International Journal of Agricultural Technology 2018 Vol. 14(7): 1731-1742
Available online http://www.ijat-aatsea.com
ISSN: 2630-0613 (Print) 2630-0192 (Online)

Efficiency of cytokinin for propagation of *Gluta usitata* (Na-pong3) *in vitro*

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Rakrawee, R., Kittibanpacha, K., Chareonsap, P. and Poeaim, A. (2018). Efficiency of cytokinin for propagation of *Gluta usitata* (Na-pong3) *in vitro*. International Journal of Agricultural Technology 14(7): 1731-1742.

Abstract The result was explained an effect of Plant Growth Regulators (PGRs) for young seeds induction and shoot multiplication of *Gluta usitata* (Na-pong3) by plant tissue culture technique to preserve and increased plant propagation. The young seeds were induced by comparison to 0.5, 1, 1.5, 2 and 3 mg/L 6-Benzyl amino purine (BAP), Thidiazuron (TDZ) or *meta*-Topolin (*m*T) on Woody Plant Medium (WPM). The results showed the highest percentage of seed regeneration was 1.5 mg/L BAP which was100% germination. The shoots was highest induced by 0.5 mg/L BAP after culture for 6 weeks. The shoots were transferred to WPM medium containing with BAP, TDZ or *m*T for multiple shoot induction. The maximum number of shoots were observed on WPM medium combined with 1.5 mg/L BAP (about 6 shoots per explant) after subcultured for three to four times. The best of root induction was observed on WPM medium without PGRs.

Keywords: *Gluta usitata*, shoot multiplication, young seeds regeneration

Introduction

Gluta usitata (Rak Yai), commonly known as Lacquer varnish tree or Burmese tree, which belong to Anacardiceae, is a mixed deciduous forest and deciduous dipterocarp forest in the northern, southeastern and upper south region of Thailand (Fine Art Department., 2008). Rak lacquer varnish is the natural lacquer from Lacquer varnish tree. In Thailand, lacquer varnish has been utilized in many traditional Thai arts and crafts for its special adhesive characteristic (Tangmitcharoen *et al*., 2015). Traditionally, Rak lacquer varnish is applied or pained on wood and other surfaces for protection and durability. It is also applied to different contained and utensil surfaces, either paper or cloth, as water-proofing foundation for subsequent gold leaf application in certain forms of Thai fine arts and crafts (Fine Art Department., 2008).

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However, Rak lacquer varnish can caused dermatitis and skin irritation and synthetic lacquer has replaced lacquer varnish. Currently, its harvest has been troublesome due to the deforestation and cultivation in National park. As a result, plant preservation decreases and is extinct. (Tabtong., 2012; Wiriyaudom *et al*., 2012). Generally, the vegetative propagation of Lacquer varnish tree can be used many methods such as marcotting, layering, cutting, grafting or inarching. However, these methods caused Rak lacquer varnish getting out from incision and making the plant necrosis or death. (Kittibanpacha *et al*., 2015). Moreover, low percentage regeneration of seeds due to lose viability natural conditions and fungal contamination associated with the storage of seeds. Therefore, plant tissue culture is useful propagation of desirable trees for planting out, research and increases for rapid mass multiplication of plant (Sansberror *et al*., 2003; Tripathi and Kurami., 2010). It's can be used all parts of the plant can be harvested in a medium culture containing minerals, sugars, vitamins and plant growth regulators (PGRs). Under the conditions of microbial control and temperature control, the humidity of the plants can be developed in many ways. However, there is very few studies have been done on micropropagation of *G. usitata*. The aim of this study was to develop method for micropropagation of *G. usitata* including the optimal factors of seed germination of *G. usitata* to reduce germination time, shoot induction and shoot proliferation by tissue culture. The result of this study could be useful to develop an efficient and reproducible method for stimulating micropropagation and large-scale propagation of *G. usitata*.

