Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

European Journal of Experimental Biology, 2014, 4(4):156-163

ISSN: 2248 –9215
CODEN (USA): EJEBAU

EMS-induced mutagenesis and DNA polymorphism assessment through ISSR markers in *Neolamarckia cadamba* (kelampayan)and *Leucaena leucocephala* (petai belalang)

Mohamed ZakyZayed[1], Wei-Seng Ho[1*], Shek-Ling Pang[2] and Fasihuddin Badruddin Ahmad[3]

[1]*Forest Genomics and Informatics Laboratory (fGiL), Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan, Sarawak*
[2]*Applied Forest Science and Industry Development (AFSID), Sarawak Forestry Corporation, Kuching, Sarawak*
[3]*Department of Chemistry, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan, Sarawak*

ABSTRACT

This study was conducted to determine the effects of ethyl methanesulphonate (EMS) treatments on seed germination and the genetic diversity of EMS-induced Neolamarckia cadamba and Leucaena leucocephala seedlings using ISSR markers.The effects of soaking duration in 100^{o}C water on seed germination were also determined forN. cadambaand L. leucocephala by using the following treatments: (1) untreated (control); (2) soaking in 100^{o}C water for 20seconds followed by soaking in water for 24 hours and 48 hours, respectively at room temperature.Results showed that soaking in 100^{o}C water for 20 seconds and subsequently in water at room temperature for 48 hours had the highest seed germination rate, higher cumulative germination (CGP) and shortened the period of complete dormancy (CDP) over soaking duration of 24 hours or untreated seeds before planting. A total of 120 seeds for each treatment and three different EMS doses (0.1%, 0.3% and 0.6%) were used in the EMS-induced mutagenesis studiesof N. cadamba and L. leucocephala. The results showed that the germination percentage, survivability and seedling height were decreased, whereas lethality increased with the increasing of EMS doses. Among the N. cadamba and L. leucocephala seedlings investigated, 0.6% EMS treated samples exhibited the highest level of variability in comparison to 0.1% EMS treated samplesas revealed by using ISSR markers. This indicates that 0.6% EMS treatment is much more beneficial as compared to other EMS treatments. Further, EMS has been successfully used to produce a range of novel traits and broaden thegenetic diversityof N. cadamba and L. leucocephalaas observed in the present study.

Key words: EMS, mutagenesis, genetic diversity, *Neolamarckia cadamba*, kelampayan, *L. leucocephala*, petai belalang, ISSR

INTRODUCTION

Mutation is the ultimate source of all genetic variation. Spontaneous mutations occur at very low frequency meanwhile induced mutations facilitate the development of improved varieties at a swifter rate as these mutations typically occur at much higher frequencies than spontaneous mutations do [39]. Besides the vital role in plant breeding programmes, a new role of induced mutations in releasing of gene silencing in transgenic plants has also been reported [7]. Induced mutations have been successfully used to generate genetic variability and to improve yield components of various crops like *Oryza sativa* [4, 56], *Hordeum vulgare* [51], *Triticum durum* [54], *Sesame indicum L.* [41] and others.

Chemical mutagen, such as ethyl methane sulfonate (EMS), a compound of the alkaline sulfonate series is widely known to induce a higher frequency of mutations in higher plants. EMS usually causes high frequency of gene mutations and low frequency of chromosome aberrations [63], but loss of a chromosome segment or deletion can also be occurred in plants [46]. EMS alkylates guanine bases due to mispairing-alkylated G pairs with T instead of C, resulting in primarily G/C to A/T transitions [6]. EMS has been used in rice at chosen concentration from 0.2 % to 2.0 % for 10 h to 20 h based on the sensitivity or kill curve of the genotype used. EMS produces a large genome number of non-lethal point mutations approximately 10,000 to saturate the genome with mutations. For instance in *Arabidopsis*, EMS can produce more than four non-lethal point mutations per Mb [14]. Thus, EMS has the potential of altering loci of particular interest without inducing a great number of closely linked mutations.

