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Research Article
Mutagenic Effects of Ethyl Methanesulfonate on Morphological and Growth Characteristics of *Neolamarckia cadamba* Plantlets

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Abstract

Background and Objective: *Neolamarckia cadamba* (Roxb.) Bosser is an economically important timber species used for light construction due to its light hardwood characteristic. Ethyl methanesulfonate (EMS) is a widely used chemical mutagen in plants as it gives a high frequency of point mutations. Hence, this study aimed to determine the lethal concentration 50 (LC_{50}) of EMS and the mutagenic effects of EMS on growth characteristics and morphological variations of *N. cadamba* derived from nodal explants. **Materials and Methods:** Seeds derived from the candidate plus tree N5 were germinated *in vitro*. The nodal explants excised from the plantlets were used as the material for *in vitro* mutagenesis with different concentrations of EMS (0.1, 0.3, 0.6 and 1%) and durations (1, 2, 3 and 4 hrs). **Results:** The findings showed that the survival percentage was significantly reduced as both EMS concentration and treatment duration increased. The LC_{50} was 1.3% for 2 hrs, 0.4% for 3 hrs and 0.3% for 4 hrs. Various responses were observed in the EMS-treated explants. These include reduced and slower shoot induction, reduced number of shoot formations, reduced callus formation and lower plant height. Morphological abnormalities were also identified among the survived explants, such as unifoliate orbicular leaves, pointy leaves, small apical leaves, stunted shoot growth and distorted shoot formation. **Conclusion:** The present findings show that nodal explants can be used for *in vitro* EMS mutagenesis besides seeds as the most widely used material for mutation study. The putative mutants derived from this study could be used for future breeding and genomics research of *N. cadamba*.

Key words: *Neolamarckia cadamba*, ethyl methanesulfonate, *in vitro* mutagenesis, nodal explant, *in vitro* culture, tree breeding, antineoplastic agent

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Neolamarckia cadamba (Roxb.) Bosser, commonly known as *Kelampayan* in Sarawak, is a tropical deciduous tree with light hardwood characteristics that belongs to the Rubiaceae family. It has tremendous economic and ecological values. *Neolamarckia cadamba* has been identified as a promising plantation tree species for planted forest development in Malaysia[1-4]. Based on the chemical and physical properties analysis, *N. cadamba* wood was comparable to rubberwood and suitable for wood composite feedstock[5]. Apart from being used as timber products, it is also a well-known multi-functional plant recently cultivated as an emerging woody forage due to its high biomass yield and rich bioactive components[6]. It has been reported to have potential pharmacological significances, including antidiabetic, antitumour, anti-inflammatory and antidiarrheal[7]. Parts of the plant consisting of leaves, bark, fruits and roots are used in treating various diseases, such as fever, inflammation, ulcer, diarrhoea, gastric disorder, anaemia, skin diseases and uterine complaints[8-10].

Mutation breeding techniques using ethyl methanesulfonate (EMS) for plant improvement purposes are not new to the scientific world and have been studied intensively, especially in essential crops species, such as rice[11-13] and wheat[14,15]. The key goal of mutation-based breeding is to cultivate and refine well-adapted plant varieties by changing some significant traits to maximise their production and yield[16]. The usage of EMS as chemical mutagen has been reported to be effective in inducing phenotypically distinct new cultivars. It has also been regarded as an efficient means of supplementing existing germplasm for cultivar improvement in breeding programmes[17].

Ethyl methanesulfonate ($C_3H_8O_3S$, 124.16 g mol^{-1}) is a chemical mutagen commonly classified as an antineoplastic agent with alkylating properties, a possible carcinogenic to human, genotoxin and teratogenic agent[18-22]. Physically, EMS is described and obtainable as a clear colourless liquid at 20°C. It has a 1.206 g mL^{-1} density, which is denser than water (0.9982 g mL^{-1} at 20°C) and has a boiling point of 213-213.5°C at 761 mmHg. The half-life reduces as the temperature rises and the hydrolysis rate is unaffected by varying pH[23]. EMS is reported to cause point mutations[24]. According to Alonso and Ecker[25], some of the effects of the point mutation in plants are known as neomorphic (formation of a new or novel function of a gene), hypermorphic (increased function of a gene) and hypomorphic effects (decreased function of a gene). EMS chemically alters DNA through random reaction and modification in phosphate groups of DNA and oxygen available in nucleotide bases such as O_6 in guanine and O_4 in thymine[26]. EMS leads to changes in bases and mismatched base pairing of the nucleotides, such as the pairing of O_6-ethylguanine with thymine (T) instead of cytosine (C)[27,28]. Various studies claimed that EMS leads to the substitution of C/G to Adenine (A)/T pairing as EMS induces pairing changes between G-C into G-T[27,29,30].

EMS was reported to give immediate effect causing mispairing of O-alkyl-guanine with uracil during RNA synthesis before subsequent mispairing with thymine during the synthesis of DNA[31]. EMS caused pleiotropic effects of mutated genes or mutations on different loci within the genome[32,33]. Among the most frequently reported effects of EMS mutagenesis would be morphological mutations. As observed in *Fragaria*× *ananassa* Duch. (cv. Camarosa) strawberry, EMS treatment produced morphological variation in fruit, such as size, shape, colour, number of fruits produced and day for fruit maturation[34]. However, EMS treatment in most cases leads to high mortality percentage of treated samples due to the toxicity of EMS as a chemical mutagen, which is reported in many studies. The mortality rate is proportional to both concentration and treatment period as reported in rice[35,36] and tomato[37]. It is observed that EMS also caused inhibitory effects on many types of samples, such as delay in terms of initial growth or response toward the mutagenesis treatment. The common initial responses are the number of seed germination, shoot induction from nodal culture or callus proliferation. These effects were reported in many studies as seen in the delay of seed germination of *Bergenia ciliata*[38] and delay of shoot induction percentage in *Saintpaulia*[39]. Another interesting observation is related to changes in ploidy number was also reported in EMS-induced Chinese cabbage cv. 'Fukuda 50' in which the microspores were treated with EMS to produce homozygous double haploid mutants[40]. A similar study has also been reported in Japonica rice cv. 'Mankeumbyeo' EMS-treated seeds that produced stable mutants with the potential to be used as new breeding materials[41].

Our previous findings showed that *N. cadamba* trees are genetically less diverse compared to other tree species[1,42]. Cross-pollination between genetically related trees could reduce seed quality through inbreeding. If these seeds are used widely, it may reduce genetic diversity and over time, make the plantings less resilient to environmental changes. Hence, a pilot study has been conducted to broaden the genetic base of *N. cadamba* through EMS-induced mutagenesis[43]. *Neolamarckia cadamba* seeds were treated with three different EMS doses (0.1, 0.3 and 0.6%) for 3 hrs.

They reported that the seed germination percentage and seedling height were decreased with the increasing of EMS doses. Apart from that, 0.6% EMS treated seeds exhibited the highest level of genetic variability than other EMS treated seeds as revealed by using inter simple sequence repeat (ISSR) markers[43].

Our understanding of the effects of *in vitro* mutagenesis on morphology and growth performance of *N. cadamba* remains limited. To our knowledge, there is no report on *in vitro* mutagenesis using EMS in *N. cadamba*. Hence, an in-depth study was conducted to optimise the EMS mutagenesis treatment's effectiveness to ensure a higher mutation rate using homogenous plant materials (i.e., nodal explant). The objectives of our study were to determine the lethal concentration 50 (LC_{50}) of EMS and the effects of different concentrations and durations of EMS on growth characteristics and morphological variations of *N. cadamba* derived from nodal explants.

MATERIALS AND METHODS

Study area: The present study was carried out at the Forest Genomics and Informatics Laboratory (fGiLab), Faculty of Resource Science and Technology, UNIMAS, Sarawak, from September, 2018-April, 2021.

Culture media preparation: Four types of culture media were prepared based on Mok and Ho[44]. For seed germination, seeds were cultured on full-strength Gamborg's B5 basal media[45] without plant growth regulators (PGRs). For direct shoot induction and shoot multiplication from nodal explants, full-strength Gamborg's B5 supplemented with 1.0 and 0.8 mg L^{-1} 6-benzyl amino purine (BAP) were used, respectively. Meanwhile, for rooting, plantlets were introduced into half-strength B5 media supplemented with 0.1 mg L^{-1} paclobutrazol (PBZ). All media were supplemented with 20 g L^{-1} sucrose and 8 g L^{-1} Phyto agar. The media were adjusted to pH 5.5±0.1 and autoclaving (Hirayama) at 121°C and 15 psi for 20 min.