Materials and methods

Collection of explant, sterilization and effect of Plant Growth Regulators of seeds germination

Young seeds of *G. usitata* (Rak Yai: Na-pong3) were courtesy of Rak Yai Gene Conservation and Development Program under the Royal Initiative Projects, Ngao District, Lampang Province, Thailand. There were surface sterilized in 70% ethyl alcohol (v/v) and shook at 230 rpm. After that, dipped the seeds into 95% (v/v) ethyl alcohol (v/v) and fire burned. Then, moved the seed coat and cut half of seed to treat on WPM medium (Lloyd and Mc Cown., 1980) supplemented with 0.5, 1, 1.5, 2 or 3 mg/L of 6-Benzylaminopurine (BAP), Thidiazuron (TDZ), *meta*-Topolin (*m*T) or without Plant Growth Regulators (PGRs). The growth was crecorded by counting the number of shoots, roots and measured the length of shoot using Vernier Caliper after cultured for 6 weeks.

Shoot multiplication

The nodes from seeds in above experiment (about 1-1.5 cm) were transferred to WPM medium without PGRs for 1 week. Then, transferred to WPM medium combined with 0.5, 1, 1.5, 2 or 3 mg/L of BAP, TDZ, *m*T or without Plant Growth Regulators (PGRs) and added 2 g/L activated charcoal to reduce phenolic exudation. The nodes on shoot multiplication was periodically subcultured every 6 weeks. The media pH was adjusted to 5.8 before autoclaved at 121 ^{o}C for 15 minutes. The culture medium was supplemented with 3% sucrose and 0.26% Gellan gum. All experiments were cultured at 25±2 ^{o}C under diffused light for 16 h day^{-1} photoperiod.

The plantlets were transplanted into sterilized soil: husk (1:1) mixture in plastic cups. These were covered with plastic bags to maintain humidity for 8 weeks. Then, the plantlets were transferred to pots and maintained in greenhouse (Applied method from Panda and Hazra, 2010).

Data analysis

All experiments were repeated 3 times. Data were analyzed statistically using one-way analysis of variance and significant differences between means were assessed by Duncan's multiple range test (DMRT) at 5% probability ($P>0.05$) using SPSS (Statistics Package for the Social Sciences) version 25 (IBM SPSS Statistics 25). The results are expressed as mean ± Standard Deviation (SD).

Result

Effect of Plant Growth Regulators of seed germination

Reesult showed that sterilized seed burning method gave 66.0 % of survival rate. The plant parts were green and small shoots appreaed. Whereas some seeds died because its swollen and possible contaminated showing brown or black of the culture medium (Figure 1A). Seed germination was assessed on WPM medium combined with 3% Sucrose, 0.26% Gellan gum and different concentrations of cytokinin at 0.5, 1, 1.5, 2 and 3 mg/L of BAP, TDZ, *m*T or without PGRs after culture for 6 weeks resulted as shown in Table 1. WPM medium supplemented with BAP was the more effective in seed germination than TDZ and *m*T. The maximum percentage of seed germination was 1.5 mg/L BAP (100%) compared with all media. The greatest number of shoots induction was approximately 2 shoots/seed at 0.5 mg/L BAP. The highest length of shoot

averaged 0.5 mg/L of *m*T and 4.65 cm/shoot. In each type of cytokinin found that WPM medium combined with BAP gave the best number of shoots (2 shoots/explant) andisprouting shoot on embryo appeared (Figure 2C). The maximum of shoot length was 4.03 cm/shoot at 2 mg/L BAP. All concentrations of BAP were induced root germination. The most of root germination was 10 roots/shoot when cultured in 1 mg/L BAP. The roots form was solid brown and thick. WPM medium supplemented with TDZ showed the highest number of shoot and shoot length averaged 1.50 shoots/seed and 2.59 cm/shoot, respectively after cultured at 3 mg/L TDZ. The plants were cultured on WPM medium with TDZ had short stunting (Figure 1D). Some seeds were small shoots on embryo sprouting and not grew. All concentrations of TDZ had no root induction on seeds (Table 1). Both BAP and TDZ merged in WPM medium exhibited the growth of dark green and slightly compact calli. The calli can not be regenerated due to leach phenolics in explants and culture medium. WPM medium combined with *m*T gave the maximum number of shoots and length of shoot at 0.5 mg/L *m*T which averaged 1.75 ± 0.82 shoots/seed and 4.65 ± 0.62 cm/shoot, respectively (figure 1E). Root induction on WPM medium containing with 0.5 mg/L *m*T gave the most root induction of 10 roots/seed. WPM medium without PGRs gave number of shoots, average length of shoot and number of roots as 1.5 shoots/seed, 4.56 cm/shoot and 9.28 root/seed, respectively (figure 1B). Result of WPM medium of WPM medium without PGRs gave better shoot induction than TDZ and *m*T.

