International Journal of Agricultural Technology 2021 Vol. 17(1):47-66
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Effects of colchicine treatment on morphological variations of *Neolamarckia cadamba*

Eng, W. H.[1], Ho, W. S.[1*] and Ling, K. H.[2]

[1]Forest Genomics and Informatics Laboratory (fGiLab), Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia; [2]Sarawak Timber Association, Wisma STA, 26, Jalan Datuk Abang Abdul Rahim, 93450 Kuching, Sarawak, Malaysia.

Eng, W. H., Ho, W. S. and Ling, K. H. (2021). Effects of colchicine treatment on morphological variations of *Neolamarckia cadamba*. International Journal of Agricultural Technology 17(1):47-66.

Abstract This study described the effects of colchicine application on the morphological characteristics of *Neolamarckia cadamba* plantlets. Surface-sterilised seeds and *in vitro*-grown nodal segments were treated with different concentrations of colchicine (0.1%, 0.3%, and 0.5%) and durations (24 h and 48 h). For both seeds and nodal segment explants, mortality rates correlated positively with colchicine concentration and treatment duration. In terms of morphological characteristics, the explants showed a decrease in shoot length, but an increase in the percentage of shoots with abnormal leaves and darker green leaves over the control when treated with more severe colchicine. For seeds treated with colchicine, multiple shoot seedlings were also observed. The number of shoots proliferated correlated positively with the severity of colchicine treatment. It showed that colchicine could exert plant development *in vitro*, which is similar to plant growth regulators. The stem diameter of seedlings was larger when seeds were treated with colchicine. A higher colchicine concentration with a longer period of treatment of explants induced higher variation. The various morphological characteristics observed in the treated explants indicated genetic changes which could be useful for the genetic improvement of *N. cadamba*.

Keywords: *Kelampayan*, *In vitro* mutagenesis, Phenotype variation, Tissue culture

Introduction

Neolamarckia cadamba (Roxb.) Bosser belongs to the Rubiaceae. Its local name varies between countries: Kelempayan (Malaysia), Krathum (Thailand) and Jabon (Indonesia) (Soerianegara and Lemmens, 1993). This rapid-growing semi-hardwood forest tree can reach 45 m in height and 1.6 m in diameter. The average leaf size is 50 cm × 25 cm, opposite-leaf arrangement, oval in shape, and broader leaves on young plants (Krisnawati *et al*., 2011). *N. cadamba* has been used widely in Ayurvedic medicine to treat various types of ailments from mild to severe in India. This has led to prolific literature on the

[*] **Corresponding Author:** Ho, W. S.; **Email:** wsho@unimas.my

phytochemistry of this forest tree that focuses on its traditional medicinal uses and pharmacological effects. Three comprehensive review articles summarise all the uses of this tree in medicine (Dubey *et al*., 2011a, 2011b; Dwevedi *et al*., 2015; Pandey and Negi, 2016). In Malaysia, Indonesia, and Southern China, the silvicultural practices of *N. cadamba* are mainly aimed at timber and pulp production.

Research related to the improvement of plant genotypes, silviculture, and fibre usage of this plant has been rare in comparison with that of traditional medication. The genes responsible for lignin and cell wall synthesis in *N. cadamba* have been identified (Ho *et al*., 2014; Tiong *et al*., 2014; Pang *et al*., 2015; Tchin *et al*., 2018a, 2018b; Ho *et al*., 2020), which are essential for the production of quality timber. Genetic improvement in *N. cadamba* through mutagenesis is conducted using chemical mutagens, such as ethyl methanesulfonate (EMS) (Zayed *et al*., 2014) and physical mutagens, such as gamma rays (Zanzibar and Danu, 2015). Fibre yield from this tree is suitable for pulp production (Lal *et al*., 2010).

Colchicine is an alkaloid extracted from *Colchicum autumnale* L. whole plants (Nelson *et al*., 2007) and *Gloriosa superba* L. roots *(*Ghosh *et al*., 2002*),* both from the Liliaceae family. It has been commonly used as an effective medicine for treating gout for ages (Wallace, 1961). In plant science research, colchicine is used to produce mutants, particularly autopolyploids, due to its antimitotic actions in the cell (Rego *et al*., 2011; Dixit and Chaudhary, 2014; Niu *et al*., 2016; Noori *et al*., 2017). Apart from polyploidisation, gene mutations in explants have also been reported in several studies involving colchicine (Rauf *et al*., 2006; Khosravi *et al*., 2009; Podwyszynska *et al*., 2015). Studies on the plant morphology of a large population of explants that have undergone colchicine treatment are limited because researchers often omit this area of research. It is noteworthy that colchicine has effects similar to plant growth regulators (PGRs), which influence developmental variances, either stimulatory or inhibitory (Hayashi *et al*., 2008; El-Nashar and Ammar, 2016). Furthermore, some studies compared the effects of colchicine and PGRs on plant morphology directly (Bennici *et al*., 2006; Tank and Thaker, 2014; El-Latif *et al*., 2018).

Gene or genome mutation breeding of tropical forest trees has been limited mainly due to its slow-growing habit and recalcitrance to the plant growth regulators (PGRs). These characteristics hamper the development of an efficient *in vitro* system that is fundamental to the efficiency of mutation breeding. In this study, we described the effects of colchicine application on the *in vitro* regeneration and morphological characteristics of *N. cadamba* plantlets derived from seeds and nodal segments. Information derived from this study

could pave the way for laying the foundations in the establishment and identification of improved *N. cadamba* plants, which are more superior and economically important to both the industry and environment. Hence, the objective of this study was to determine the effects of colchicine application on the *in vitro* regeneration and morphological characteristics of *N. cadamba* plantlets derived from seeds and nodal segments. To the best of our knowledge, there are no published reports on colchicine treatment of *N. cadamba*.

Materials and methods

Seed pre-treatment and surface sterilisation

After collection from the field, the processed seeds from the selected *N. cadamba* candidate plus tree (Clone CPT-N5; N02 °00.780' E112 °03.877'), were stored in a refrigerator at 4 ℃ to ensure that they were fresh and not damaged. This experiment was conducted from February 2018 to March 2019. Seeds used in all the experiments were thoroughly cleaned from debris, and only healthy seeds were selected for pre-treatment. The seeds were soaked in sterile distilled water for 24 h at 35 ℃ in a water bath in order to overcome seed dormancy before *in vitro* germination. The seeds were surface-sterilised using the first 20% (v/v) Clorox (5.0% sodium hypochlorite) with a drop of Tween-20 for two min, and secondly 70% (v/v) ethanol for 30 seconds. The seeds were also rinsed once with sterile distilled water between the two stated sterilant treatments. Finally, after carrying out the sterilisation regime as described, the seeds were further rinsed three times in sterile distilled water.

Medium preparation

The medium used for seed germination was B5 medium (Gamborg *et al*., 1968), while for shoot regeneration, the medium used was B5 medium supplemented with 1 mg/L 6-Benzylaminopurine (BAP). Both media were supplemented with 20 g/L sucrose and solidified with 8 g/L Phyto-agar. The medium pH was adjusted to 5.5 before autoclaving at 121 ℃ for 20 min.

Colchicine treatment for seeds

Seeds were treated with colchicine at three different concentrations and two durations for each concentration: 0.1%, 0.3%, and 0.5% colchicine for 24 h and 48 h, respectively. In the control treatment, sterile distilled water was used for 24 h and 48 h. To improve contact between colchicine and explants, the

explants submerged in the colchicine solution were agitated at 100 rpm on an orbital shaker. The whole process of treatment was conducted in the dark to avoid deterioration of colchicine. Seeds treated with colchicine were surface-sterilised before being cultured on basal B5 medium in a disposable Petri dish. The culture was incubated for four weeks to determine the germination percentage. Germinated seedlings were transferred to glass jars with fresh basal B5 medium for eight weeks with 4-weekly subcultures before morphological data were recorded.