ISSR (Inter simple sequence repeat) has been found to be the most economical among PCR based markers recently. It has many advantages like low quantities of template DNA, no need of sequence data for primer construction, random distribution throughout the genome, high level of polymorphic bands, even using only a few primers [47], less investment in time and labour than other markers, and exhibit Mendelian inheritance [25, 26, 62]. Therefore, ISSR markers have been widely used for DNA fingerprinting studies [23], genetic diversity studies [22, 47, 48], phylogenetic studies [27] and identification of closely related cultivars [21, 43].

Neolamarckia cadamba or locally known as kelampayan belongs to the family of Rubiaceae, has been selected as one of the fast growing plantation species for planted forest development in Sarawak [28]. It is praised as a “miracle tree” in China due to its fast growing characteristics and an ideal tree species to study genetic functions related to tree growth and cell wall development [38]. It is one of the best sources of raw material for the plywood industry, besides pulp and paper production. It can also be used as a shade tree for dipterocarp line planting, whilst its leaves, barks, roots and fruits have medical applications [30]. It also has high potential to be utilized as one of the renewable resource of raw materials for bioenergy production such as cellulosic biofuels in the near future.

Leucaena leucocephala or locally known as petai belalang belongs to family Leguminosae. It is a perennial leguminous tree native to Central America with a wide distribution in the tropics and subtropics, and suitable for growing in marginal and sub-marginal lands with a wide assortment of uses. It is cultivated for multipurpose uses, e.g. forage or fodder, lumber, fence posts, fuelwood, charcoal, pulp and to soil improvement [37]. All parts of *L. leucocephala* plants are edible to animals, including leaves, young stem, flowers, young and mature pods, and seeds [11]. *Leucaena* foliage (leaflets plus stems) contains both nutrients and roughage and makes a ruminant feed roughly comparable to alfalfa forage. It is a rich source of protein (15% to 38%) and the foliage is highly digestible [10]. The leaves are also a rich source of carotenoids and vitamins.

Induced mutations have not been carried out in *N. cadamba* and *L. leucocephala* though there are important multi-purpose tree species for agroforestry either for timber industry or as a feed for ruminants and non-ruminants livestock, respectively. Our recent findings have documented that *N. cadamba* trees are genetically less diverse or closely related to each other compared to other tree species [28, 61]. Cross-pollination between these closely related trees can reduce seed quality through inbreeding. If these seeds used widely, it may actually reduce genetic diversity and over time, make the plantings less resilient to changes in the environment. *L. leucocephala* is a highly self-compatible tetraploid (2n = 4x = 104) and has a relatively narrow genetic base [10]. Induced mutations can be used to facilitate the genetic enhancement of various qualitative and quantitative characters besides widening the genetic base of both species.

Thus, the present study was aimed to broaden the genetic base of *N. cadamba*and *L. leucocephala*via EMS-induced mutagenesis. Various parameters such as seed germination, survival after germination, lethality and seedling height were used to evaluate the effects of EMS doses on both species. The genetic variation of those EMS-induced *N. cadamba* and *L. leucocephala*seedlings was also determined by using ISSR markers.

MATERIALS AND METHODS

Plant materials and experimental design:

The seeds of*N. cadamba* were obtained from the Seed Bank of Sarawak Forestry Corporation, Sarawak. *L. leucocephala* seeds were obtained from the nursery of Forestry and Wood Technology Department, Faculty of Agriculture, Alexandria University, Egypt. The completely randomized design containing four replicates was used in the EMS study. Each replicate contained four treatments and 120 seeds. Meanwhile for the soaking duration in 100^{o}C water study, a completely randomized design containing three replicates was used and each replicate contained three treatments and 30 seeds for each species.

The seed germination test:
The germination test was carried out according to the methods outlined in the "International Rules for Seed Testing Rules" as published by the International Seed Testing Association ISTA. The seeds from both species were exposed to the following treatments: (1) untreated (control); (2) soaking in 100^{o}C water for 20 seconds followed by soaking in water for 24 hours and 48 hours at room temperature, respectively. Three replicates were used each with 30 seeds. 30 seeds were soaked in water for 24 hours, 30 seeds were soaked in water for 48 hours and 30 seeds for the control were not given any treatment for each species. After the treatment, those seeds were sown in trays of 50 holes and contained sand and compost (3:1). The trays were watered for 25 days. Records consisted of daily count of germinated seeds; complete dormancy period (CDP) and mean daily germination (MDG). Seeds were scored as having germinated when the radical length was 1.5 mm long. Other parameters recorded were days to the appearance of first true leaf and coefficient velocity of germination (CVG) which was calculated using the formula outlined by Kotowski [35].