Shoot multiplication

Shoot multiplication was used the node from seedlings in previous experiment and cultured on WPM medium combined with different concentrations of PGRs. The effect of PGRs concentrations from four repeated subcultures on shoot multiplication of *G. usitata* is shown in Table 2. Shoots responded a differentl on various concentrations of PGRs in WPM medium mixed with 2 g/L activated charcoal. The result revealed that the concentrations of three tested cytokinins found to be significantly affected on shoot proliferation. The maximum number of shoots were treated on WPM medium supplemented with 1.5 mg/L BAP (approximately 6 shoots/explant) after fourth subculture. The new shoots were small in young leaves (figure 2B), which similarly with 3 mg/L BAP (5 shoots/explant). The number of shoots increased slightly up after the third or fourth transferred.

It is observed that the interaction of BAP gave a shoot multiplication better than TDZ and *m*T. Overall of TDZ. The number of shoots were less than WPM medium obtained with BAP, *m*T and without PGRs (approximately 1.5

to 2.5 shoots/explant) (Table 2). WPM medium including with TDZ found that the best number of shoots were 2.66 shoots/explant at 0.5 mg/L TDZ. Characteristic of shoot regeneration was short stunting, swollen and green sprouting on base of shoot (Figure 2C, 2E). The first and second subcultured on WPM medium combined with TDZ was a new slowly growth. While, third and fourth subculture had begun to grow. Shoot multiplication on WPM medium combined with *m*T, the fourth subculture gave the best response in 1.5 mg/L *m*T (3.75 shoots/explant). The elongated shoots were tall, swollen stem and white fiable compact (figure 2D). The concentrations of 0.5, 1, 2 and 3 mg/L *m*T gave similar averaged number of shoots . In the other hand, the highest length of shoot observed in 3 mg/L BAP was 3.26 cm/shoot (Table 2). Total BAP concentrations gave the length of shoot higher than TDZ or *m*T. The length of shoot on TDZ was similar with *m*T. It showed the length less than the control medium. The cultured on WPM medium without PGRs (control medium) gave the number of shoot and length of shoot averaged 2.64 shoots/explant and 2.47 cm/shoot. The shoots were shapely and large leaves on shoot tips (Figure 2A). After four repeated subcultures, shoots were cultured on WPM medium without PGRs that the roots emerged around 1-2 roots/shoot (Figure 2F). For acclimatization of plantlets, the plants were grew for 28 days in plastic cups and it died due to rot.

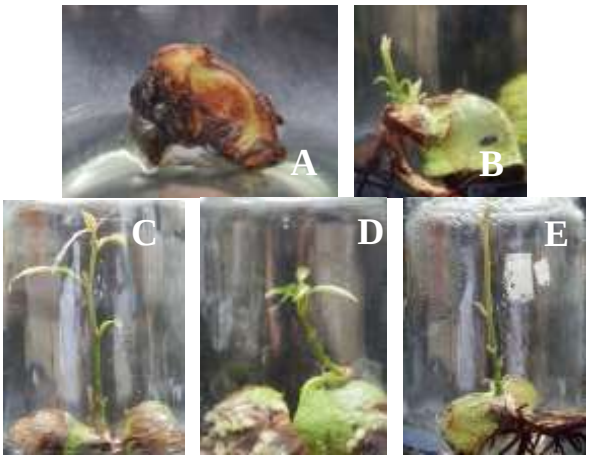

Figure 1. Characteristics of seedling of *G. usitata* treated on WPM medium supplemented with different concentrations of PGRs after culture 6 weeks. Seed had brown and swollen deu to leaching of phenolics, death seed (A). Seedling cultured on WPM medium without PGRs, it had white callus on germinated area (B). Seedling cultured on WPM medium combined with 0.5 mg/L BAP (C), 1.5 mg/L TDZ (D) and 0.5 mg/L *m*T (E), respectively. Seedling had high shoots and many leave