Colchicine treatment for nodal segments

The following procedure was used to obtain nodal segments for colchicine treatment. Surface-sterilised seeds were inoculated onto basal B5 medium (Gamborg *et al*., 1968) for seed germination. After two months, a healthy seedling was chosen as the explant for multiple shoot regeneration. The seedlings were excised into individual nodal segments and transferred onto shoot regeneration medium (B5 medium + 1.0 mg/L BAP). After a month, individual nodal segments were excised again from newly formed shoots. This process was repeated until a sufficient number of nodal segments were attained. Finally, individual shoots were excised from shoot clumps and cultured on B5 medium for eight weeks (sub-cultured every four weeks) to eliminate the effects of BAP before colchicine treatment. The nodal segments harvested after the process, as described above, were treated with the same colchicine concentrations and durations as the treatment conditions for seeds. Treated nodal segments were cultured on shoot regeneration medium in a glass jar for eight weeks with 4-weekly subcultures before morphological data were recorded.

Culture conditions

All cultures were incubated at 25 ± 1 ℃ with a 16 h photoperiod (40 $\mu molm^{-2}s^{-1}$) provided by LEDs (Light Emitting Diodes) horticultural lighting. The cultures were incubated for four weeks to determine the surviving explants. Morphological characters of seedlings were recorded, which include plant height, number of leaves, number of shoots, and leaf greenness after eight weeks of colchicine treatment.

Experimental design and data analysis

There were eight treatments, including two controls for both seeds (100 seeds per treatment) and nodal segments (ten nodal segments per treatment). Both experiments were repeated thrice. Morphological characters of seedlings

and shoots were recorded, including plant height, stem diameter, number of nodes, number of leaves, number of shoots, and leaf greenness. Leaf greenness of this study was determined using the IRRI (International Rice Research Institute) leaf colour chart. This chart is used by the IRRI to measure the greenness of rice leaves before determining the nitrogen fertiliser requirement of rice crops (www.knowledgebank.irri.org). Data were subjected to analysis of variance (ANOVA) using IBM SPSS version 23 programme. ANOVA was carried out, followed by Duncan New Multiple Range Test (DNMRT) ($P < 0.05$).

Results

Seed and nodal segment mortality

In the first experiment, seeds were treated with different colchicine concentrations and durations before inoculation on seed germination medium, namely basal B5 medium. The seed germination percentage was the first variation assessed before the morphological characteristics (Figure 1a). The data collected showed the extent of toxicity of different concentrations of colchicine for different durations of treatment on the seeds (Figure 2). The highest seed germination percentage attained was the control treatment (distilled water, 24 h) at 15.67% (Figure 1a).

In the second experiment, uniform nodal segments derived from a seedling were treated with the same colchicine regime as the seeds in the first experiment. The highest surviving nodal segments were from the control treatment (distilled water, 24 h) at 86.67%. Meanwhile, the lowest surviving percentage of nodal segments (23.33%) was from 0.5% colchicine treatment at 48 h. In all colchicine-treated nodal segments and control, more prolonged submersion in either colchicine solution or sterile distilled water tended to increase mortality (Figure 1b). In the control treatment, buds started to emerge after the second week of culture on the shoot regeneration medium. However, colchicine-treated explants only started to produce buds after three to four weeks. Generally, nodal segments treated with more severe colchicine took longer time to regenerate (Figure 3a, 3b, 3c, and 3d). This could be due to the injury inflicted by colchicine, and therefore, time is required for the explants to recover before regeneration can take place. Explants that did not survive were bleached before they turned brown (Figure 3e).

Physical and chemical-induced mutagenesis studies often used lethal dose 50 (LD50) to determine the specific dosage of mutagens that likely to induce mutations. LD50 means the concentration or duration of mutagen treatment that

can reduce the living population of explants by half. In this study, control treatment (0.0% colchicine, 24 h, and 48 h) was used as the baseline to determine the LD 50 of colchicine-treated explants (Figure 1a and b). In seeds, half of the germination percentage of the first control (0.0% colchicine, 24 h) was 7.84%, while second control (0.0% colchicine, 48 h) was 7.16% (Figure 1a). Therefore, the LD50 for *N. cadamba* seeds was 0.3% colchicine for 48 h, 0.5% colchicine for 24 h, and 0.5% colchicine for 48 h. In nodal segments, half of the surviving of the first control (0.0% colchicine, 24 h) was 45.0%, while second control (0.0% colchicine, 48 h) was 33.35% (Figure 1b). Therefore, the LD 50 for *N. cadamba* nodal segments for the first control was 0.5% colchicine for 24 h, while the second control was 0.5% colchicine for 48 h.

Shoot length and node number

In the first experiment, when seeds were treated with different concentrations of colchicine for different durations, the results showed that the seedlings with the longest mean shoot length (2.83 cm) were from 0.0% colchicine for 24 h (control), whereas the shortest mean shoot length (1.81 cm) was from 0.5% colchicine for 24 h (Table 1). In terms of the number of nodes, 0.0% colchicine for 24 h (control) had the highest mean number of nodes per shoot (5.06), while treatment with 0.5% colchicine for 24 h had the lowest mean number of nodes per shoot (2.53). For the second experiment involving the nodal segments, the longest mean shoot length (0.73 cm) was from the control treatments, while the shortest mean shoot length (0.41 cm) was from 0.5% colchicine for 48 h (Table 2). In the case of the number of nodes, the highest mean number of nodes per shoot (2.08) was from treatment with 0.3% colchicine for 48 h, whereas treatment with 0.5% colchicine for 24 h had the lowest mean number of nodes per shoot (1.22). Therefore, morphological variation concerning the mean shoot length, either the colchicine-treated seeds or nodal segments, produced shoots shorter than those in the control treatment due to delayed growth after colchicine treatment.

Stem diameter and multiple shoot production

The mean stem diameters of shoots from all colchicine-treated seeds were larger than those of the control treatment. The largest mean stem diameter was 2.09 mm found on seedlings from seeds treated with 0.5% colchicine for 48 h, while the smallest was 1.15 mm from control for 48 h (Table 1). The stems of seedlings from the control treatment were weak and thin (Figure 2a). However, seedlings from seeds treated with colchicine had thicker and stronger stems

(Figure 2c and 2d). In the case of nodal segments, no significant difference was found in stem diameter between shoots regenerated from colchicine-treated or control nodal segments (Table 2). No morphological variation was observed in terms of stem diameter of the regenerated shoots from the colchicine-treated nodal segment. This could be due to the effect of the regeneration medium used in forcing multiple shoot regeneration from colchicine-treated nodal segments.

Seeds treated with colchicine produced multiple-shoot seedlings, while very few of the seedlings in the control treatments showed multiple shoot traits. The data showed that 0.3% colchicine for 48 h treatment induced the highest mean number of shoots per seedling (2.59) and the highest mean percentage of seedlings with multiple shoots (75.0%) (Table 1). Among all seeds treated with 0.1% colchicine for 24 h and 48 h, the mean number of shoots per seedling was lower than that of 0.3% colchicine regardless of treatment duration. This result indicates that more severe colchicine treatments (0.3% for 24 h and 48 h) tend to stimulate seeds to form a higher number of shoots per seedling. Seedlings from seeds treated with colchicine exhibited immense morphological variation through the development of seedlings with multiple shoots (Figure 2b and 2c). The extent of this variation is, therefore, colchicine-dosage dependent. However, in the nodal segment study, all nodal segments treated with colchicine from control to 0.1% colchicine for 48 h produced multiple shoots, but at 0.5% for 24 h and 48 h, only about 50% of them produced shoots even though nodal segments were cultured on the same shoot regeneration medium (Table 2). Half of the nodal segment produced only a single shoot with stunted growth. The result of nodal segment culture was in contrast with seed culture as colchicine seems to be inhibitive to the growth and development of nodal segments, but simulative to seeds.