The mutagenic treatments:
Seeds were pre-soak in distilled water for one hour and then air dried before soaked in different EMS concentrations in petri dishes for three hours. Laboratory temperature during the treatment was 21^{o}C, whereas the relative humidity was about 59%. Three different concentrations of EMS were used, i.e. 0% (distilled water), 0.1%, 0.3% and 0.6%. After the treatment, seeds were washed in distilled water for 15 minutes; air dried and then soaked in 100^{o}C water for 20 seconds followed by soaking for 24 hours to 48 hours in 25^{o}C water. The treated seeds were then sown in seed trays for recording the germination behavior such as germination percentage, survival after germination and maturation, and lethality over control (LOC). The germination percentage per treatment with four replicates was counted and recorded on 21st day after seed sowing. Percentage of inhibition or stimulation over control (lethality over control, LOC) was calculated as [Control – Treated / Control] x 100.Plant height was measured in centimeters from the soil surface to the top of the longest branch. Measurements were taken after six months of planting.

DNA extraction and ISSR-PCR amplification:
Total genomic DNA was isolated from the fresh leaves of 47 *N. cadamba* and 51 *L. leucocephala* seedlings derived from the EMS-induced mutagenesis study by using a modified CTAB method [19]. The PCR amplification was carried out by using a Master Cycler Gradient PCR (eppendorf, Germany). 25 µl reaction mixture containing 20 ng genomic DNA, 1x PCR buffer, 0.2 mM of each dNTPs, 2.5 mM $MgCl_2$, 0.5 unit *Taq* DNA polymerase (Promega, USA) and 10 pmol of $(GTG)_6$ and $(AC)_8G$ ISSR primers for *N. cadamba*, and $(GTG)_6$, and $(GA)_5GC$ ISSR primers for *L. leucocephala*. The thermal cycling condition using $(GTG)_6$ primerwas as follows: an initial denaturation period of 2 minutes at 94 ºC was followed by 38 cycles of 30 seconds at 94 ºC, 30 seconds at 55.7 ºC, 1 minute at 72 ºC, and then 10 minutes at 72 ºC for final extension. However, it was programmed at 94°C for 2 minutes as the initial denaturation step, 40 cycles of 30 seconds at 94°C, 30 seconds at 53°C for $(AC)_8G$ and 44°C for $(GA)_5GC$, 1 minute at 72°C and final extension step at 72°C for 10 minutes. The amplification products were separated on a 1.5% agarose gel and 1kb DNA ladder (Promega, USA) was ran simultaneously. The agarose gel was documented by using the UV-gel image acquisition camera (Geliance 200, Perkin Elmer).

ISSR data analysis:
The DNA bands produced at different loci were determined and named for each DNA sample. Banding profiles generated were converted into a binary data matrices on the basis of present (1) or absent (0) of bands. Data scoring is based on several criteria [67]: (1) locus is assumed as independent or non-allelic, (2) there is no bias in scoring monomorphic fragments versus polymorphic fragments, (3) amplified loci are expected to be in the range of 250 bp to 1500 bp, and (4) the similarity of fragment size is assumed to be the indicator of homology. The binary matrices were used to estimate genetic diversity of *N. cadamba* and *L. leucocephala*.Genetic data analysis was performed by using POPGENE version 1.32 software [68]by assuming Hardy-Weinberg equilibrium to calculate percentage of polymorphic loci (*P*), Shannon's diversity index (*I*), and Nei's gene diversity [44].