Plant materials and *in vitro* culture establishment: Nodal explants of *in vitro* cultures were preferred as the plant material for the mutagenesis of *N. cadamba*. For establishing the *in vitro* culture, the selected candidate plus tree (CPT) clone N5 from the Kelampayan planted forest in Kanowit, Sarawak, (N02°00.780' E112°03.877') was chosen and seeds were obtained as the starting plant material. Upon collection, fresh and processed seeds were cleaned and stored at 4°C under dark conditions for prolonged freshness and good storage until required. Once required, seeds were added into each 1.5 mL tubes containing preheated 45°C sterile distilled water (dH_2O). Seed mixtures in tubes were mixed thoroughly by pipetting to ensure complete coverage and these tubes were then incubated at a 45°C water bath for 30 min. Then, under sterile laminar hood condition, dH_2O was pipetted out completely. The sterilisation process proceeded with 15% commercial bleach (Clorox)+2 drops of Tween 20 for 15 min, rinsing with sterile dH_2O for once, short immersion in 70% ethanol for 30 sec (vigorous pipetting) and rinsing with sterile dH_2O for four times. Seeds were then air-dried and directly cultured on petri dishes containing seed germination media for 90 days. Germinated *in vitro* seedlings were transferred into culture bottles containing the same media within 90 days after culture.

Culture condition: Cultures were kept in the Forest Genomics and Informatics Laboratory (fGiLab), Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS), under sterile conditions. Under optimum circumstances, the temperature of the laboratory was maintained at room temperature (25±2°C) and LED growth lights were used for growing *in vitro* cultures. Specifically, for seed germination, sterile cultures were kept at room temperature and in complete darkness for the initial seven days after culture (DAC). After 7 DAC, cultures were kept under growth light (approx. 1,000 lux) for 16:8 hrs of light and dark photoperiod for three months. Constant culture condition was applied thereafter for shoot induction, shoot multiplication and rooting stages. For acclimatisation, cultures were kept under similar conditions for 30 days before being gradually introduced into *ex vitro* conditions, e.g., higher light intensity (approx. 23,000 lux) and lower external atmospheric humidity.

Shoot induction and multiplication: After 90 days after culture, three aseptic *in vitro* seedlings with at least four nodes with healthy characteristics were selected and labelled as Clone 1 (C_1), Clone 3 (C_3) and Clone 5 (C_5). Nodal explants were then excised and were cultured on a Petri dish containing shoot induction media for direct shoot organogenesis. After 2 weeks, explants were transferred into culture bottles containing the same shoot induction media for another 2 weeks. Regenerated shoots (height ≈1 cm) from nodal cultures were then excised and cultured into shoot multiplication media for a period of 3-4 weeks. Multiplications of shoots were repeated until sufficient homogenous nodal explants for EMS mutagenic treatments.

EMS mutagenic treatment: Chemical mutagen ethyl methanesulfonate (Sigma-Aldrich) and a completely randomised experimental design were used in the present study. For *in vitro* EMS mutagenesis using nodal explants, all the replicates consisted of four treatments and control with a total of 180 nodal explants per replicate. The treatment parameters were four gradually increasing concentrations of EMS (i.e., 0 (control), 0.1, 0.3, 0.6 and 1.0% (v/v) at four different durations (i.e., 1, 2, 3 and 4 hrs). Nodal explants for the mutagenic treatments were excised from two to three months old *in vitro* regenerated shoots with three to four nodes per explant. Freshly excised nodal explants with approximately 3 mm width and 1 cm length were added into sterile tubes and EMS solutions at different concentrations were then pipetted into each tube, respectively. Tubes were then placed securely on a rotary shaker and were shaken at 150 rpm for the specified duration of treatments as stated previously. After the treatment, nodal explants were taken out using sterile forceps and directly soaked in tubes containing 0.1 M sodium thiosulphate for 5 min, rinsing in sterile dH_2O three times and were let to soak in liquid basal B5 media before being cultured on a Petri dish containing solid shoot inducing media. Observations and data collections were continuously made up to 90 DAC and each surviving nodal explant was transferred into fresh shoot inducing media every 3-4 weeks. After the 4th week, the regenerated new shoots were sub-cultured by excising the nodal explants from the regenerated shoots and cultured on the shoot induction media again for 3-4 weeks. The sub-culturing stage was repeated five times. Then, the regenerated shoots were excised and grown on shoot multiplication media for 3-4 weeks.

Rooting and acclimatisation of regenerated explants: After the shoot multiplication stage, healthy shoots with approximately 2 cm height were excised, detached from the shoot clumps and transferred into new culture bottles containing rooting media. Each culture bottle had three shoots and was allowed to be grown for 4 weeks. Then, rooted shoots were introduced into the *ex vitro* environment. For acclimatisation purposes, the samples were transferred into each 10×8 cm degradable non-woven bag containing topsoil placed in a 15 cm tall plastic drinking cup. The lids of the cups were kept closed for three to four weeks and were gradually opened until each sample is well hardened.

Data analysis: Data in this study were collected from all mutation lines (C_1, C_3 and C_5) and were analyzed through the multivariate test and Tukey's Honest Significant Difference test (Tukey's HSD) at $p<0.05$ using SPSS (version 22). The Lethal Concentration (LC_{50}) of EMS on *N. cadamba* cultures were calculated and determined based on the percentage of survival/lethality of treated nodal explants using probit analysis.

RESULTS

Survival percentage and lethal concentration 50 (LC_{50}) identification: The mean survival percentage of three *N. cadamba* lines at four different concentrations of EMS (0.1, 0.3, 0.6 and 1%) and durations (1, 2, 3 and 4 hrs) is summarised in Table 1. The survival percentages were recorded progressively from as early as 14 days after culture (DAC) until 90 DAC (12 weeks). Nodal explants showing no response within 90 DAC were considered lethal (dead) and were not carried forward for subsequent micropropagation stages. The EMS usage as a chemical mutagen in this study was expected to cause increasing lethality proportional to the increasing concentrations and treatment durations. After 12 weeks of culture, the survival percentages for all the EMS concentrations and durations were significantly lower than control samples (100%) at $p = 0.05$ according to Duncan Multiple Range Test (Table 1). The lowest survival percentage was recorded at 0%, which showed a total of 100% lethality of nodal explants, which was observed from 3 and 4 hrs of 1% EMS treatments. This result indicates that the combination of higher EMS concentration and longer treatment duration leads to a higher lethality percentage.

For the determination of lethal concentration (LC_{50}) of EMS on nodal explants of *N. cadamba*, the concentrations of EMS, which were initially in percentage (v/v), were converted into log concentrations. Similarly, the lethality percentage of various treatment durations was converted into probit values. Graphs of lethality probit values responding to log concentrations of EMS were plotted (Fig. 1 and 2). The LC_{50} values were determined by taking antilog values of the log concentration values at a probit value of 5.00. The LC_{50} value for 1 hr EMS treatment could not be accurately estimated due to the high survival percentage observed within 14 DAC (Table 1 and Fig. 1a). Based on the graphs of lethality probit values (Fig. 1b, Fig. 2a-b), the LC_{50} of EMS treatments were 1.2589, 0.3715 and 0.3311% for 2, 3 and 4 hrs treatment, respectively.