Leaf morphology

In seeds, seedlings with the highest mean number of leaves per shoot (10.11) were from 0.0% colchicine for 24 h, while the lowest mean number of leaves per shoot (5.24) was from 0.5% colchicine for 24 h (Table 1). The mean number of leaves per shoot reduced when the severity of colchicine increased to 0.5% colchicine for 24 h but decreased on 0.5% colchicine for 48 h. In nodal segments, shoots regenerated with the highest mean number of leaves per shoot (4.15) was from 0.3% colchicine for 48 h, while the lowest mean number of leaves per shoot (2.47) was on 0.5% colchicine for 24 h. Although there was a significant difference in terms of the mean number of leaves per shoot between treatments (Table 2), no noticeable trend was observed between the number of leaves per shoot when the severity of colchicine treatment increased.

Abnormal leaves occur on the shoots of seeds and nodal segments treated with colchicine. Several types of morphologically aberrant leaves occur, such as curled leaves (Figure 2e), orbicular leaves (Figure 2f), and unexpanded leaves (Figure 3f). For both explants derived from seeds and nodal segments without colchicine treatment, no abnormal leaf was observed. Seeds treated with 0.3% colchicine for 48 h produced the highest mean percentage of the explants with abnormal leaves (45.83%) (Table 1). However, nodal segments treated with 0.5% colchicine for 48 h produced the highest mean percentage of the explants with abnormal leaves (50.00%) (Table 2). Over the passage of *in vitro* conditions, the abnormal leaves will age and senesce, and the plant may grow new leaves that are normal. This phenomenon appears to be temporary in nature. In the experiment using seeds, seedlings with darker green leaves were not significantly different (Table 1). In nodal segments, the mean percentage of darker green leaves was significant among the colchicine-treated treatments. However, none of the shoots from nodal segments in control treatments showed a darker green leaf trait. The highest mean percentage of dark green leaves (61.11%) was obtained at 0.5% colchicine for 24 h (Table 2).

Table 1. Morphological effects after colchicine treatment on seeds under *in vitro* condition

Colchi-cine (%, w/v)	Time (H)	Shoot length (cm) ± S.E	No. of nodes per shoot ± S.E	Stem diameter (mm) ± S.E	No. of shoots per seedling ± S.E	Multiple shoots (%) ± S.E	No. of leaves per shoot ± S.E	Abnormal leaves (%) ± S.E	Dark green leaves (%) ± S.E
0.0	24	2.83 ± 0.05^{a}	5.06 ± 0.07^{a}	1.34 ± 0.08^{cd}	1.07 ± 0.04^{c}	6.99 ± 4.13^{d}	10.11 ± 0.13^{a}	0.00 ± 0.00^{c}	11.06 ± 2.76^{a}
	48	2.80 ± 0.09^{a}	4.68 ± 0.12^{ab}	1.15 ± 0.02^{d}	1.04 ± 0.02^{c}	6.85 ± 4.14^{d}	9.36 ± 0.24^{ab}	0.00 ± 0.00^{c}	12.27 ± 6.38^{a}
0.1	24	2.19 ± 0.13^{bc}	3.92 ± 0.32^{cd}	1.73 ± 0.04^{abc}	1.30 ± 0.08^{bc}	15.50 ±2.57^{cd}	7.99 ± 0.49^{bcd}	16.30 ± 9.57^{abc}	12.16 ± 1.64^{a}
	48	2.65 ± 0.12^{ab}	4.29 ± 0.39^{bc}	2.06 ± 0.14^{ab}	1.25 ± 0.09^{bc}	25.00 ± 8.66^{c}	8.59 ± 0.78^{bc}	12.78 ± 3.64^{bc}	16.11 ± 3.89^{a}
0.3	24	2.26 ± 0.16^{bc}	3.29 ± 0.27^{de}	1.80 ± 0.14^{ab}	2.20 ± 0.10^{a}	53.33 ± 3.33^{b}	6.65 ± 0.58^{def}	38.89 ± 11.11^{ab}	16.39 ± 2.17^{a}
	48	1.94 ± 0.14^{c}	3.04 ± 0.22^{ef}	1.85 ± 0.15^{ab}	2.59 ± 0.18^{a}	75. 00 ± 0.00^{a}	6.08 ± 0.43^{ef}	45.83 ± 4.17^{a}	27.78 ± 2.78^{a}
0.5	24	1.81 ± 0.30^{c}	2.53 ± 0.24^{f}	1.65 ± 0.17^{bc}	2.39 ± 0.31^{a}	55.56 ± 5.56^{b}	5.24 ± 0.45^{f}	41.67 ± 17.35^{ab}	27.78 ± 11.11^{a}
	48	2.49 ± 0.11^{ab}	3.71 ± 0.11^{cde}	2.09 ± 0.18^{a}	1.59 ± 0.05^{b}	41.11 ± 4.84^{b}	7.42 ± 0.21^{cde}	23.33 ± 14.53^{abc}	38.33 ± 21.67^{a}

S.E is Standard error. Data are means of 3 replicates with 100 explants per replicate. Means followed by different alphabet denote significant differences within a column based on DNMRT (P = 0.05).

Table 2. Morphological effects after colchicine treatment on nodal segments under *in vitro* condition

Colchi-cine (%, w/v)	Time (H)	Shoot length (cm) ± S.E	No. of nodes per shoot ± S.E	Stem diameter (mm) ± S.E	No. of shoots per nodal segment ± S.E	Multiple shoots (%) ± S.E	No. of leaves per shoot ± S.E	Abnormal leaves (%) ± S.E	Dark green leaves (%) ± S.E
0.0	24	0.73 ± 0.01^{a}	1.85 ± 0.04^{a}	1.15 ± 0.02^{a}	4.81 ± 0.19^{a}	100.00 ± 0.00^{a}	3.71 ± 0.08^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
	48	0.73 ± 0.00^{a}	1.83 ± 0.07^{a}	1.16 ± 0.01^{a}	4.00 ± 0.17^{b}	100.00 ± 0.00^{a}	3.67 ± 0.13^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
0.1	24	0.66 ± 0.04^{ab}	1.88 ± 0.08^{a}	1.28 ± 0.08^{a}	2.40 ± 0.13^{d}	77.46 ± 5.64^{b}	3.77 ± 0.16^{a}	17.78 ± 1.11^{bc}	10.32 ± 5.21^{bc}
	48	0.48 ± 0.03^{cd}	1.40 ± 0.05^{b}	1.09 ± 0.04^{a}	3.04 ± 0.22^{c}	100.00 ± 0.00^{a}	2.80 ± 0.10^{b}	31.11 ± 5.88^{ab}	18.89 ± 1.11^{bc}
0.3	24	0.67 ± 0.03^{ab}	1.82 ± 0.05^{a}	1.16 ± 0.02^{a}	3.25 ± 0.26^{c}	78.33 ± 1.67^{b}	3.64 ± 0.11^{a}	28.33 ± 6.01^{ab}	35.00 ± 5.00^{ab}
	48	0.58 ± 0.05^{bc}	2.08 ± 0.23^{a}	1.28 ± 0.05^{a}	2.15 ± 0.08^{de}	73.89 ± 3.89^{b}	4.15 ± 0.45^{a}	45.56 ± 13.65^{a}	50.56 ± 12.92^{a}
0.5	24	0.47 ± 0.06^{cd}	1.22 ± 0.03^{b}	1.19 ± 0.10^{a}	1.78 ± 0.22^{ef}	50.00 ± 9.62^{c}	2.47 ± 0.17^{b}	50.00 ± 9.62^{a}	38.89 ± 5.56^{ab}
	48	0.41 ± 0.04^{d}	1.50 ± 0.10^{b}	1.19 ± 0.10^{a}	1.44 ± 0.06^{f}	44.44 ± 5.56^{c}	3.00 ± 0.19^{b}	44.44 ± 5.56^{a}	61.11 ± 20.03^{a}

S.E is Standard error. Data are means of 3 replicates with 10 explants per replicate. Means followed by different alphabet denote significant differences within a column based on DNMRT (P = 0.05).