Table 1. Effect of WPM medium supplemented with different concentration of BAP, TDZ or *m*T on seed germination of *G. usitata*. Values represent number of shoots, shoot length and number of roots on seedling after culture for 6 weeks

PGRs (mg/L)	Seed germination (%)	Number of shoot[1,2,3] mean ± SD	Shoot length[1,2,3] mean ± SD in cm	Number of Root mean ± SD
0 (control)	73.34	1.50^{ab} ± 0.62	4.56^{a} ± 0.23	9.28 ± 2.57
BAP 0.5	86.67	1.93^{a} ± 0.99	2.70^{abcde} ± 0.10	6.86 ± 1.19
BAP 1	46.67	1.23^{abc} ± 0.00	2.62^{abcde} ± 0.76	10.00 ± 0.00
BAP 1.5	100.00	1.42^{ab} ± 0.82	3.08^{abc} ± 0.12	8.00 ± 3.85
BAP 2	86.67	1.00^{abc} ± 0.00	4.03^{ab} ± 0.26	8.53 ± 3.05
BAP 3	26.67	1.50^{abc} ± 0.50	2.94^{abcd} ± 0.70	5.25 ± 2.75
TDZ 0.5	60.00	1.00^{abc} ± 0.00	0.56^{cde} ± 0.08	0
TDZ 1	46.67	1.00^{abc} ± 0.00	1.71^{bcde} ± 0.37	0
TDZ 1.5	46.67	1.33^{ab} ± 0.33	1.22^{bcde} ± 0.18	0
TDZ 2	60.00	1.00^{abc} ± 0.00	1.13^{cde} ± 0.47	0
TDZ 3	66.67	1.50^{abc} ± 0.5	2.59^{abcde} ± 0.13	0
*m*T 0.5	40.00	1.75^{a} ± 0.82	4.65^{a} ± 0.62	10.00 ± 0.00
*m*T 1	0	0^{c}	0^{e}	0
*m*T 1.5	20.00	1.00^{bc} ± 0.37	0.34^{de} ± 0.12	0
*m*T 2	46.67	1.00^{abc} ± 0.00	0.87^{cde} ± 0.32	0
*m*T 3	60.00	1.00^{abc} ± 0.00	1.18^{bcde} ± 0.43	2.00 ± 1.15
ANOVA		S5%	S5%	

[1]/Three repeats and five explants in each repeated.

[2]/Each value represents the mean ± SD of three repeats per treatment.

[3]/The data were statistically analysis using Duncan's multiple range test (DMRT). In the same column, significant differences according to significant difference at the $P \leq 0.05$ level are indicated by different letters.

Discussion

Seeds of *G. usitata* (Rak Yai: Na-Pong3) are large seeds and thick bark. In nature, the seeds must be mature seed and dried for planting. The cultivation of this method took a long time. The thick bark of seeds affected to seed dormancy and the efficacy of seeds to germinate (Velempini *et al.*, 2003). A thick seed coat and phenolics compound in seed had an influence in poor germination by formation a barrier that interferes with the water impermeability of pericarp and gaseous exchange for respiration (Panda and Hazra., 2009; Baskin and Baskin., 1998). This experiment was used immature seeds, the sterilization can be done by burned and cut off the seed coat because of it had soft seed coat and easily removed. A similar approach has been followed for explant establishment in *Tectona grandis* Linn. F. (Pianhanuruk., 2007) and *Khaya senegalensis* (Darwesh *et al*., 2017). In methodology of *in vitro* seedling

germination took short periods less than natural germination (In this experiment, seedling germinated after culture 10-12 days). This experiments ware used cytokinins such as BAP, TDZ and *m*T, cytokinins are known to induce both axillary and adventitious shoot formation from meristematic explant and to engender green compact callus. The result exhibited influence of PGRs showed that, BAP was more efficiency of *in vitro* seedling germination and shoot proliferation than *m*T and TDZ. On the same of Bonga and Von Aderkas (1992) mentioned to the best efficient and commonly used cytokinin in plant tissue culture is BAP.