Effects of EMS mutagenesis on shoot induction and multiplication: EMS-treated nodal explants at various concentrations and durations showed different responses

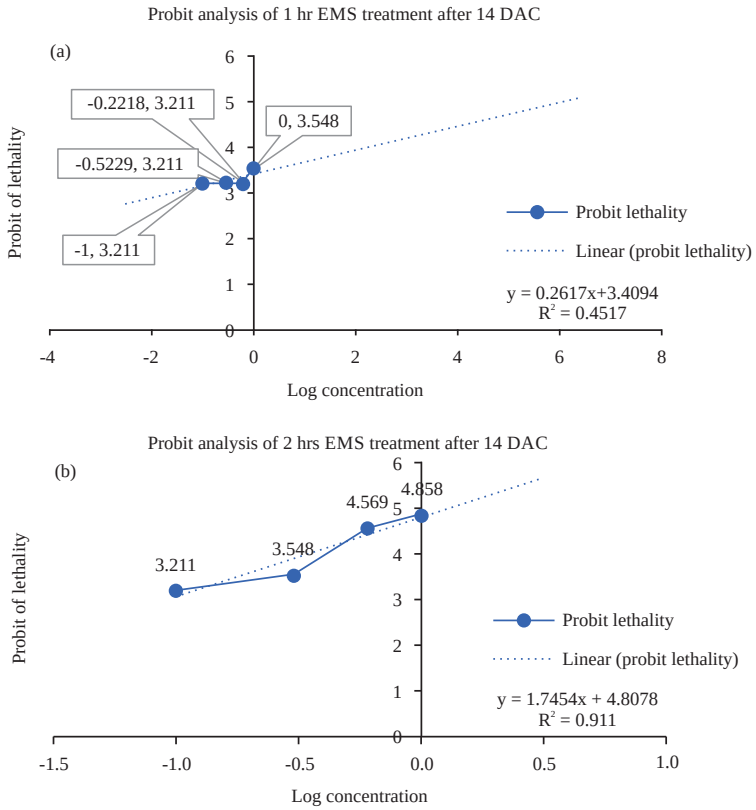

Fig. 1(a-b): Trend of lethality probit values responding to log concentrations of EMS treatments at (a) 1 and (b) 2 hrs
Data shown were collected after 14 DAC

Table 1: Survival (%) of *N. cadamba* after EMS mutagenesis treatments

		Survival (%)						
			2 weeks		8 weeks		12 weeks	
Duration (hrs)	EMS concentration (%)	N	n	(%)	n	(%)	n	(%)
1	0	27	27	100.0^{a}	27	100.0^{a}	27	100.0^{a}
	0.1	27	26	96.3^{a}	21	77.8^{b}	12	44.4^{b}
	0.3	27	26	96.3^{a}	22	81.5^{ab}	9	33.3^{b}
	0.6	27	26	96.3^{a}	20	74.1^{b}	10	37.0^{b}
	1	27	25	92.6^{a}	18	66.7^{b}	9	33.3^{b}
2	0	27	27	100.0^{a}	27	100.0^{a}	27	100.0^{a}
	0.1	27	26	96.3^{a}	23	85.2^{a}	12	44.4^{b}
	0.3	27	25	92.6^{a}	22	81.5^{a}	10	37.0^{b}
	0.6	27	18	66.7^{b}	15	55.6^{b}	11	40.7^{b}
	1	27	15	55.6^{b}	8	29.6^{c}	2	7.4^{c}
3	0	27	27	100.0^{a}	27	100.0^{a}	27	100.0^{a}
	0.1	27	26	96.3^{a}	17	63.0^{b}	7	25.9^{c}
	0.3	27	18	66.7^{b}	15	55.6^{b}	12	44.4^{b}
	0.6	27	5	18.5^{c}	5	18.5^{c}	2	7.4^{d}
	1	27	3	11.1^{c}	1	3.7^{c}	0	0.0^{d}
4	0	27	27	100.0^{a}	27	100.0^{a}	27	100.0^{a}
	0.1	27	26	96.3^{a}	17	63.0^{b}	12	44.4^{b}
	0.3	27	13	48.1^{b}	9	33.3^{c}	8	29.6^{bc}
	0.6	27	6	22.2^{c}	7	25.9^{c}	5	18.5^{cd}
	1	27	2	7.4^{c}	0	0.0^{d}	0	0^{d}

Means within a column with the same letter are not significantly (DMRT, $\alpha = 0.05$)

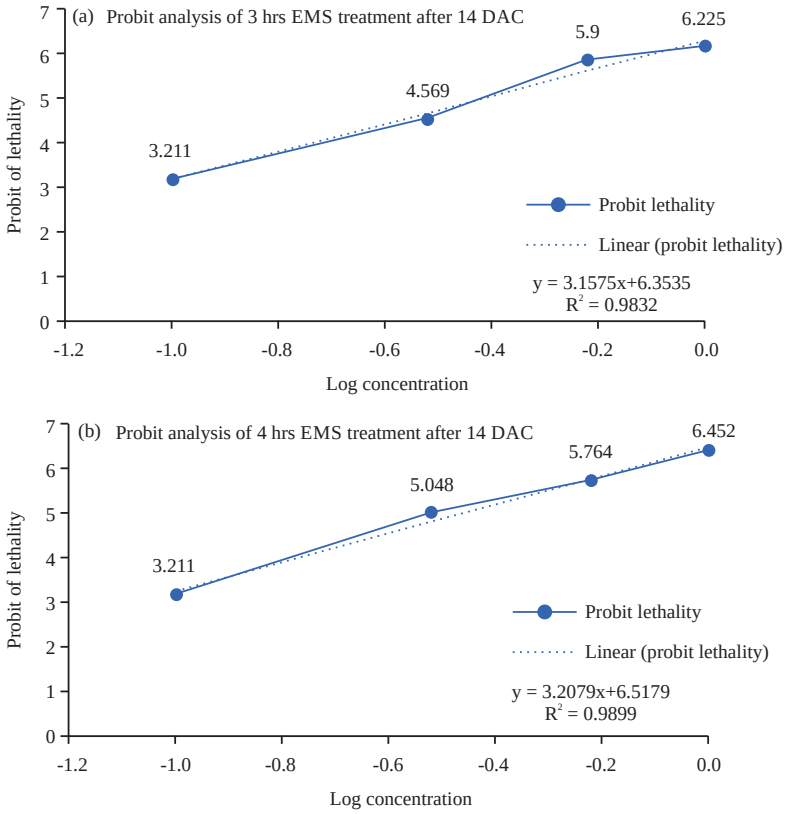

Fig. 2(a-b): Trend of lethality probit values responding to log concentrations of EMS treatments at (a) 3 and (b) 4 hrs
Data shown were collected after 14 DAC

toward the EMS treatments with comparison to untreated explants in the control samples (Fig. 3). In control samples, nodal explants remained in green colour throughout the observation period and upon culturing on B5 media supplemented with 1 mg L^{-1} BAP, a direct shoot organogenesis process could be seen to be successfully induced within the first 7-14 DAC. The axillary meristems were observed to show approximately 1-2 mm expansion and the development of micro shoots from axillary buds located at both axils was initiated. Under constant medium, continuous elongation and increased heights of the shoot stem were observed within 42 DAC (Fig. 4a-l). Untreated nodal explants in control samples showed a consistent percentage of shoot inductions. On average, the shoots induced ranged from 2.48-3.11 within 4 weeks and 5.22-9.07 within 56 DAC in control samples (Table 2).

In the present study, nodal explants of *N. cadamba* were cultured only on B5 media supplemented with 1 mg L^{-1} BAP to assess differences in response. The comparison showed that the number of shoots formed in control samples was within the range of the mean number of shoots and remained constant in subsequent sub-cultures. Meanwhile, some EMS-treated nodal explants showed a different number of shoot multiplications after each sub-culture. For instance, treated nodal explants in 1 hr EMS treatment that survived the process showed generally high percentages (%) of shoot inductions within 28 DAC, i.e., 88.9 (0.1%), 92.6 (0.3%), 85.2 (0.6%) and 70.4% (1%) (Table 2). By comparing to the shoot induction percentage of control samples, only nodal explants treated with 1% showed significantly lower shoot induction (%), according to Tukey's HSD. As the treatment duration and concentration of EMS increases, the declining trend of shoot induction percentage became clearer.