Discussion

Screening for mutants from explants treated with mutagen (chemical or physical) using morphological markers on induced putative mutants is termed as indirect assessment. Mutants can be morphologically determined by comparing control plants and mutagen-treated plants. Many articles analysed the morphological characteristics of the population of regenerants derived from explants treated with colchicine and its control plants. The experiments evaluated various plant morphological characteristics, such as plant height, stem diameter, number of shoots, number of leaves, leaf colour, and abnormal leaf (Bennici *et al*., 2006; Amiri *et al*., 2010; Baig *et al*., 2016; Feng *et al*., 2017; El-Latif *et al*., 2018). Screening methods, such as indirect assessment is essential because the population of surviving plants treated with mutagen is large. The extent of variation in plant morphological characteristics could reflect the genetic changes and show the effectiveness of mutagen treatments.

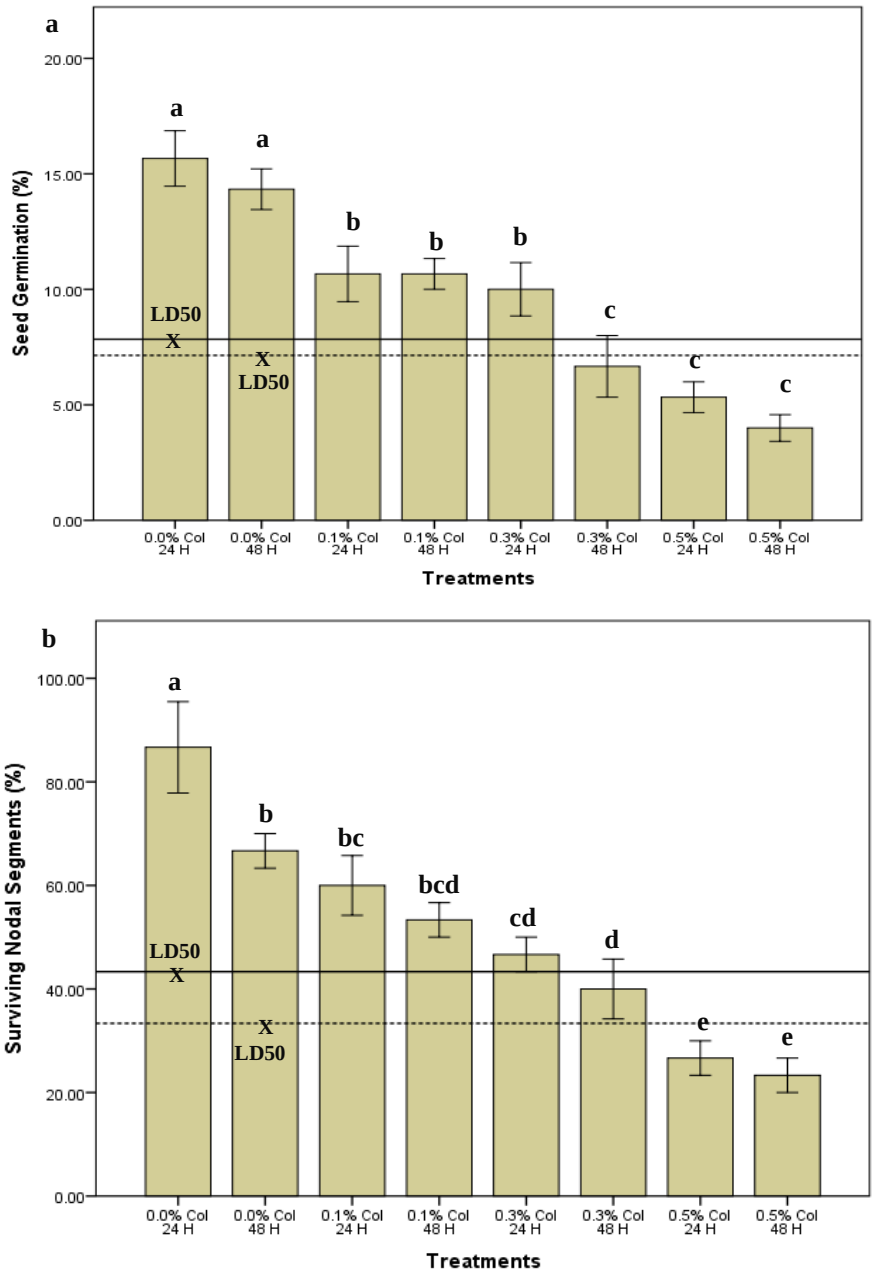

Figure 1. Effects of colchicine on (a) seed germination percentage and (b) surviving nodal segments (%). The solid line across the bars is 24 h treatment duration, The dotted line across the bars is 48 h treatment duration