In study of shoot multiplication, the result summarized that 1.5-3 mg/L BAP had the most of shoot proliferation in third or fourth subculture of explant and length of shoots were longer in each subculture. This is consistent with many researchers such as Darwesh (2017) observed that culturing of African Mahokany (*Khaya senegalensis*) on MS medium supplement with 0.8 ppm BA caused the best of shoots per explant and repeated subculture for three times brought to the highest number of shoots. Gupta *et al.* (2014) revealed that the A maximum number of shoot and length of shoot were increased steadily up to the third repeated transfer at MMS medium merged with 1.5 mg/L BAP and 0.1 mg/L NAA and decline slightly in the next subculture. In the same of Phulwaria *et al.* (2012) noted that the shoot number increased significantly for four repeated transfers and reduced thereafter on cultured of *Terminalia bellirica*. In many researchers showed that repeated transferring has been used and suggested as an efficient method for shoot multiplication of woody plants. The increase in shoot may be because of suppression of apical dominance during subculture that induced basal dormant meristametic cells to form new shoot (Shukla *et al*. 2008). Furthermore, many researches for supported that BAP induced shoots regeneration in the same with this research, Panda and Hazra (2010) described that BAP singly produced a greater number of multiple shoots, the most of shoot proliferation was 7-8 buds/explant on 4.44-8.88 µL BAP after culture 12 weeks and some multiple buds failed to grow and changed necrotic in the PGRs free medium. Effectiveness of BAP as the most active cytokinin for stimulating shoot proliferation can be found in Guava (Psidium guajava L.) at MS medium consist of 1 mg/L BAP (Rai *et al*., 2009), *Spondias mangifera* cultured on MS medium containing 1 mg/L BAP had the most number of shoot (10.6 shoots/explant) after transfer mother explant up to fourth subculture (Tripathi and Kurami, 2010). In the same way, our experiments exhibited that number of shoots on WPM medium combined with *m*T was similarly with BAP. In contrast of some researchers mention that *m*T was nearly twice as effective as BAP in the in duction of shoot proliferation (Koetle *et al*., 2010; Benmahioul *et al*., 2012).