Shoot formations were observed to be delayed in some EMS-treated nodal explants, albeit staying in green colour. An example could be seen in Fig. 3, whereby the nodal explant treated with 0.6% EMS showed delayed shoot organogenesis compared to untreated explants in control samples. The nodal explants were also observed to show browning within the first 14 DAC but were then observed to have shoot forming within 28 DAC. In another example, explants were also recorded to have survived the treatments and showed shoot growth within 14 and 28 DAC, nonetheless showing browning and finally lethal within 84 DAC. The final survival percentage

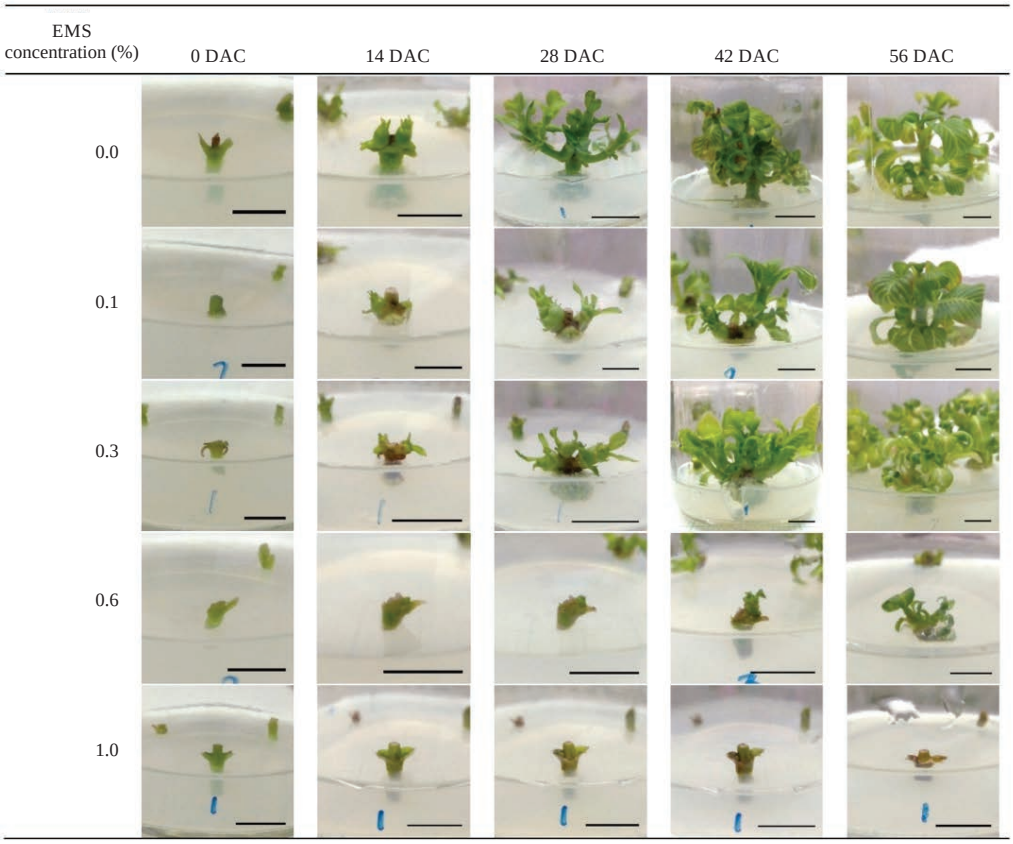

Fig. 3: Response of nodal explants at 2, 4, 6 and 8 weeks after treatment with 0.1, 0.3, 0.6 and 1% EMS at 4 hrs exposure duration
*Media used was Gamborg's B5 supplemented with 1.0 mg L^{-1} BAP, DAC: Days after culture, bar = 10 mm)

Table 2: Response of nodal explants on shoot induction and multiplication at 4 and 8 weeks after EMS treatment

			28 DAC (4 weeks)		56 DAC (8 weeks)	
Duration (hrs)	EMS concentration (%)	Shoot induction (%)	Mean number of shoots/explant ($\bar{x}\pm$SD)	Total shoot multiplication/ explant	Mean number of shoots/explant ($\bar{x}\pm$SD)	Total shoot multiplication/ explant
1	0	100.00^{a}	3.111±2.309^{a}	8	6.444±6.930^{a}	14
	0.1	88.9^{a}	1.815±1.001^{b}	5	5.444±10.353^{a}	39
	0.3	92.6^{a}	1.889±1.577^{b}	9	4.333±9.257^{a}	44
	0.6	85.2^{ab}	1.741±1.163^{b}	4	4.519±7.763^{a}	30
	1	70.4^{b}	1.630±1.363^{b}	5	6.333±11.939^{a}	54
2	0	100.00^{a}	2.482±1.988^{a}	9	5.222±7.607^{ab}	15
	0.1	88.9^{a}	2.148±1.379^{a}	6	5.482±7.552^{a}	23
	0.3	85.2^{ab}	2.185±1.882^{a}	9	8.148±15.066^{a}	57
	0.6	66.7^{b}	1.296±1.295^{b}	5	4.148±8.448^{ab}	41
	1	37.0^{c}	0.519±0.753^{b}	2	0.630±1.214^{b}	5
3	0	100.00^{a}	3.037±2.667^{a}	10	7.519±8.182^{ab}	14
	0.1	70.4^{b}	2.037±2.227^{b}	10	8.148±14.984^{a}	46
	0.3	66.7^{b}	1.407±1.575^{b}	6	3.111±7.159b^{c}	26
	0.6	29.6^{c}	0.407±0.694^{c}	2	0.889±2.592^{c}	12
	1	11.1^{c}	0.111±0.320^{c}	1	0.037±0.192^{c}	1
4	0	100.00^{a}	2.741±1.723^{a}	8	9.074±7.746^{a}	12
	0.1	74.1^{b}	1.926±1.920^{b}	7	7.741±11.834^{a}	34
	0.3	51.9^{c}	1.148±1.460^{c}	5	4.778±10.559^{ab}	47
	0.6	22.2^{d}	0.333±0.679^{d}	2	1.889±3.955b^{c}	16
	1	7.4^{d}	0.074±0.267^{d}	1	0^{c}	0

Means within a column with the same letter in each EMS treatment period are not significantly different according to Tukey HSD at $\alpha = 0.05$