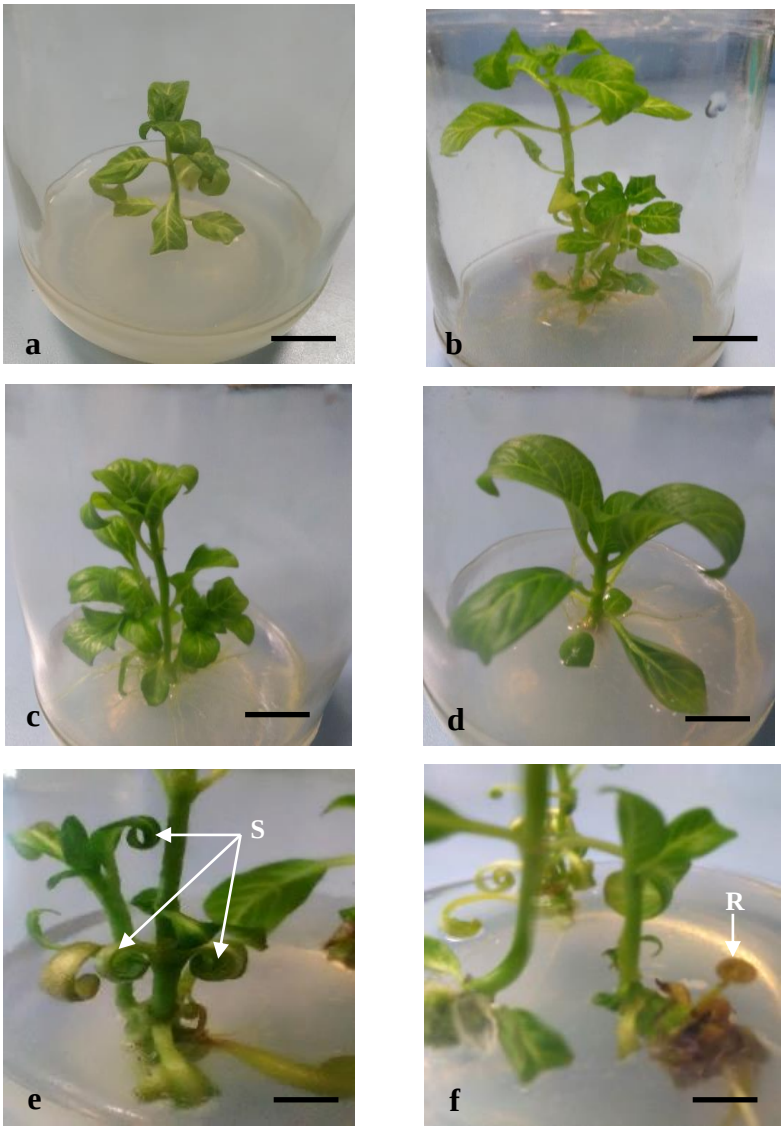

Figure 2. Seeds treated with different concentrations of colchicine and durations after 12 weeks of culture: (a) Seedling from seed treated with 0.0% colchicine for 24 h (control); (b) Seedling from seed treated with 0.1% colchicine for 24 h; (c) Seedling from seed treated with 0.3% colchicine for 24 h; (d) Seedling from seed treated with 0.5% colchicine for 48 h; (e) Seedling with curled-leaves (S) from seed treated with 0.3% colchicine for 24 h, and (f) Seedling with orbicular shape leaf (R) from seed treated with 0.5% colchicine for 24 h. (Bar = 1 cm)

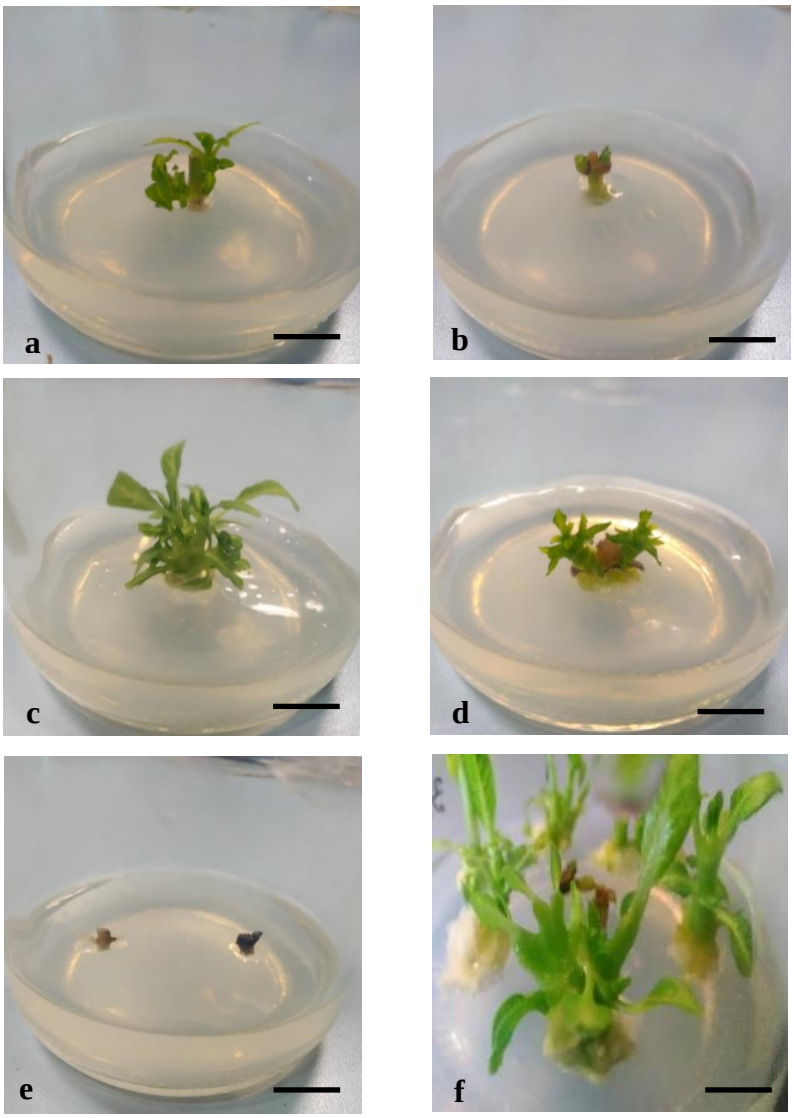

Figure 3. Nodal segments treated with different concentrations of colchicine and durations: (a) Shoots regenerated from nodal segment treated with 0.0% colchicine for 24 h (control) after four weeks of culture; (b) Shoots regenerated from nodal segment treated with 0.5% colchicine for 24 h after four weeks of culture; (c) Shoots regenerated from nodal segment treated with 0.0% colchicine for 24 h (control) after eight weeks of culture; (d) Shoots regenerated from nodal segment treated with 0.5% colchicine for 24 h after eight weeks of culture, (e) Bleached and browned explants, and (f) Shoots with unexpanded leaves from nodal segments treated with 0.5% colchicine for 48 h after eight weeks of culture. (Bar = 1 cm)

According to Martawijaya *et al*. (1989), the percentage of seed germination in the soil of fresh seed varied, but it was relatively low at approximately 25.0%. In this study, at a concentration of 0.3% colchicine for 24 h or higher, the seed germination percentage was less than 10.0%. As colchicine concentration and treatment duration increased, the germination percentage reduces (Figure 1a). In most cases, at the same colchicine concentration, the longer the treatment, the less successful the seed germination. The seeds started to germinate the first week after culturing on seed germination medium, but no new seed germination after a month of culture. One-month-old germinated seedlings were 1.0 - 1.5 cm in length, and they were transferred to the basal medium in bigger culture vessels for further growth.

The results of this study are in agreement with the results of the study of other species, such as *Bletilla striata* (Li *et al*., 2018), *Eriobotrya japonica* (Blasco *et al*., 2015), and *Sesamum indicum* (Anbarasan *et al*., 2014), where the higher the colchicine concentration, and the longer the duration of colchicine treatment, the lower the percentage of seed germination success under *in vitro* conditions. On the contrary, in the case of *ex vitro* conditions carried out by other researchers, the more severe the colchicine treatment, the higher is the success of seed germination, for example in *Calendula officinalis* (El-Nashar and Ammar, 2016), *Carica papaya* (El-Latif *et al*., 2018), and *Dianthus caryophyllus* (Roychowdhury and Tah, 2011). Therefore, different results in seed germination after colchicine treatment are species-dependent. Seed freshness and methods of colchicine treatment (*in vitro* or *ex vitro*) are also relevant contributing factors. Different species have different seed sizes, dormancy lengths, and existing protecting surfaces such as testa thickness and waxy surface. These factors contribute to the success of colchicine penetration into cells, thereby affecting cell cycles.

The seeds of *N. cadamba* are tiny and light. One million air-dried seeds only weight 38-56 g. A single fruit of *N. cadamba* contains 8000 seeds (Soerianegara and Lemmens, 1993). In nature, the seeds are transmitted by either air or water over a wide geographical area. Wild *N. cadamba* trees usually colonise river banks in the deep forests of Borneo. Plants often produce many seeds to offset the low survival rate after germination. In our opinion, the smaller the seeds, the easier for colchicine to penetrate the cell to induce mutation and polyploidisation. Apart from the abundance of seeds, seed germination either *in vitro* or *ex vitro* is relatively easier to conduct and straightforward as the medium for seed germination generally consists of basal agar medium and soil mix.