RESULTS AND DISCUSSION

Effects of soaking duration in 100^{o}C water at room temperatureforN. cadamba*and*L. leucocephala seeds:
The results obtained from the complete dormancy period, the cumulative germination percentage until nine days after planting, the mean daily germination (MDG), the co-efficient velocity of germination (CVG), the days to 50% germination and the days to first true leaf of these seeds which soaked in 100^{o}C water for 20 seconds and then for durations of 0 hour, 24 hours and 48 hours are summarized in **Table 1**. The results showed that the parameters were significantly differed and affected. The complete dormancy period (CDP) of these seeds which soaked for durations of 0 hour, 24 hours and 48 hours was 19.6%, 14.3% and 6.3% for*N. cadamba* and 12.3%, 10.3% and 4.3% for*L. leucocephala*, respectively. The cumulative germination percentage (CGP) until nine days after planting was 0%,

0% and 92.2% for*N. cadamba* and 0%, 0% and 95.9% for*L. leucocephala*, respectively. The different treatments showed substantial variation in germination percentage at nine days after sowing.

Table 1:Effects of soaking duration in 100^{o}C water at room temperature for*N. cadamba* and *L. leucocephala* seeds

Soaking duration in water (hours)	CDP	CGP	MDG	CVG	Days to 50% germination	Days to first true leaf
			N. cadamba			
0	19.6^{a}	0.0	1.0	0.05	22.7^{a}	31.3^{a}
24	14.3^{b}	0.0	1.3	0.06	17.0^{b}	19.7^{b}
48	6.3^{c}	92.2	2.7	0.11	8.0^{c}	13.7^{c}
			L. leucocephala			
0	12.3^{a}	0.0	1.0	0.05	20.5^{a}	28.5^{a}
24	10.3^{b}	0.0	0.8	0.06	15.5^{b}	19.0^{b}
48	4.3^{c}	95.9	0.5	0.13	7.0^{c}	13.5^{c}

$LSD_{0.05}$ means in the same column within each item having different superscript are significantly different ($p< 0.05$)

Seeds soaked after 48 hours showed germination percentage of 92.2% and 95.9%, while seeds soaked after 24 hours and the control both had no germination at 9 days after sowing for *N. cadamba* and *L. leucocephala*, respectively. While various pre-treatment methods have been advocated to reduce dormancy and hasten germination, no single pre-treatment technique has been found to be equally effective for all seeds in both species. The soaking in 100^{o}C water for 20 seconds and then soaked in water for 48 hours had the highest speed of germination, higher cumulative germination percentage (CGP) and shortened the period of complete dormancy over soaking duration of 24 hours and the control (**Table 1**) for *N. cadamba* and *L. leucocephala*. This result suggests that the seed germination rate increases with the soaking duration of seeds as observed in this study.

The production of the first true leaf was earliest with 48 hours soaking and least with the untreated seeds. Those untreated *N. cadamba* seeds normally take two to three weeks to germinate[57]. Argel and Paton [2] reported that *L. leucocephala* seeds were scarified and then immersed in 60^{o}C water for 15 to 30 minutes without affecting seed viability and seedling vigor, but this was not as effective as soaking of theseeds in 100^{o}C water for 4 seconds, resulting in 82% germination [24]. Warrage and Eltigani [65] showed that the length of soaking period of seeds in water was the most critical factor such as when soaking *Acacia nilotica* seeds in water for 18 weeks increased germination and then decreased when increased the length of soaking. Water treatment of seeds was the best alternative to sulfuric acid treatment, which is expensive and hazardous compared to water treatment.

Several reports had suggested that hot water treatments can be used to improve germination of hard coated seeds [5]. Hot-water treatments have been used successfully on large number of tropical and sub-tropicalseeds [17, 18, 50], and in forestry for rural development programmes [40, 42]. This treatment has yielded additional benefit of controlling coat-borne pathogens [29]. Sharma et al. [55]found that soaking seeds in 100^{o}C water for 1 to 10 minutes increasing the germination percentage 7 to 9 fold (94% to 100%) in a very short period of 4 to 6 days compared to the control. They concluded that the optimum soaking duration of seedsin 100^{o}C water was 1 minute with germination percentage of 94% for *A. lebbek*, 5 minutes with 100% germination for *L. leucocephala*, and 10 minutes with 94% and 98% germination for *A. procera*and *A. auriculiformis*, respectively. Soaking the seeds of *L. leucocephala* and *A. farnesiana* in 70°C water for 20minutes and then soaked for 72hours was more effective in breaking seed dormancy than scarification, and the germination rate was above 97% [59].