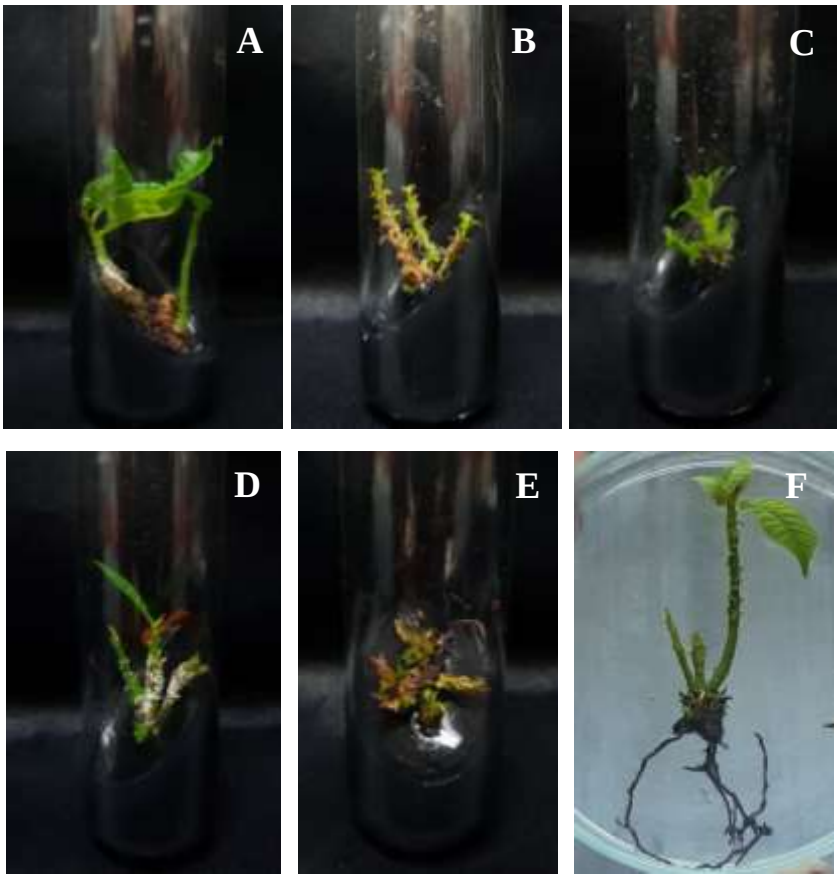

Figure 2. Characteristics of shoot multiplication of *G. usitata* treated on WPM medium supplemented with different concentration of PGRs after four repeated subculture every 6 weeks. Shoots induced on WPM medium without PGRs (A). Shoots proliferating on WPM medium combined with 1.5 mg/L BAP, shoots had small leaves and brown callus occurred at the trunk (B). Shoots regenerated on WPM medium combined with 0.5 mg/L TDZ, the explant was short and stunting (C). Shoots emerged on WPM medium combined with 1.5 mg/L *m*T, shoots had tall and white callus occurred at the trunk (D). Multiple shoots induced on WPM medium with 2 mg/L TDZ, shoots were shoot and small (E). Roots development on *in vitro* regenerated shoot after 6 months (F)

Table 2. Effect of WPM medium supplemented with different concentration of BAP, TDZ or *m*T on shoot multiplication of *G. usitata*. Values represent number of shoots and length of shoot after four repeats subculture every 6 weeks

PGRs (mg/L)	Characters							
	Number of shoots in each subculture[/1,2,3] (mean ± SD)				Length of shoots in cm in each subculture[/1,2,3] (mean ± SD)			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th
0	1.69^{abcd} ±1.40	2.00^{b} ±0.91	2.31^{bc} ±1.64	2.64^{abc} ±1.45	1.61^{a} ±1.02	1.74^{ab} ±0.16	1.85^{ab} ±0.91	2.47^{a} ±1.60
BAP 0.5	1.16^{bcd} ±0.55	1.50^{bc} ±0.80	1.90^{bc} ±0.92	2.63^{abc} ±0.88	1.30^{ab} ±0.40	1.37^{abc} ±0.16	1.58^{ab} ±0.49	2.21^{a} ±1.03
BAP 1	0.50^{ab} ±0.10	0.75^{c} ±0.11	1.00^{c} ±0.00	2.33^{abc} ±1.24	1.32^{ab} ±0.16	1.58^{abc} ±0.16	1.79^{ab} ±0.45	2.04^{a} ±0.18
BAP 1.5	2.67^{abc} ±0.47	4.57^{a} ±1.84	5.28^{a} ±0.95	6.00^{a} ±1.68	1.48^{ab} ±0.43	1.48^{abc} ±0.16	1.49^{ab} ±0.29	2.20^{a} ±0.79
BAP 2	2.15^{abc} ±0.87	2.33^{b} ±0.67	2.45^{ab} ±0.73	2.75^{abc} ± 1.09	1.05^{c} ±0.49	1.35^{abc} ±0.46	1.52^{ab} ±0.86	2.05^{a} ±0.71
BAP 3	1.50^{abcd} ±0.50	2.50^{b} ±0.50	3.75^{ab} ±0.42	5.00^{a} ±2.00	1.69^{a} ±0.71	1.72^{abc} ±0.36	1.96^{a} ±0.12	3.26^{a} ±1.9
TDZ 0.5	1.00^{e} ±0.00	1.33^{bc} ±0.00	1.33^{c} ±0.47	2.66^{abc} ±1.24	1.17^{abc} ±0.52	1.42^{abc} ±0.43	1.64^{ab} ±0.16	2.33^{a} ±0.20
TDZ 1	1.00^{e} ±0.00	1.00^{c} ±0.00	1.67^{bc} ±0.94	2.00^{abc} ±0.00	1.05^{c} ±0.04	1.09^{d} ±0.32	1.12^{ab} ±0.41	1.59^{a} ±0.38
TDZ 1.5	1.00^{e}+0.00	1.00^{c} ±0.00	1.00^{c} ±0.00	1.67^{bc} ±0.47	1.00^{c} ±0.24	1.12^{c} ±0.21	1.34^{ab} ±0.04	1.64^{a} ±0.44
TDZ 2	1.00^{e} ±0.00	1.00^{c} ±0.00	1.00^{c} ±0.00	1.33^{bc} ±0.47	1.01^{c} ±0.32	1.14^{abc} ±0.47	1.41^{ab} ±0.49	1.64^{a} ±0.47
TDZ 3	1.00^{cd} ±0.00	1.00^{c} ±0.00	2.00^{bc} ±0.19	1.50^{bc} ±0.00	1.01^{c} ±0.02	1.23^{abc} ±0.21	1.56^{ab} ±0.10	2.51^{a} ±0.73
*m*T 0.5	1.33^{bcd} ±0.47	1.50^{bc} ±0.86	2.00^{bc} ±0.00	2.50^{abc} ±0.50	1.49^{ab} ±0.21	1.54^{abc} ±0.24	1.59^{ab} ±0.16	1.67^{a} ±0.70
*m*T 1	1.00^{e} ±0.00	1.00^{c} ±0.00	1.90^{bc} ±1.30	2.00^{bc} ±0.70	1.08^{c} ±0.36	1.12^{c} ±0.32	1.25^{ab} ±0.33	1.26^{a} ±0.42
*m*T 1.5	3.00^{a} ±0.00	3.15^{a} ±0.00	3.33^{ab} ±0.94	3.75^{ab} ±1.77	1.57^{ab} ±0.06	1.83^{ab} ±0.24	2.02^{a} ±0.41	2.30^{a} ±0.1
*m*T 2	1.00^{e} ±0.00	1.50^{bc} ±0.00	2.33^{ab} ±0.94	3.00^{ab} ±0.00	1.02^{c} ±0.24	1.14^{c} ±0.49	1.37^{ab} ±0.41	1.53^{a} ±0.41
*m*T 3	1.00^{e} ±0.00	1.00^{c} ±0.00	1.80^{bc} ±0.00	2.00^{abc} ±0.00	1.24^{bc} ±0.54	1.42^{abc} ±0.32	1.62^{ab} ±0.49	1.74^{a} ±0.16
ANOVA	S5%	S5%	NS5%	NS5%	S5%	S5%	NS5%	S5%