The mortality of nodal segments was positively correlated with the colchicine concentration and treatment duration (Figure 1b). This is supported by other studies on explants derived from *in vitro* systems with preformed shoot buds, such as nodal segments of *Manihot esculenta* (Zhou *et al*., 2017), shoot tips of *Citrus sp*. (Aleza *et al*., 2009), *Gerbera jamesonii* (Gantait *et al*., 2011), *Thymus persicus* (Tavan *et al*., 2015), and *Vitis* x *Muscadinia* hybrids (Xie *et al*., 2015). Unlike seeds, nodal segments are not protected from seed coats or testa. As a result, nodal segments are more permeable to colchicine, which could lead to explant mortality.

In vitro regeneration of either auxiliary or adventitious shoots requires the intervention of PGRs in order to establish an efficient regeneration protocol for a specific plant. Nodal segments with preformed shoot-buds enable the auxiliary shoot to regenerate more readily as compared to adventitious shoots. Both nodal segments and shoot tips contained buds that could be forced to regenerate in the regeneration medium. In our study, treated nodal segments were transferred to regeneration medium (B5 + 1 mg/L BAP) to ensure higher surviving explants and induce a higher number of regenerants (Fig. 3b and 3d). In *Manihot esculenta*, colchicine-treated nodal segments were transferred to shoot proliferation medium consisting of Murashige and Skoog (1962) medium supplemented with 0.05 mg/L BAP, 0.02 mg/L naphthalene acetic acid (NAA), 20 mg/L sucrose, and 6.3 mg/L agar (Zhou *et al*., 2017). This medium helps to reduce the number of chimeric (mixoploid) regenerants of *Manihot esculenta* after four cycles of subcultures of nodal segments from regenerants. Therefore, the optimised regeneration medium is vital for the production of solid polyploids.

Slower growth of shoots from either colchicine-treated seeds or nodal segments could be due to the gene or genome changes after colchicine treatment, leading to biosynthesis or biosynthesis inhibition of PGRs in the cell. However, long-term observation is needed to ascertain whether changes in plant height are due to genetics or epigenetics. In the case of the mango tree, mutants induced by EMS showed a significant reduction in plant height yielding, a population of dwarf mango plants (Rime *et al*., 2019). Further analysis showed that plants treated with a high concentration of EMS have altered plant hormones with reduced gibberellic acid and elevated abscisic acid in leaves. With changes in these plant hormones, plant morphological development might be altered. In this study, shoots regenerated from colchicine-treated nodal segments showed a reducing trend in shoot length when colchicine treatment became more severe (Table 2).

Shorter shoot length after colchicine treatment has been described in other studies, such as *Boesenbergia siphonantha* and *Curcuma inodora*

(Prabhukumar *et al*., 2015), *Dianthus caryophyllus* (Roychowdhury and Tah, 2011), *Datura stramonium* (Amiri *et al*., 2010) and *Sesamum indicum* (Anbarasan *et al*., 2014). However, some studies are in contrast with these studies, namely *Carica papaya* (El-Latif *et al*., 2018) and *Larsenianthus careyanus* (Prabhukumar *et al*., 2015). Different results in shoot length from various studies showed that the effects of colchicine could be different for different species. However, for the number of nodes, several studies have shown that colchicine-induced polyploids do not affect the number of nodes such as *Eriobotrya japonica* (Blasco *et al*., 2015), *Petunia axillaris* (Regalado *et al*., 2017) and *Thymus persicus* (Tavan *et al*., 2015). The number of nodes per shoot indicates the growth rate of a plant and plant compactness.

Our results are similar to Feng *et al*. (2017), where the stem diameters of the entire population of seedlings from seeds treated with colchicine were recorded and analysed. The largest stem diameter of *Rosa multiflora* and *Rosa roxburghii* were found in seedlings from seeds treated with 0.5% colchicine for 12 h and 6 h, respectively. Another similar study on *Dianthus caryophyllus* explants from colchicine-treated seeds also showed that these plants had marked an increase in stem diameter (Roychowdhury and Tah, 2011). However, no significant difference was found in stem diameter between shoots regenerated from colchicine-treated or control nodal segments of *N. cadamba* (Table 2). No morphological variation was observed in terms of stem diameter of the regenerated shoots from the colchicine-treated nodal segments. This could be due to the effect of the regeneration medium used in forcing multiple shoot regeneration from colchicine-treated nodal segments.

Larger stem diameter is an important morphological feature as the larger stem will result in higher productivity, especially for timber tree species. Stem diameter studies are typical in colchicine-induced polyploids. In a study involving stem diameter of colchicine-induced autopolyploid *Salix viminalis*, a destructive cross-section of the stem was conducted to analyse its bark, wood, and pith thickness. The bark and wood of the autopolyploids of *Salix viminalis* were thicker than its diploids (Dudits *et al*., 2016). Many similar studies showed that colchicine-treated explants produced polyploid plants with larger stem diameters. These studies include *Pogostemon cablin* (Widoretno, 2016) and *Vitis* sp. (Sinski *et al*., 2014).

The present result of *N. cadamba* seeds treated with colchicine is similar to *Datura stramonium* seeds treated with colchicine and trifularin under *ex vitro* conditions, where an increase in mutagen doses will lead to seedling with more branches (Amiri *et al*., 2010). However, multiple shoot formation of colchicine-treated seeds under *in vitro* conditions free from PGR has never been published to the best of our knowledge. This study demonstrated that morphological

variation with regards to the multiple shoot formation on seedlings could be due to growth regulatory effects exerted by colchicine treatment on seeds of *N. cadamba*. The regulatory effect was similar to the effects of cytokinin like BAP, which is widely used to induce more multiple shoots per explant. According to Bennici *et al*. (2006), colchicine could stimulate the growth and development process of plant cells and tissues. This effect of colchicine in respect should be further explored and must not be ignored and underestimated. However, in nodal segments, the number of shoots and multiple shoot percentage reduced when colchicine concentration increased.

Leaf morphology is an important characteristic that can be easily observed due to its abundance. Two factors affect the mean number of leaves per shoot in this study, namely the number of nodes and the number of shoots. In almost all cases, every node of *N. cadamba* has two leaves arranged on opposite sides of the stem. However, some nodes have more than two leaves, and when this phenomenon occurs, the frequent occurrence is three leaves per node. In explants producing multiple shoots, the overall growth for every shoot is slower, which results in a lower number of leaves. Some studies showed that colchicine at lower concentrations caused the plants to produce more leaves, while the elevated concentration of colchicine caused the plants to produce a smaller number of leaves (El-Nashar and Ammar, 2016; El-Latiff *et al*., 2018).

Darker green leaves have been observed in several colchicine-induced polyploids, such as *Anthurium andraeanum* (Chen *et al*., 2011), *Manihot esculentum* (Zhou *et al*., 2017), and *Pinellia ternate* (He *et al*., 2012). The darker green colour among the leaves from explants treated with colchicine can be related to the chloroplast number in cells and leaf thickness. An increase in the number of chloroplasts in the cell and thicker leaves cause the leaves to become darker in colour. The chloroplast number is often determined by the anatomical studies of the leaf stomata. Several polyploidisation studies have shown that the number of chloroplasts has increased in the stomata cells of polyploids (He *et al*., 2012; Xie *et al*., 2015; Yang *et al*., 2015; Xu *et al*., 2016; Zhou *et al*., 2017). In *Manihot esculentum* polyploids, increased photosynthetic capacities were observed (Zhou *et al*., 2017). Many published reports have shown that polyploid plants have thicker leaves (Chen *et al*., 2011; Huang *et al*., 2015; Xu *et al*., 2016; Zhou *et al*., 2017). Leaf thickness can be analysed using transverse sections of paraffin with embedded leaves (Zhou *et al*., 2017).