Effects of EMStreatments in N. cadamba and L. leucocephala:

A total of 420 seeds for each species were used in this study and the seed germination ranged from 36.7% and 9.2% in 0.6% EMS to 80.8% and 50.0% in the control samples for *N. cadamba* and *L. leucocephala*, respectively (**Table 2**). At the treatments level, the germination percentage was the highest (75.0% and 18.3%) in 0.1% EMS and the lowest (36.7% and 9.2%) in 0.6% EMS for *N. cadamba* and *L. leucocephala*, respectively.These results indicate that the germination percentage in all EMS treated(0.1%, 0.3% and 0.6%) samples were lower than the control samples.The low germination percentage among the EMS treated samples might be due to the genotoxic effect of EMS which could be arrested the cell cycle or genetic damage such as chromosomal aberrations [12]and thus reducing the seed germination.

The survival of *N. cadamba* and *L. leucocephala* in the control samples was 35.0% and 37.5%, respectively (**Table 2**). However, it was decreased with the increasing doses of EMS. It was highest (29.2% and 18.3%) in 0.1% EMS and lowest (15.0% and 9.2%) in 0.6% EMS for *N. cadamba* and *L. leucocephala*, respectively. The lethality was highest (57.1% and 75.6%) in 0.6% EMS and lowest (16.7% and 51.1%) in 0.1% EMS for *N. cadamba* and *L. leucocephala*, respectively. Similar findings were also reported in several other plant species such aspigeonpea [58], *Cyamopsis tetragonoloba* variety Sharada [20], sorghum [52] and wheat [16].

Table 2: Germination percentage of the EMS treatments for *N. cadamba* and *L. leucocephala* seeds

Treatment	No. of seeds treated	Survival seedlingafter germination	Germinationpercentage(%)	Survivalseedlingafter 6 months	Survival percentage(%)	Seedling height(cm)	Lethalityovercontrol(%)
			N. cadamba				
Control	120	97[a]	80.8[a]	42[a]	35.0[a]	80.0[a]	0.0
0.1	120	90[b]	75.0[b]	35[b]	29.2[b]	64.8[b]	16.7[c]
0.3	120	70[c]	58.3[c]	25[c]	20.8[c]	51.3[c]	40.5[b]
0.6	120	44[d]	36.7[d]	18[d]	15.0[d]	37.5[d]	57.1[a]
			L. leucocephala				
Control	120	60[a]	50.0[a]	45[a]	37.5[a]	149.0[a]	0.0
0.1	120	22[b]	18.3[b]	22[b]	18.3[b]	138.0[b]	51.1[c]
0.3	120	15[c]	12.5[c]	15[c]	12.5[c]	127.7[c]	66.7[b]
0.6	120	11[d]	9.2[d]	11[d]	9.2[d]	126.4[d]	75.6[a]

$LSD_{0.05}$ means in the same column within each item having different superscript are significantly different ($p< 0.05$)

The seedling height in the control samples was 80 cm and 149 cmfor *N. cadamba* and *L. leucocephala*, respectively (**Table 2**). It was reduced with an increase in the concentration of EMS, being highest (64.8 cm) in 0.1% EMS and lowest (37.5 cm) in 0.6% EMSfor *N. cadamba* and also the gradual decrease in seedling height was recorded with an increase in the concentration of EMS for*L. leucocephala*. The highest seedling height (138 cm) was observed in 0.1% EMS while the lowest (126.4 cm) was noted in 0.6% EMS. These results are in good agreement with Bhosale and More [8], who reported that the seedling height was gradually decreased with an increase in EMS concentrations in *Withania somnifera*. Similar reports were given in other plant species such astomato [34], chilly plants [45], pigeonpea [58], barley[32] and rice [12, 31, 60, 64].

The survival of plants depends on the nature and extent of chromosomal damage. The increasing frequency of chromosomal harm with increasing mutagenic doses may be responsible for the reduction in germination inability, plant growth and survival [33]. Changes in the germination percentage were attributed to mutagenic treatments. Furthermore, the genes near to the centromere are more sensitive and prone to mutagenic treatment than others. Chlorophyll mutants were frequently observed among EMS doses but were rare among those treated with physical mutagens [13]. The stimulating effect of physical mutation on germination may be credited to the activation of RNA or protein synthesis. It may occur during the early stage of germination after the seeds are treated [1].