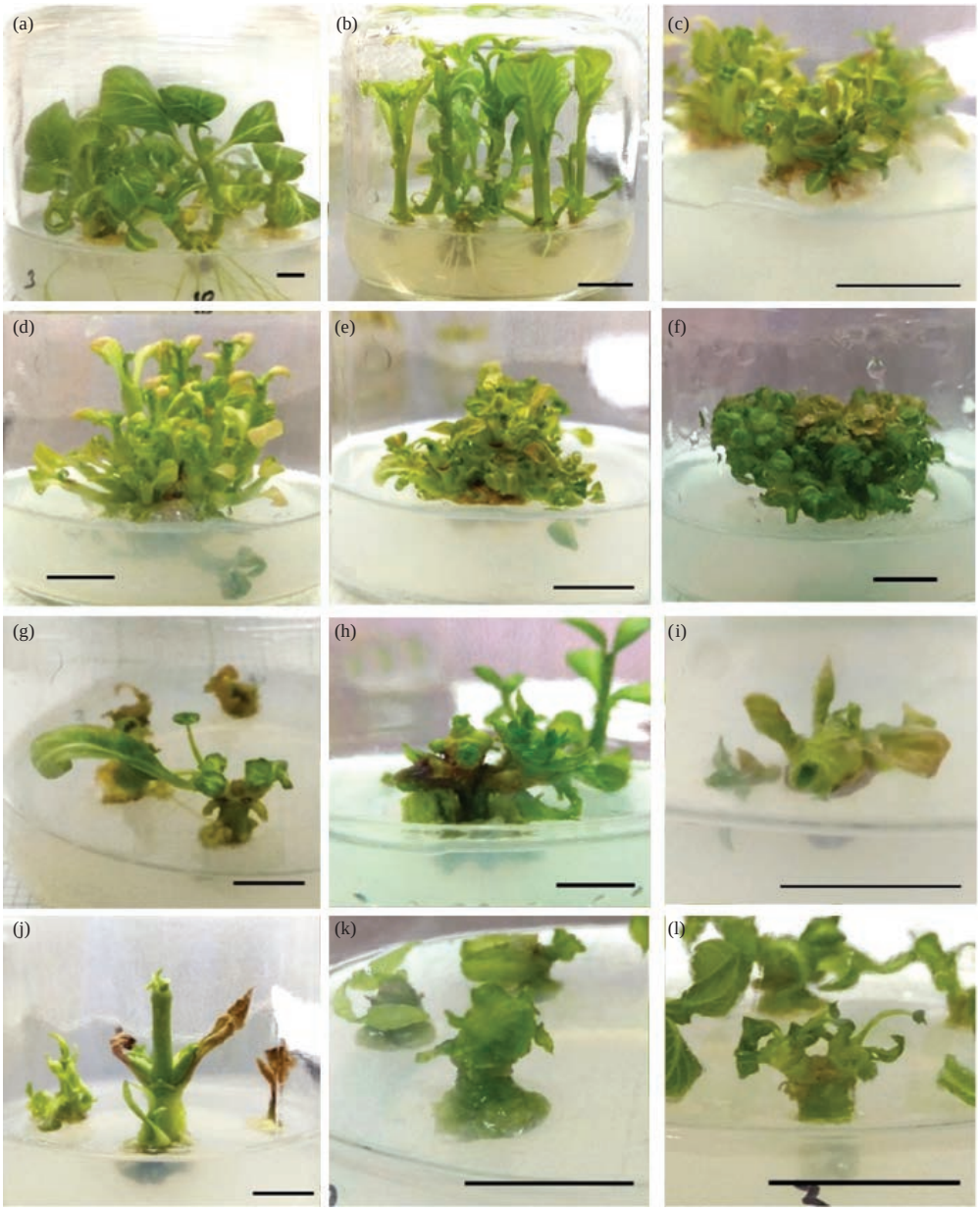

Fig. 4(a-l): Shoot multiplication of various EMS-treated nodal explants of *N. cadamba*. (a) Control untreated shoots in control sample at 42 DAC, (b) control sample after 56 DAC, (c) treated samples in 0.3% EMS at 4 hrs, (d) 0.3% EMS at 2 hrs, (e and f) 0.3% EMS at 3 hrs, (g) 1% EMS at 2 hrs, (h) 0.1% EMS at 2 hrs, (i) 0.3% EMS at 4 hrs, (j and k) 0.6% EMS at 2 hrs, (l) 0.6% EMS at 1 hr
Bar = 10 mm

could be affected by potential mutations, which later affected the growth. The average number of shoots induced in EMS-treated nodal explants decreased proportionally to EMS concentrations within 28 DAC. However, data recorded after 56 DAC showed that the declining trend is no longer applicable to every EMS-treated nodal explant as each surviving nodal explant from different EMS treatments showed different shoot multiplication rates. For instance, in 1 hr EMS treatment, the mean number of shoots per explant of EMS-treated nodal explants was not significantly different from the control samples ($p<0.05$). Nodal explants treated with 1% EMS showed a higher mean number of shoots formed, i.e., 6.33, followed by 5.44 in 0.1%, 4.52 in 0.6% and 4.33 in 0.3% EMS (Table 2). The highest total number of shoot

multiplication was 57 shoots as seen in nodal explants treated with 0.3% EMS at 2 hrs treatment, while the lowest was one shoot in 1% EMS treatment at 3 hrs (Table 2). No shoot multiplication was recorded in 1% EMS at 4 hrs treatment because no nodal explants survived from the EMS treatment.

Subsequent sub-culturing showed variation in shoot multiplication rates from low to high regardless of EMS concentrations. For comparison, shoot multiplication rates in untreated nodal explants is demonstrated in Fig. 4a (28 DAC) and Fig. 4b (56 DAC). Some EMS-treated nodal explants showed more vigorous shoot multiplication than the other sub-cultured explants. For instance, some of the nodal explants formed a high number of stunted shoots with approximately 5 mm in height and globular shoot clumps within 28 DAC after sub-culture (Fig. 4c), the formation of a higher number of shoots with different heights (Fig. 4d) and formation of a high number of shoots with deformed small leaves with reduced stem elongation (Fig. 4e-f). Treated nodal explants at various EMS concentrations and durations were also observed, producing morphological abnormalities that include the formation of unifoliate orbicular leaf induced from the apical meristem of the regenerated axillary shoot (Fig. 4g), thickening of the stem with the induction of pointy leaves that showed growth inhibition (Fig. 4h), stunted growth of shoots even after transferred to fresh media (Fig. 4i), the formation of small apical leaves with abnormal shoot developments (Fig. 4j) and distorted shoot formation with irregular first axillary shoots (Fig. 4k-l). However, these regenerants did not survive before maturity due to the inability to induce multiplication in constant media composition after sub-culture. They were not carried forward due to the selection of putative mutants in favour of potential superior traits.

Effects of EMS mutagenesis on callus formation: Callus formations were mainly induced at the incision area of the nodal explants facing downward. Formation of compact and friable callus was observed at the elongating shoots' base within 4 weeks (Fig. 5a, b). The latter was produced in both the stem base and sometimes loosely at the axil of the petiole maturing leaves, leaf surfaces and on nodal roots. Callus formation percentage and types of calli formed are recorded in Table 3. Observation within 56 DAC after treatments showed increasing percentages of callus formation in all EMS-treated nodal explants, except in 3 hrs treatment at 0.6 and 1% EMS and in 4 hrs treatment at 1% EMS. Darkening of the stem at the base of the incision site was observed at these nodal explants. EMS-treated *N. cadamba* regenerants that survived the treatments were sub-cultured up to five cycles to reduce the possibility of having chimeric somatic cells. Sub-cultures were repeated every three to four weeks via both apical and lateral meristem incisions at the nodal explants. Incised nodal explants were repeatedly cultured in shoot inducing media (B5 media+1 mg L^{-1} BAP). Data collected inferred that callus formation was high and not significantly different from the control samples. Compact and friable loose calli were induced in both untreated (or control) and EMS-treated explants within five cycles of sub-cultures.

Table 3: Response of shoot explants on callus formation in B5 medium+1 mg L^{-1} BAP

Duration (hrs)	EMS concentration (%)	N	Callus inductions after 4 weeks (%)	Callus inductions after 8 weeks (%)	N	Callus inductions after S5 (%)	*Type of callus
1	0	27	70.4^{a}	74.1^{a}	57	100^{a}	C, F
	0.1	27	44.4^{ab}	55.6^{ab}	213	100^{a}	C, F
	0.3	27	37.0^{ab}	48.2^{ab}	107	100^{a}	C, F
	0.6	27	29.6^{b}	37.0^{b}	79	100^{a}	C, F
	1	27	14.8^{b}	33.3^{b}	77	100^{a}	C, F
2	0	27	51.9^{a}	55.6^{a}	92	100^{a}	C, F
	0.1	27	33.3^{ab}	63.0^{a}	184	100^{a}	C, F
	0.3	27	18.5^{bc}	48.2^{a}	106	100^{a}	C, F
	0.6	27	11.1^{bc}	29.6^{ab}	54	100^{a}	C, F
	1	27	0.0^{c*}	3.7^{b}	11	100^{a}	C, F
3	0	27	63.0^{a}	77.8^{a}	80	100^{a}	C, F
	0.1	27	18.5^{b}	33.3^{b}	93	100^{a}	C, F
	0.3	27	3.7^{b}	14.8^{bc}	147	100^{a}	C, F
	0.6	27	0.0^{b}	0.0^{c}	18	100^{a}	C, F
	1	27	0.0^{b}	0.0^{c}	0	-	-
4	0	27	55.6^{a}	55.6^{a}	63	100^{a}	C, F
	0.1	27	7.4^{b}	33.3^{ab}	80	100^{a}	C, F
	0.3	27	7.4^{b}	22.2^{bc}	101	100^{a}	C, F
	0.6	27	0.0^{b}	7.4^{bc}	44	100^{a}	C, F
	1	27	0.0^{b}	0.0^{c}	0	-	-

Means within a column with the same letter in each EMS treatment period are not significantly different according to Tukey HSD at $\alpha = 0.05$. (*Type of callus, C: Compact, F: Friable)