The finding suggested that the optimal colchicine treatment threshold for both seed and nodal segment explants was between 0.3% and 0.5% colchicine for the duration of 48 h. The colchicine-induced putative polyploids of *N. cadamba* exhibited apparent differences in growth and morphology, such as a decrease in shoot length, larger stem diameter, abnormal leaves, and darker

green leaves. Variations in plant morphological characteristics reflect the genetic changes and the effectiveness of colchicine treatment on seeds and nodal segments of *N. cadamba*. The colchicine-induced putative polyploids could facilitate the development of new germplasm for *N. cadamba*, which in turn increases the probability of selecting useful germplasm for tree planting and breeding programmes that can withstand the change in climate for future tree planting programmes.

Acknowledgements

This study was supported by funding from the Sarawak Timber Association (STA) to Universiti Malaysia Sarawak (GL/F07/STA01/2019). The Ministry of Education Malaysia is also acknowledged for granting financial assistance to the first author. The authors would like to acknowledge WTK for providing the plant materials for this research project.

References

Aleza, P., Juárez, J., Hernández, M., Pina, J. A., Ollitrault, P. and Navarro, L. (2009). Recovery and characterisation of a *Citrus clementina* Hort. ex Tan. 'Clemenules' haploid plant selected to establish the reference whole Citrus genome sequence. BMC Plant Biology, 110.

Amiri, S., Kazemitabaar, S. K., Ranjbarm, G. and Azadbakht, M. (2010). The effect of trifluralin and colchicine treatments on morphological characteristics of jimsonweed (*Datura stramonium* L.). Trakia Journal of Sciences, 8:47-61.

Anbarasan, K., Rajendran, R., Sivalingam, D. and Chidambaram, C. A. (2014). Studies on the effect of EMS and colchicine in M1 generation of Sesame (*Sesamum indicum* L.) Var. TMV3. International Letters of Natural Sciences, 11:209-214.

Baig, M. M. Q., Ahmad, T., Hafiz, I. A., Abbasi, N. and Tareen, M. J. (2016). *In vitro* improvement of characters in *Rosa spp*. through colchicine. Journal of Animal and Plant Sciences, 26:1666-1674.

Bennici, A., Schiff, S. and Moru, B. (2006). Morphogenic effect of colchicine in *Cichorium intybus* L. root explants cultured *in vitro*. Caryologia, 59:284-290.

Blasco, M., Badenes, M. L. and Naval, M. M. (2015). Colchicine-induced polyploidy in loquat (*Eriobotrya japonica* (Thunb.) Lindl.). Plant Cell, Tissue and Organ Culture 120:453-461.

Chen, C., Hou, X., Zhang, H., Wang, G. and Tian, L. (2011). Induction of *Anthurium andraeanum* ''Arizona'' tetraploid by colchicine *in vitro.* Euphytica 181:137-145.

Dixit, V. and Chaudhary, B. R. (2014). Colchicine-induced tetraploidy in garlic (*Allium sativum* L.) and its effect on allicin concentration. The Journal of Horticultural Science and Biotechnology, 89:585-591.

Dubey, A., Nayak, S. and Goupale, D. C. (2011a). A review on phytochemical, pharmacological and toxicological studies on *Neolamarckia cadamba*. Der Pharmacia Lettre, 3:45-54.

Dubey, A., Nayak, S. and Goupale, D. C. (2011b). *Anthocephalus cadamba*: A review. Pharmacognosy Journal, 2:71-76.

Dudits, D., Török, K., Cseri, A., Paul, K., Nagy, A. V., Nagy, B., Sass, L., Ferenc, G., Vankova, R., Dobrev, P., Vass, I. and Ayaydin, F. (2016). Response of organ structure

and physiology to autotetraploidization in early development of energy willow *Salix viminalis*. Plant Physiology, 170:1504-1523.
Dwevedi A., Sharma K. and Sharma, Y. K. (2015). *Cadamba*: A miraculous tree having enormous pharmacological implications. Pharmacogn. Reviews, 9:107-113.
El-Latif, F. M. A., El-Gioushy, S. F., Islam, S. E. and Zakry, T. A. (2018). Impact of papaya seed soaking in different BA, colchicine and EMS solutions on germination, growth and chromosomal behaviour. Asian Journal of Biotechnology and Genetic Engineering, 1:1-17.
El-Nashar, T. I. and Ammar, M. H. (2016). Mutagenic influences of colchicine on phenological and molecular diversity of *Calendula officinalis* L. Genetics and Molecular Research, 15.
Feng, H., Wang, M., Cong, R. and Dai, S. (2017). Colchicine- and trifluralin-mediated polyploidisation of *Rosa multiflora* Thunb. var. inermis and *Rosa roxburghii* f. normalis. *The* Journal of Horticultural Science and Biotechnology, 92:279-287.
Gamborg, O. L., Miller, R. A. and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cell. Experimental Cell Research, 50:151-158.
Gantait, S., Mandal, N., Bhattacharyya, S. and Das, P. K. (2011). Induction and identification of tetraploids using *in vitro* colchicine treatment of *Gerbera jamesonii* Bolus cv. Sciella. Plant Cell, Tissue and Organ Culture, 106:485-493.
Ghosh, B., Mukherjee, S., Jha T. B. and Jha, S. (2002). Enhanced colchicine production in root cultures of *Gloriosa superba* by direct and indirect precursors of the biosynthetic pathway. Biotechnology Letters, 24:231-234.
Hayashi, L., Yokoya, N. S., Kikuchi, D. M. and Oliveira, E. C. (2008). Callus induction and micropropagation improved by colchicine and phytoregulators in *Kappaphycus alvarezii* (*Rhodophyta, Solieriaceae*). Journal of Applied Phycology, 20:653-659.
He, L., Ding, Z., Jiang, F., Jin, B., Li, W., Ding, X., Sun J. and Lv, G. (2012). Induction and identification of hexadecaploid of *Pinellia ternate.* Euphytica, 186:479-488.
Ho, W. S., Pang, S. L. and Julaihi, A. (2014). Identification and analysis of expressed sequence tags present in xylem tissues of kelampayan (*Neolamarckia cadamba* (Roxb.) Bosser). Physiology and Molecular Biology – Plants, 20:393-397.
Ho, W. S., Pang, S. L. and Ling, K. H. (2020). Silviculture, Genetics and Molecular Breeding of *Neolamarckia cadamba* (Kelampayan) in Sarawak. ISBN 978-967-2298-28-1. UNIMAS Publisher.
Huang, R., Liu, D., Zhao, M., Li, Z., Li, M. and Sui, S. (2015). Artificially induced polyploidisation in *Lobularia maritima* (L.) Desv. and its effect on morphological traits. HortScience, 50:636-639.
Khosravi, A. R., Kadir, M. A., Kadzemin, S. B., Zaman, F. Q. and De Silva, A. E. (2009). RAPD analysis of colchicine induced variation of the *Dendrobium* Serdang beauty. African Journal of Biotechnology, 8:1455-1465.
Krisnawati, H., Kallio, M. and Kannine, M. (2011). *Anthocephalus cadamba* Miq.: Ecology, Silviculture and Productivity. Center for International Forestry Research (CIFOR), Bogor, Indonesia.
Lal, M., Dutt, D., Tyagi, C. H., Upadhyay, J. S. and Upadhyay, S. (2010). Characterisation of *Anthocephalus cadamba* and its delignification by kraft pulping. TAPPI Journal, 9:30-37.
Li, M., Ding, B., Huang, W., Pan, J., Ding, Z. and Jiang, F. (2018). Induction and characterisation of tetraploids from seeds of *Bletilla striata* (Thunb.) Reichb.f. BioMed Research International, 2018:1-8.
Martawijaya, A., Kartasujana, I., Mandang, Y. I., Prawir, S. A. and Kadir, K. (1989). Atlas kayu Indonesia Jilid II. Pusat Penelitian dan Pengembangan Hasil Hutan, Bogor, Indonesia.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiolgia Plantarum, 15:473-497.