Phytochromes that are responsible for normal growth might have affected pre-synthetic level of DNA-RNA and reduced the seedling height in rice [12]. Similarly, EMS reduced the seedling height in *N. cadamba* and *L. leucocephala* might be due to the effect of EMS which can disrupt the synthesis of new DNA may have led to the inhibition of seedling height in the present study. The germination, seedling growth and other plant characters can be increased or decreased depend on the mutagenic doses [66, 69, 70]. The high-dose treatment that caused growth inhibition has been ascribed to the cell cycle arrest during somatic cell division and/or various damages in the entire genome [9].It can be concluded that the genetic damages increase with an increase in EMS doses and this has led to the chances of getting more variables may also be increased.

Table 3:Estimates of standard genetic diversity parameters of *N. cadamba* and *L. leucocephala* by using ISSR markers. Figures in parentheses are standard errors

Parameters	Sample size N	Polymorphic loci P (%)	Gene diversity h	Shannon diversity index I
		N. cadamba		
Control	14	72.38	0.298 (0.055)	0.4336 (0.077)
0.1% EMS	10	78.09	0.307 (0.062)	0.4488 (0.087)
0.3% EMS	10	83.81	0.337 (0.056)	0.4914 (0.078)
0.6% EMS	13	91.90	0.383 (0.038)	0.5562 (0.052)
Mean		81.54	0.331 (0.052)	0.4825 (0.073)
		L. leucocephala		
Control	14	64.61	0.239 (0.056)	0.354 (0.079)
0.1% EMS	12	71.79	0.290 (0.058)	0.425 (0.082)
0.3% EMS	14	78.97	0.328 (0.052)	0.475 (0.072)
0.6% EMS	11	89.49	0.383 (0.045)	0.552 (0.062)
Mean		76.21	0.310 (0.052)	0.451 (0.074)

Genetic diversity of EMS-induced N. cadamba and L. leucocephala seedlings using ISSR markers:

In this study, 15 and 21 loci were generated by $(GTG)_6$ primer respectively for *N. cadamba* and *L. leucocephala*, meanwhile 15 loci generated by $(AC)_8G$ primer for *N. cadamba* and 13 loci were generated by $(GA)_5GC$ primer for *L. leucocephala.*At the population level, the percentage of polymorphic loci (P) ranged from 64.61% and 72.38% in the control samples to 89.49% and 91.90% in 0.6% EMS treated samples (**Table 3**), with an average of 81.54% and 76.21% for*N. cadamba* and*L. leucocephala* seedlings,respectively. The mean Nei's gene diversity (h) varied

between 0.2387 to 0.3832 with an average of 0.3100 and 0.3313 for *L. leucocephala* and *N. cadamba* seedlings,respectively. Meanwhile, the mean Shannon's diversity indices varied from 0.3536 to 0.5562, with an average of 0.4514 and 0.4825 for *L. leucocephala* and *N. cadamba* seedlings,respectively. Among the samples investigated, the highest and lowest levels of genetic variability occurred in the control samples (P: 71.79% and 78.09%; h: 0.2387 and 0.2985; I: 0.3536 and 0.4336) and 0.6% EMS treated samples (P: 89.49% and 91.90%; h: 0.3826 and 0.3832; I: 0.5520 and 0.5562) for *L. leucocephala* and *N. cadamba* seedlings,respectively. These results indicate that all EMS treated (0.1%, 0.3% and 0.6%) samples were higher than the control in genetic variability.