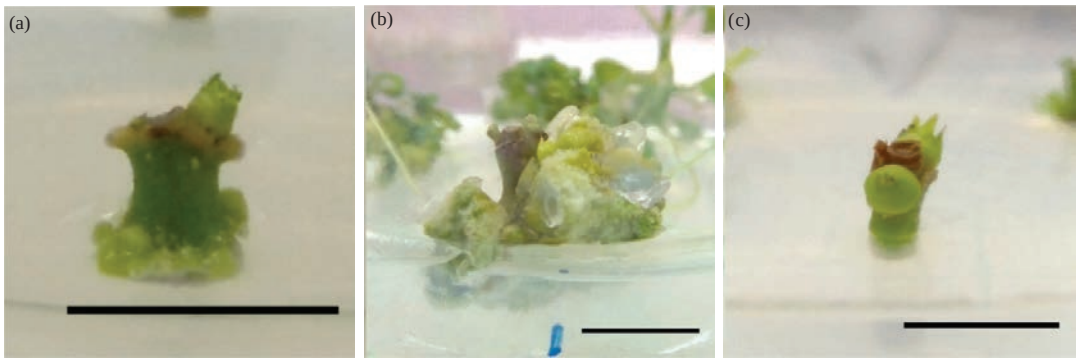

Fig. 5(a-c): Callus formation in nodal explants of *N. cadamba* at various EMS treatments. (a) Response of nodal explants at 28 DAC at various treatments, i.e. formation of compact callus in 1-hr 1% EMS treatment, (b) friable callus in 2 hrs 0.3% EMS treatment, (c) no callus in 1 hr 1% EMS treatment
Bar = 10 mm

Table 4: Plant height response in *N. cadamba* shoots regenerated from nodal explants

Duration (hrs)	EMS concentration (%)	Mean plant heights after S5 (cm) ($\bar{x}\pm$SD)	Mean plant heights after rooting (cm) ($\bar{x}\pm$SD)	Mean plant heights after acclimatisation ($\bar{x}\pm$SD)
1	0	2.5667 ± 1.0293^{a}	3.900 ± 0.787^{ab}	5.067 ± 1.029^{b}
	0.1	1.9127 ± 0.3535^{b}	3.850 ± 1.024^{b}	9.150 ± 0.919^{a}
	0.3	1.8495 ± 0.5971^{bc}	4.332 ± 0.845^{ab}	5.450 ± 1.485^{b}
	0.6	1.6519 ± 0.3231^{c}	4.552 ± 1.149^{ab}	7.136 ± 2.287^{ab}
	1	1.7676 ± 0.6133^{bc}	4.739 ± 0.925^{a}	5.700 ± 0.173^{b}
2	0	2.5783 ± 1.0117^{a}	4.240 ± 0.768^{a}	5.007 ± 0.966^{b}
	0.1	1.9370 ± 0.5694^{c}	4.480 ± 1.155^{a}	6.356 ± 1.356^{ab}
	0.3	2.0425 ± 0.4869^{bc}	4.492 ± 1.093^{a}	-
	0.6	1.9870 ± 1.0815^{bc}	4.200 ± 1.287^{a}	6.550 ± 1.583^{a}
	1	2.4273 ± 0.0^{ab}	3.967 ± 0.692^{a}	6.050 ± 1.261^{ab}
3	0	2.3013 ± 0.8773^{a}	3.640 ± 0.682^{a}	4.801 ± 0.877^{b}
	0.1	1.5613 ± 0.2370^{b}	3.624 ± 1.276^{a}	7.750 ± 0.354^{a}
	0.3	1.7116 ± 0.5517^{b}	4.682 ± 1.065^{a}	8.140 ± 0.972^{a}
	0.6	2.100 ± 0.6670^{a}	4.600 ± 0.265^{a}	-
	1	-	-	-
4	0	2.0238 ± 0.8568^{a}	3.800 ± 0.932^{a}	4.524 ± 0.857^{b}
	0.1	1.4213 ± 0.3011^{b}	3.320 ± 0.850^{a}	3.800 ± 1.100^{b}
	0.3	1.4475 ± 0.4005^{b}	3.894 ± 1.240^{a}	-
	0.6	1.3205 ± 0.1306^{b}	3.715 ± 0.966^{a}	6.736 ± 1.548^{a}
	1	-	-	-

Means within a column with the same letter in each EMS treatment period are not significantly different according to Tukey HSD at $\alpha = 0.05$

Effects of EMS mutagenesis on plant height and growth: The height of the *in vitro* clones was also recorded at the end of the fifth sub-culture (S5) (Table 4). The mean plant height in most EMS-treated cultures was significantly lower compared to the average height of untreated (or control) cultures (Tukey HSD, $\alpha = 0.05$). The data, however, provided no distinct trend in terms of the average plant height. This result indicates no correlation between the increasing EMS concentration and duration with the plant height in the present study. For instance, in 3 hrs EMS treatment, the highest average plant height was 2.10 cm (0.6%) and lowest at 1.56 cm (0.1%).

Effects of EMS mutagenesis on rooting: Shoots approximately more than 2 cm in length and at least four leaves were then excised and selected for rooting. Half-strength B5 medium supplemented with 0.1 mg L^{-1} paclobutrazol (PBZ) were used for root induction. The root formation capabilities and growth of shoot regenerants from EMS-treated nodal explants are summarised in Table 5. The percentages of root formation were observed to be high in shoots regenerated from the nodal cultures of both 1 and 2 hrs EMS treatments in all concentrations. The data indicated no noticeable trend in the percentage of root formation with increasing EMS concentration and duration in

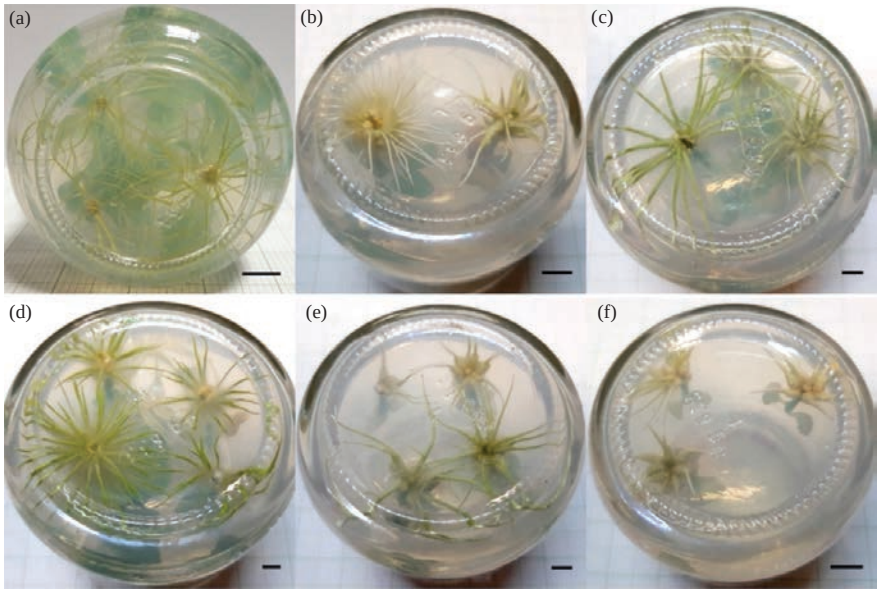

Fig. 6(a-f): Various types of root formation of EMS-treated nodal explants of *N. cadamba* cultured on ½ B5 medium supplemented with 0.1 mg L^{-1} PBZ

Table 5: Response of shoot explants at 4 weeks after culture in rooting media (½ B5 medium+0.1 mg L^{-1} paclobutrazol)