Nelson, L. S., Shih, R. D. and Balick, M. J. (2007). Handbook of Poisonous and Injurious Plants. 2nd ed, Springer, USA.

Niu, L., Tao Y., Chen, M., Fu, Q., Dong, Y., He, H. and Xu, Z. (2016). Identification and characterisation of tetraploid and octoploid *Jatropha curcas* induced by colchicine. Caryologia, 69:58-66.

Noori, S. A. S., Norouzi, M., Karimzadeh, G., Shirkool, K. and Niazian, M. (2017). Effect of colchicine-induced polyploidy on morphological characteristics and essential oil composition of ajowan (*Trachyspermum ammi* L.). Plant Cell, Tissue and Organ Culture, 130:543-551.

Pandey, A. and Negi, P. S. (2016). Traditional uses, phytochemistry and pharmacological properties of *Neolamarckia cadamba*: A review. Journal of Ethnopharmacology, 181:118-135.

Pang, S. L., Ho, W. S., Mat-Isa, M. N. and Julaihi, A. (2015). Gene discovery in the developing xylem tissue of a tropical timber tree species: *Neolamarckia cadamba* (Roxb.) Bosser (Kelampayan). Tree Genetics & Genomes, 11:47.

Podwyszynska, M., Gabryszewska, E., Dyki, B., Stepowska, A. A., Kowalski, A. and Jasinski, A. (2015). Phenotypic and genome size changes (variation) in synthetic tetraploids of daylily (Hemerocallis) in relation to their diploid counterparts. Euphytica, 203:1-16.

Prabhukumar, K. M., Thomas, V. P., Sabu, M., Prasanth, A. V. and Mohanan, K. V. (2015). Induced mutation in ornamental gingers (Zingiberaceae) using chemical mutagens viz. colchicine, acridine and ethyl methanesulphonate. Journal of Horticulture, Forestry and Biotechnology, 19:18-27.

Rauf, S., Khan, I. A. and Khan, F. A. (2006). Colchicine-induced tetraploidy and changes in allele frequencies in colchicine-treated populations of diploids assessed with RAPD markers in *Gossypium arboreum* L. Turkish Journal of Biology, 30:93-100.

Regalado, J. J., Carmona-Mart ń, E., Querol, V., Vel éz, C. G., Encina, C. L. and Pitta-Alvare, S. I. (2017). Production of compact petunias through polyploidisation. Plant Cell, Tissue and Organ Culture, 129:61-71.

Rego, M. M., Rego, E. R., Bruckner, C. H., Finger, F. L. and Otoni, W. C. (2011). *In vitro* induction of autotetraploids from diploid yellow passion fruit mediated by colchicine and oryzalin. Plant Cell, Tissue and Organ Culture, 107:451-459.

Rime, J., Dinesh, M. R., Sankaran, M., Shivashankara, K. S., Rekha, A. and Ravishankar, K. V. (2019): Evaluation and characterisation of EMS derived mutant populations in mango. Scientia Horticulturae, 254:55-60.

Roychowdhury, R. and Tah, J. (2011). Chemical mutagenic action on seed germination and related agro-metrical traits in M1 *Dianthus generation*. Current Botany, 2:19-23.

Sinski I., Bosco D. D., Pierozzi N. I., Maia, J. D. G., Ritschel, P. S. and Quecini, V. (2014): Improving *in vitro* induction of autopolyploidy in grapevine seedless cultivars. Euphytica, 196:299-311.

Soerianegara, I. and Lemmens, R. H. (1993) Plant resources of South-East Asia 5(1): Timber Trees: Major Commercial Timbers. Pudoc Scientific Publishers, Wageningen, Netherlands.

Tank, J. G. and Thaker, V. S. (2014). Systemic control of cell division and endoreduplication by NAA and BAP by modulating CDKs in root tip cells of *Allium cepa*. BioMed Research International, 2014:1-13.

Tavan, M., Mirjalili, M. H. and Karimzadeh, G. (2015). *In vitro* polyploidy induction: changes in morphological, anatomical and phytochemical characteristics of *Thymus persicus* (Lamiaceae). Plant Cell, Tissue and Organ Culture, 122:573-583.

Tchin, B. L., Ho, W. S. and Pang, S. L. (2018a). Isolation and characterisation of *cinnamate 4-hydroxylase* (*C4H*) gene controlling the early stage of phenylpropanoid biosynthetic pathway in developing xylem tissues of kelampayan (*Neolamarckia cadamba*, Rubiaceae). Asian Journal of Agriculture and Biology, 6:278-286.

Tchin, B. L., Ho, W. S. and Pang, S. L. (2018b). Isolation and *in silico* characterisation of full-length cinnamyl alcohol dehydrogenase gene involved in lignin biosynthesis in *Neolamarckia cadamba*. Journal of Applied Biology and Biotechnology, 6:1-5.

Tiong, S. Y., Ho, W. S., Pang, S. L. and Ismail, J. (2014). *In silico* analysis of *cellulose synthase* (*NcCesA1*) gene in developing xylem tissues of *Neolamarckia cadamba*. American Journal of Bioinformatics, 3:30-44.

Wallace, S. L. (1961). Colchicine - Clinical pharmacology in acute gouty arthritis. The American Journal of Medicine, 439-448.

Widoretno, W. (2016). *In vitro* induction and characterisation of tetraploid Patchouli (*Pogostemon cablin* Benth.) plant. Plant Cell, Tissue and Organ Culture, 125:261-267.

Xie, X., Aguero, C. B., Wang, Y. and Walker, M. A. (2015). *In vitro* induction of tetraploids in *Vitis* x *Muscadinia* hybrids. Plant Cell, Tissue and Organ Culture, 122:675-683.

Xu, C., Huang, Z., Liao, T., Li, Y. and Kang, X. (2016). *In vitro* tetraploid plants regeneration from leaf explants of multiple genotypes in *Populus*. Plant Cell, Tissue and Organ Culture, 125:1-9.

Yang, N., Rong, E., Li, Q., Dong, J., Du, T., Zhao, X. and Wu, Y. (2015). Tetraploid Induction and Identification of *Gossypium arboreum*. Agricultural Science, 6:436-444.

Zayed, Z. M., Ho, W., Pang, S. and Ahmad, F. B. (2014). EMS-induced mutagenesis and DNA polymorphism assessment through ISSR markers in *Neolamarckia cadamba* (kelampayan) and *Leucaena leucocephala* (petai belalang). European Journal of Experimental Biology, 4:156-163.

Zanzibar, M. and Danu. (2015). Aplikasi iradiasi sinar gamma untuk peningkatan produktivitas tanaman jabon putih. *In* Bunga Rampai Teknologi Pembenihan dan Pembibitan Jabon Putih (*Neolamarckia cadamba* (Roxb.) Bosser) (ed. N. Mindawati, I. Mansur and P. Setio). Forda Press, Indonesia, pp.137-157.

Zhou, H. W., Zeng, W. D. and Yan, H. B. (2017). *In vitro* induction of tetraploids in cassava variety 'Xinxuan 048' using colchicine. Plant Cell, Tissue and Organ Culture, 28:723-729.

(Received: 10 July 2020, accepted: 30 December 2020)