The high genetic variation among the EMS treated samples may be explained by the disappearance of normal bands and the appearance of new bands in comparison to the control due to the effects of EMS. Plants treated with different concentrations of EMS showed differences in terms of disappearance of normal bands and the appearance of new bands in comparison to the control and thus increase the genetic variability [15]. The new bands could be related to mutation, while the disappearance of bands could be related to DNA damage, DNA methylation and chromosomal damage, both of which resulted in generation of DNA polymorphism in the treated population [3]. The lower EMS concentration (0.1% EMS) had lower polymorphic loci (%), Shannon's diversity index and Nei's gene diversity. The higher number of bands was lost in 0.1% EMS treated samples than 0.6% EMS treated samples. However, it was more or less equal number of lost bands in the intermediate concentrations (0.3% EMS). Our results are in good agreement with Saba and Mirza [53]whofound that with an increase in EMS concentration, there was an increase in the genetic variation in *Lycopersicon esculentum***.** Similar results have also been reported for several plants such as *Capsicum annuum* [49] and *Avena sativa* [36].

CONCLUSION

Mutation induction technology is widely used for developing improved new varieties through improving character of direct importance, early maturity and tolerance to biotic and abiotic stresses. In addition, EMS has been used to obtain high frequency of gene mutation and chromosomal alteration in different crops which led to evolve a number of mutant varieties. For the purpose of plant breeding programme in tree species particularly in *L. leucocephala* and *N. cadamba*, the treatments of chemical mutagen (EMS) with strong genetic effects are desirable. Hence, we used EMS concentrations ranging from 0.1% to 0.6 % for plant genetic improvement in *L. leucocephala* and *N. cadamba* of which higher concentrations, particularly 0.6% concentration of EMS was effective to influence the genetic variation from our research. The effect of EMS on the genetic variation of the mutated seedlings for *L. leucocephala* and *N. cadamba* with 0.6% EMS treatment is much more beneficial as compared to 0.1% and 0.3% EMS treatments. From the current findings we concluded that the genetic variability was increased with an increase in the EMS doses. Further, EMS has been successfully used to produce a range of novel traits and broadening of genetic diversityof both species studied in the present study.

Acknowledgements
The authors would like to thank all the lab assistants and foresters involved in this research project for their field assistance in sample collection. This work is part of the joint Industry-University Partnership Programme, a research programme funded by the Sarawak Forestry Corporation (SFC), Sarawak Timber Association (STA) and Universiti Malaysia Sarawak under grant no. 02(DPI09)832/2012(1), RACE/a(2)/884/2012(02) and GL(F07)/06/ 2013/STA-UNIMAS(06).

REFERENCES

[1] Abdel-Hady, MS, Okasha EM, Soliman SA, Talaat M, *Australian Journal of Basic and Applied Sciences*, **2008**, 2(3), 401-405.
[2] Argel PJ, Paton CJ, In: LochDS, Ferguson JE (Ed.),*Forage seed production: tropical and subtropical species*, CAB International, Wallingford, **1999**, pp 247-265.
[3] Atienzar FA, Cordi B, Donkin ME, Evenden AJ, Jha AN, Depledge MH, *Aquatic Toxicology*, **2000**, 50, 1-12.
[4] Awan MA, Konzak CF, Rutger JN, Nilan RA, *Crop Science*, **1980**, 20, 663-668.
[5] Aydın I, Uzun F, *Pakistan Journal of Biological Sciences*, **2001**, 4, 181-183.
[6] Bhat TA, Sharma M, Anis M, *Asian. J. Plant. Sci.*,**2007**, 6,1051-1057.
[7] Bhatia CR,*Mutation Breeding Newsletters*,**1999**, 44, 3-5.
[8] Bhosale RS, More AD, *Int. J. of Life Sciences*, **2013**, 1(2), 158-160.
[9] Borzouei A, Kafi M, Khazaei H, Naseriyan B, Majdabad A, *Pakistan Journal of Botany*, **2010**, 42(4), 2281–2290.
[10] Brewbaker JL, *Leucaena leucocephala-a versatile nitrogen fixingtree*, Winrock International, Morrilton, AK., **1997**.
[11] Chanchay N, Poosaran N, *Asian Journal of Food and Agro-Industry (Special Issue)*, **2009**, S137-S144.

