 g/L was the best to reduce phenolic compounds due to the adsorption undesirable inhition substances from the plant. Effect of 2 g/L avtivated charcoal has been reported in tree species of Anacardiaceae such as *Schinopsis balansae* (Sansberro *et al*., 2003), *Semecarpus anacardium* (Panda and Hazra., 2010), Pistacia vera L. (Benmahioul *et al*., 2016). Moreover, Boggetti (1999) described as either activated charcoal or cultivation in darkness for the initial 7 days had positive effects on shoot elongation. However, Dunuwila (2010) said activated charcoal absorb some PGRs which may lead to unsuccessful cultures due to reduction in PGRs activity. Similarly, the high concentration of ascorbic acid and critric acid can be reduced phenolic compounds (Table 2) because acid treatment caused cautury of the pericarp, which possibly allowed excretion of inhibitory phenolics and entry of water into the seed to encourage germination of embryo (Panda and Hazra., 2009) in the same with *Anacardium occidentale* (Sija *et al*., 2015; Dunuwila *et al*., 2010), *Mangifera indica* (Krishna *et al*., 2008). In addition to, in the presence of low concentration of PVP showed that the release of phenolics was increased resulting (Table 2) while the high concentration of PVP Inhibited phenolic more as well as *Mangifera indica* (Raghuvanshi and Srivastava., 1995). In contrast of Thimmappaiah (2002b) observed that PVP at 1 g/L was better the most effective with a higher percentage of bud and inhibited phenolic compounds than 0.2% activated charcoal and 100 mg/L ascorbic acid in cashew (*Anacardium occidentale* L.). There are also other researchs such as Gannoun (1995) found that browning of *Pistacia terebinthus* explants *in vitro* was successfully controlled by adding 100 mg/l ascorbic acid in the medium and by soaking the explants prevoius culture for 30 minutes in 10 g/L PVP solution. Thimmappaiah (2002a) reported scions culture on MS medium containing 2 mg/L activated charcoal and scions immersed in solution of 0.01% ascorbic acid and 0.015% citric acid reduced phenolic compounds and oxidative browning of scion was further prevented by incubation in low light in the initial stage.

Seed germination by tissue culture on young seed of *Gluta usitata* take in short periods (In this experimented, seedling germination after culture 2 weeks) while growth in natural using long-term and mature seed for germinate. PGRs used in the experiment was cytokinins, cytokinins are known to induce both axillary and adventitious shoot formation from meristematic explant (George., 1993). According to the result of influence of PGRs showed that, *m*T was more

suitable for effective in vitro proliferation of seed germination than BAP and TDZ. There is research reported *m*T was nearly twice as effective as BAP and its positive effected on several parameters of tissue culture, such as high rate of shoot multiplication and better rooting and acclimatization (Benmahioul *et al*., 2012; Benmahioul *et al*., 2016). In our results found that the high percentages of seed germination were 2 mg/L *m*T, its has been reported in tree species of Anacardiaceae such as *Sclerocarya burea* (Moyo *et al*., 2012), *Pistacia vera* (Benmahioul *et al*., 2012). However, TDZ was similary effective with *m*T, its produced more number of shoots than *m*T due to low concentration of TDZ promoted shoot multiplication or somatic embryogenesis in several plants such as *Acer pseudoplatanus* (Wilhelm., 1999), *Mangifera indica* L. var. Darshehari (Kidwai *et al*., 2009), whereas higher concentrations than 20 µM and above stimulated somatic embryogenesis, callus proliferation or adventitious shoots (Huetteman and Preece., 1993), which is similar in this study on *Azadirachta indica* A. Juss. (Murthy and Saxena., 1998), *Tectona grandis* L. (Kozgar and Shahzad., 2012). In addition to BAP produced more number of multiple shoot and was very effective in shoot proliferation (Benmahioul *et al*., 2016; Panda and Hazra., 2010; Tilkat *et al*., 2005). Many researchs including Eng (2015) reported BAP at 2.22-6.66 µM produced the highest number of shoot of *Citus hystrix* DC, whereas high concentrations (13.32 µM) of BAP was inhibited shoot regeneration, which is similarly to this study on Tilkat (2005) described that the best multiple shoot initiation of *Khinjuk pistachio* was cultured on MS medium supplemented with 2-4 mg/L BAP and all microshoots initiated by 1 mg/L BAP.

This research describing *in vitro* propagation of Burmese lacquer (*Gluta usitata*, 217 Mae Ka), which is the experiments to inhibit the phenolic compounds and to accelerate the growth of young seedlings to enhance the growth of plants can be improved. This research can be used as the basic for the development and conservation of this plant species. It also led to studies in other research as well.

Acknowledgement

This research was funded by the Royal Thai Government's annual budget in 2017.

The authors were grateful to Mrs. Kaewnapa Kittibanpacha for providing the seed of Gluta usitata (217 Mae Ka).

The authors would like to offer thanks to King Mongkut's Institute of Technology Ladkrabang responsible for scholarship to carry out our research program.

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(Received: 24 August 2018, accepted: 20 October 2018)