Duration (hrs)	EMS concentration (%)	Root inductions (%)	Mean number of roots/explant ($\bar{x}$±SD)	Mean root length (cm), ($\bar{x}$±SD)	Mean nodal number ($\bar{x}$±SD)
1	0	100.00^{a}	8.700±6.129^{a}	3.370±1.265^{a}	4.500±0.850^{a}
	0.1	87.5^{ab}	7.321±2.611^{a}	3.039±0.777^{a}	4.357±1.129^{a}
	0.3	70.4^{b}	7.421±1.924^{a}	3.047±0.733^{a}	4.526±0.905^{a}
	0.6	95.5^{ab}	9.476±4.895^{a}	3.043±0.714^{a}	4.524±0.928^{a}
	1	100.00^{a}	9.839±4.569^{a}	2.894±0.707^{a}	4.452±0.810^{a}
2	0	100.00^{a}	9.500±4.743^{a}	2.860±1.320^{a}	4.200±0.919^{b}
	0.1	98.1^{a}	8.628±4.907^{a}	3.145±0.838^{a}	4.549±1.119^{b}
	0.3	72.7^{a}	10.458±5.579^{a}	3.179±0.613^{a}	4.500±0.885^{b}
	0.6	84.9^{a}	8.533±5.716^{a}	2.963±0.703^{a}	4.500±1.009^{b}
	1	100.00^{a}	12.833±6.145^{a}	1.867±0.266^{b}	5.833±1.169^{a}
3	0	100.00^{a}	8.400±6.150^{a}	3.490±1.424^{a}	4.300±0.675^{a}
	0.1	65.6^{a}	8.667±4.800^{a}	3.071±0.641^{a}	4.048±0.865^{a}
	0.3	65.1^{a}	11.321±4.173^{a}	3.150±0.729^{a}	4.179±0.863^{a}
	0.6	75.0^{a}	13.000±5.568^{a}	3.400±0.458^{a}	4.667±0.577^{a}
	1	-	-	-	-
4	0	100.00^{a}	9.546±5.956^{a}	3.227±1.218^{a}	4.273±0.647^{a}
	0.1	50.0^{b}	5.800±4.324^{a}	3.680±1.057^{a}	3.400±0.894^{a}
	0.3	45.0^{b}	8.412±3.776^{a}	2.853±0.897^{a}	4.177±0.951^{a}
	0.6	100.00^{a}	9.385±4.857^{a}	2.915±0.586^{a}	4.154±0.689^{a}
	1	-	-	-	-

Means within a column with the same letter in each EMS treatment period are not significantly different according to Tukey HSD at $\alpha = 0.05$

EMS-treated *N. cadamba* cultures. The highest average number of roots induced per explant was 13 observed in a 0.6% EMS at 3 hrs, while the lowest was 5.8 roots per explant recorded in a 0.1% EMS at 4 hrs. All average number of roots induced per explant were not significantly different from data collected from untreated cultures, ranging from 8.4-9.5 roots per explant. However, the average number of roots showed an increasing trend following increasing EMS concentrations. Meanwhile, for the average root length, only the average root length recorded in 1% EMS at 2 hrs treatment was significantly lower and the lowest compared to control. Similarly, there was once again no trend showing proportional response due to EMS concentrations.

After 4 weeks of culture in half-strength B5 medium supplemented with paclobutrazol (0.1 mg L^{-1}), the roots formed were approximately 1 mm in width and green in colour in most successfully rooted shoots (Fig. 6a). Some rooted shoots in the treated cultures at higher EMS

Table 6: Survival percentage and plant growth after acclimatisation

Duration (hrs)	EMS concentration (%)	Survival (%)	Number of nodes ($\bar{x}\pm$SD)	Stem diameter ($\bar{x}\pm$SD)
1	0	81.8±0.405[a]	6.000±1.000[a]	2.293±0.338[a]
	0.1	57.1±0.535[a]	5.000±0.000[a]	2.250±0.354[a]
	0.3	50±0.707[a]	5.500±2.121[a]	2.333±0.471[a]
	0.6	90.5±0.301[a]	6.455±0.820[a]	2.379±0.381[a]
	1	100±0.000[a]	5.333±0.577[a]	2.056±0.096[a]
2	0	80±0.422[a]	6.567±1.609[a]	2.664±0.531[a]
	0.1	85.7±0.378[a]	6.667±1.323[a]	2.778±0.363[a]
	0.3	0[b]	-	-
	0.6	77.3±0.429[a]	6.100±1.729[a]	2.417±0.492[a]
	1	100±0.000[a]	7.500±2.380[a]	2.792±1.066[a]
3	0	90±0.316[a]	6.213±1.393[a]	2.533±0.445[a]
	0.1	90.5±0.301[a]	8.000±1.414[a]	2.333±0.000[a]
	0.3	92.9±0.262[a]	6.000±1.414[a]	2.733±0.417[a]
	0.6	0[b]	-	-
	1	0[b]	-	-
4	0	90±0.316[a]	5.810±1.120[a]	2.312±0.382[a]
	0.1	40±0.548[b]	5.333±1.528[a]	2.056±0.962[a]
	0.3	0[b]	-	-
	0.6	100±0.000[a]	5.786±0.975[a]	2.250±0.351[a]
	1	0[b]	-	-

Means within a column with the same letter in each EMS treatment period are not significantly different according to Tukey HSD at $\alpha = 0.05$

Fig. 7: Plant growth after acclimatisation stage (3 months in *ex vitro*)

concentrations showed differences in root colours, thickness, number of roots and length. Among the observed differences are the formation of white colour roots (Fig. 6b), thick pale green roots (Fig. 6c), the formation of a high number of roots (Fig. 6d), the formation of a reduced number of roots (Fig. 6e) and the formation of shorter roots with callus (Fig. 6f). Green and thin roots were deemed healthy and suitable for acclimatisation. Meanwhile, it was observed that both thicken roots and roots with callus were unsuitable for acclimatisation. This is because the roots detached easily upon transfer into soil mix for acclimatisation to *ex vitro* conditions.

Survival rate and plant growth after acclimatisation: Survival of plants in the control samples was high after 4 weeks gradually introduced into *ex vitro* conditions, ranging from 80-90% (Table 6). There is no correlation observed between the increasing EMS concentration and duration at this acclimatisation stage. Successfully acclimatised *N. cadamba* seedlings after 3 months in *ex vitro* are shown in Fig. 7. The average number of nodes and stem diameter from the EMS-treated seedlings were not significantly different from the untreated control seedlings. The average number of nodes in control samples ranged from 5.8-6.5. Meanwhile, in EMS-treated seedlings, the highest average number of nodes was 8.0 from 0.1% EMS in 3 hrs treatments and the lowest was 5.0 from 0.1% EMS in 1 hr treatment. For stem diameter, the average ranged from 2.293-2.664 mm in control samples. Meanwhile, EMS-treated seedlings ranged from 2.056 mm (1% EMS, 1 hr treatment) to 2.792 mm (1% EMS, 2 hrs treatment).

DISCUSSION

The development of planting materials with novel genetic variability and desired traits of interest using mutagen has increased recently. The application of chemical mutagenesis, in general, is deemed to be advantageous due to the ability in generating increased genetic variability, which is applicable in many species with ease, albeit being inexpensive[23,46]. EMS alkylates the position of guanine residues in O-6, resulting in replication errors in which Thymine (T) is incorporated into the newly synthesized strand rather than Cytosine (C), resulting in changes in the transition from G/C-A/T[22]. These transformations produce several alleles in each

gene, resulting in many mutation types, including nonsense, missense and slicing mutants[47]. Mutation breeding using EMS has been previously reported to effectively induce mutation through *in vitro* methods in numerous important plant species as seen in coffee[48], rice[49], tomato[37] and wheat[50].

Before the mutagenesis process, the homogeneity of the plant material is essential. For mutation breeding of sexually propagated plants, seeds are predominantly preferred as seen in various species, such as arabidopsis[51], rice[35] and tomato[37]. Homogeneity can be maintained by choosing an initial plant that is derived from self-fertile seeds, followed by self-fertilisation for the subsequent generation of seeds[52]. Meanwhile, other plant tissues, such as callus, nodes and leaves, were also reported in many mutation studies. Homogeneity population in the vegetatively propagated plant could be achieved via clonal propagation of common fixed heterozygosis[23]. These explants were considered as the regular plant materials commonly used in *in vitro* EMS-mutagenesis. Upon taking all those factors into account, multiple *N. cadamba* explant's options have been considered for the EMS treatment, i.e., seeds, leaves, shoot tips, nodes and callus. Seeds of *N. cadamba* are known to be recalcitrant, which can affect the outcomes of the subsequent observations in mutation breeding studies as reported in our previous study[43]. Hence, *in vitro* nodal explants were selected due to the known ability of each nodal explant in inducing shoot formations and enabling rapid micropropagation of *N. cadamba* in mass number consistently adequate for EMS mutagenesis study.