[1]/Three repeats and five explants in each repeated.

[2]/Each value represents the mean ± SD of three repeats per treatment.

[3]/The data were statistically analysis using Duncan's multiple range test (DMRT). In the same column, significant differences according to significant difference at the $P \leq 0.05$ level are indicated by different letters.

In this study, WPM medium combined with 1.5-2 mg/L *m*T was induced shoot multiplication around 3-4 shoots/explant after forth subculture. Which is consistent with Benmahioul (2012) found that the maximum shoot induction was achieved on MS medium amended with 2 mg/L *m*T and *m*T established a greater number of elongated shoots. The effectiveness of TDZ found that the shoots were short stunting, green sprouting and callus on the base. Deu to, high concentrations TDZ (approximately 20 µM or more) were somatic embryogenesis and callus proliferation (Murty and Saxena, 1998), in the same of Mneney and Mantell (2015) said TDZ were stunted and showed marked hyperhydricty. It found in this experiment at 2-3 mg/L TDZ (Figure 2C, 2E). Nevertheless, callusing on incision of base shoot caused by the shoots were cut and transferred to new medium, its has been reported in *Buchanania lanzan* (Spreng) (Shende and Rai, 2005). The aspect of micropropagation technology is its successful execution from lab to field conditions. Rooting of shoot is a critical factor in the micropropagation of woody tree species (Durkovic and Lux, 2010). For acclimatization of this research unsuccessfully because root system of plants was not stable and cultivated area was high humidity. In many researchers suggested that shoot cultured on shoot transferred to substate (perlite, vermiculite) with MS liquid medium combined IBA or NAA can be induced strong root and transplant to green house (Sansberror *et al.*, 2003; Moncousin, 1991).

Acknowledgement

This research was funded by the Royal Thai Government's annual budget in 2017-2018.

The authors were grateful to Mrs. Kaewnapa Kittibanpacha and Rak Yai Gene Conservation and Development Program under the Royal Initiative Projects, Ngao District, Lampang Province, Thailand for providing the seed of *Gluta usitata*.

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: 10 September 2018, accepted: 2 November 2018)