Since somaclonal variation is more likely to occur because of the tissue culture process, screening of EMS-induced mutations is becoming a primary concern recently on the genetic integrity of *N. cadamba* seedlings derived from tissue culture using nodal explants. However, our recent finding confirmed the genetic fidelity of micropropagated *N. cadamba* seedlings via nodal explants compared to stock plants after sixth subcultures using ISSR markers and RAPD markers[53]. No polymorphic bands were observed in 9,334 bands from RAPD markers and 2,760 bands from ISSR markers. Single nucleotide polymorphism (SNP) also was not detected in all the amplified *sucrose synthase* (*SuSy*) genomic sequences from micropropagated *N. cadamba* seedlings after sixth subcultures compared to the stock plants[54]. This result indicates that nodal explants can be used as ideal plant material for EMS mutagenesis in the present study. Hence, we used the *in vitro* propagation protocol of *N. cadamba* established earlier by Mok and Ho[44] in the present study to reduce the possibility of somaclonal variations.

Directly after each EMS treatment, nodal explants were cultured on shoot inducing media to reinitiate the ontogenetic pathways for direct shoot organogenesis from the pre-existing meristem. Application of EMS treatments at different concentrations and durations indicated that nodal explants of *N. cadamba* were highly susceptible to the chemical mutagen treatments. As both EMS concentrations and durations increased, the survival percentage was significantly reduced. This declining trend is normal in EMS mutagenesis studies that correlate with the efficacy of the EMS in inducing mutations. The survival of explants and the capability to further initiate growth were considered crucial parameters[49]. Similar declining trends were reported in other woody plant species such as *Prunus persica* L. Batsch[54] and *Coffea arabica* L. var. Catuaí[48].

Through the survival and lethality percentage, the lethal concentration 50 (LC_{50}) of EMS in *N. cadamba* nodal explants was determined and estimated to be 1.2589, 0.3715 and 0.3311% EMS for 2, 3 and 4 hrs treatments, respectively. Determining LC_{50} of mutagen is important because mutagen concentrations that lead to 50% survival or lethality in EMS-treated explants are estimated to provide a well-saturated mutagenized population[35]. The determination of LC_{50} offers a benchmark for future mutagenesis of the same species, which is applicable only on equal plant materials. Various LC_{50} were reported from various EMS-induced mutagenesis, e.g., 0.3% EMS for 3 hrs in saffron (*Crocus sativus* L.)[55], 0.5% EMS for 12 hrs in seeds of Malaysian, rice variety (cv. MR219)[35] and 0.5% EMS at 9 hrs treatments in Echeveria "Brave" leaf cuttings[56].

The percentage of shoot induction and the average number of shoot induction within 4 weeks after culture showed a declining trend with increasing EMS concentration and treatment duration to control. The untreated nodal explants (or control) showed similar consistency in shoot induction percentages (100%) and the highest average number of shoot induced, which provide distinct differences in shoot induction versus EMS-treated nodal explants. This indicated that EMS mutagenesis significantly impacted the *N. cadamba* shoot induction percentage and the average number of shoots induced. A similar trend was reported in the shoot induction percentage of rice[35]. However, observation within 8 weeks showed an increasing average of shoot induction in the EMS-treated explants. This response indicated the delay effect and inhibition of the shoot induction in treated *N. cadamba* nodal explants due to EMS mutagenesis. A similar delayed response was reported in embryo development of EMS-mutagenized *Phaseolus vulgaris* L.[57].

During shoot multiplication, nodal explants at various EMS concentrations showed different responses under a constant cytokinin level. These responses include both lower and higher numbers of shoot proliferation in comparison to the control samples. Changes in response could be due to increase or reduce sensitivity toward exogenous cytokinin requirements in shoot-inducing media or potential changes in the production level of endogenous phytohormones. There is a lack of data published regarding both changes stated earlier, resulting specifically from EMS-induced point mutations. However, in the cytokinin-regulated plant regeneration signalling pathway study of *Arabidopsis*, defected *Arabidopsis histidines kinase* 4 (*AHK4*) receptor contributes to a declined reaction to cytokinin and inhibit shoot induction[58]. Shoot regeneration capacity is repressed while the primary member of type-B *Arabidopsis* response regulators (ARRs) is mutated, i.e., *arr1* single mutant[59]. Type-B ARRs are essential transcription factors primarily responsible for controlling the transcription of cytokinin response genes[60]. With the presence of exogenous cytokinin in the medium, continuous axillary shoots of *N. cadamba* were induced continuously, resulting in the clump of micro shoots with reduced height. Besides, there were also possibilities of modifications in either signalling pathways or biosynthesis of endogenous phytohormones. The development of plant stem elongation is known to be regulated by complex signalling pathways and various phytohormones are involved[61]. Mutants with modification in genes encoding gibberellin biosynthesis caused the reduction of stem cell length, resulting in the development of dwarf plants[61,62]. Dwarf mutants were reported to occur in various species, e.g., maize *Dwarf1* (*D1*)[63], *Arabidopsis Dwarf12* (*dwf12*)[64] and rice *Dwarf18* (*D18*)[65], which resulted from mutations in *gibberellin 3-oxidase* (*GA3ox*) genes. Besides, the *sucrose synthase* (*Susy*) gene was also suggested to be involved in shoot apical meristem (SAM) development. Suppressed *SlSUS1*, *SlSUS3* and *SlSUS4* in transgenic tomato were reported to develop abnormality in leaf morphology and cotyledons, with altered auxin-related genes expression in the SAM[66].

A slight increase of calli formation in EMS-treated explants was recorded in eight weeks while remained significantly lower to control samples. This result indicates the delay and inhibition effects due to EMS mutagenesis on surviving EMS-treated explants. The calli formed at the incision sites are known as wound-induced callus, which is regulated by cytokinin[67]. In *Arabidopsis*, the development of the wound-induced callus is regulated by the AP2 transcription factors wound induced dedifferentiations (WINDs), primarily by modifying the cytokinin signalling mediated by ARR1 and ARR12[68]. The repressed formation of callus in increasing EMS concentration and treatment duration in the present study suggests the possibilities of having modifications or damages in these types of response regulators.

In the present study, half-strength B5 supplemented with 0.1% paclobutrazol was used as a rooting medium. Paclobutrazol is a retardant that inhibits the concentrations of gibberellic acid and endogenous indole acetic acid and therefore inducing the production of the other endogenous plant growth hormones, such as abscisic acid, cytokinin and ethylene in plants. The development of roots was commonly observed to be generated at the base of the stem, located at the incision site within 7 DAC. The variations in root developments might be due to modifications in phytohormones biosynthesis or pathway related to the root apical meristem (RAM) development. Defective mutations in the biosynthesis or signalling of cytokinins, such as *ipt3*, *ahk3* or *arr1*, exhibit the increased size of RAM[69].

Ethyl methanesulfonate (EMS) is a widely used chemical mutagen in plants. Hence, an optimum EMS dose is required to produce a high frequency of non-bias irreversible mutations and cause minimum killing. As observed in this study, the mutagenesis treatments at different EMS concentrations and treatment durations resulted in considerable growth and morphological differences than untreated *N. cadamba* cultures. Among others are leaves with unifoliate orbicular shapes, plants with thickened stems and small pointy leaves showing growth inhibition. However, these putative mutants are still at their early development stage *in vitro* and field trials of putative mutants of *N. cadamba* should be carried out to assess their growth and productivity performance in the field.

CONCLUSION

The findings obtained in the present study suggest that direct treatment of *in vitro* derived nodal explants using EMS via soaking method is an efficient method to induce *in vitro* mutation of *N. cadamba*. Various responses were observed in EMS-treated cultures derived from nodal explants and the present findings agree with EMS mutagenesis studies reported over the years. Hence, combining both plant tissue culture technique and *in vitro* EMS mutagenesis can significantly reduce the time required to produce mutant clones and broaden the genetic base of *N. cadamba*, a woody plant species with a slightly longer reproductive lifespan. It is hoped that this newly established *in vitro* EMS mutagenesis approach can be used in the future to speed up the tree improvement of *N. cadamba*.

SIGNIFICANCE STATEMENT

In vitro EMS mutagenesis is reported to cause point mutations easily and these morphological variants also resulted from combinations of mutations as observed in the present study. To the best of our knowledge, this is the first report on the effects of *in vitro* EMS mutagenesis using nodal explants of *N. cadamba*. This approach is simple and fast to perform and therefore can be implemented in any laboratory equipped with basic tissue culture facilities. The putative mutants derived from the present study can benefit future breeding and genomics research of *N. cadamba*.

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