[12] Cheema AA, Atta BM, *Pakistan Journal of Botany*, **2003**, 35(2), 197-207.
[13] Chopra VL, *Current Science*, **2005**, 89(2), 353-359.
[14] Comai L, Henikoff S, *Plant J*,**2006**, 45, 684-694.
[15] Dhakshanamoorthy D, Selvaraj R, Alagappan C, *Journal Crop Science Biotechnology*, **2013**, 16(3), 201-207.
[16] Din R, Khan MM, Qasim M, Jehan S, Khan MI, *Asian Journal of Plant Sciences*, **2003**, 2(17), 1179-1182.
[17] Doran JC, Turnbull JW, Boland DJ, Gunn BV, *Handbook of seeds of dry-zone Acacias. A guide for collecting, cleaning, extracting and storing the seed to provide germination of dry-zone Acacias*, FAO, Rome, Italy, **1983**.
[18] Doussi MA, Thanos CA, *Proceeding of the 2nd Conference on Forest Fire Research*, **1994**, 1035-1044.
[19] Doyle J, Doyle L, *Focus*, **1990**, 12, 13-15.
[20] Dube KG, Bajaj AS, Gawande AM,*Asiatic Journal of Biotechnology Research*, **2011**, 2(6), 747-754.
[21] Esselman EJ, Jianqiang L, Crawford DL, Windus JL, Wolfe AD, *Molecular Ecology*, **1999**, 8, 443-451.
[22] Gajera B, Kumar BN, Singh AS, Punvar BS, Ravikiran R, Subhash N, Jadejam GC, *Industrial Crops and Products*, **2010**, 32(3), 491–498.
[23] Gilbert JE, Lewis RV, Wilkinson MJ, Caligari PDS, *Theoretical and Applied Genetics*, **1999**, 98, 1125-1131.
[24] Gosling PG, Samuel YK, Jones SK, *Seed Science Technology*, **1995**, 23, 521-532.
[25] Gupta M, Chyi YS, Severson RJ, Owen JL, *Theoretical and Applied Genetics*, **1994**, 89, 998-1006.
[26] Harris J, In:Hollingsworth PM, Bateman RM, Gornall.MolecularRJ (Ed.),*Systematics and plant evolution*, Taylor and Francis, London,UK, **1999**.
[27] Hess J, Kadereit JW, Vargas P,*Molecular Ecology*, **2000**, 9, 857-868.
[28] Ho WS, Pang SL, Julaihi A, *Physiology and Molecular Biology of Plants*, **2014**, 20(3), 393-397.
[29] Hoersten DV, Luecke W, *American Society of Agricultural and Biological Engineers*, **2001**, 12(1), 21-26.
[30] Joker D, *Seedleaflet*:*Neolamarckia cadamba*(Roxb.) Bosser (*Anthocephalus chinensis*(Lam.) A. Rich. ex Walp.) (http://curis.ku.dk/portal-life/files/20648324/ neolamarckia_cadamba_int.pdf), **2000**.
[31] Katosh PC, Massar JE, *Indian Journal of Genetics and Breeding*,**1992**, 52, 213-218.
[32] Khalatkar AS, Bhatia CR,*Radiation Botany*, **1975**, 15, 223-229.
[33] Kiong ALP, Lai AG, Hussein S, Harun AR, *American-Eurasian Journal of Sustainable Agriculture*, **2008**, 2(2), 135-149.
[34] Kostov K, Batchvarova R, Slavov S, *Bulgarian. Journal of Agricultural Science*, **2007**, 13, 505-513.
[35] Kotowski F, *Proc. Am. Soc. Hortic. Sci.*, **1926**, 23, 176-184.
[36] Krishna-Murthy CS, Vasudevan K, *Crop Improvement*, **1984**, 11, 128-31.
[37] Lefroy SC, Dann PR, Wildin JI, Smith RN, McGowan AA, *Agroforestry Systems*, **1992**, 20, 117-139.
[38] Li W, Zhu SL, Li N, Chen XY, *Forestry Studies in China*, 2011, 13(1), 45-51.
[39] Maluszynski M,*Gene manipulation in plant improvement*, Plenum press, New York, **1990**.
[40] Masamba C, *Forest Ecology and Management*, **1994**, 64, 105-109.
[41] Mensah JK, Obadoni BO, Akomeah PA, Lkhajiagbe B, Ajibolu J, *African Journal of Biotechnology*,**2007**, 6(5), 534-538.
[42] Muhammad S, Amusa NA, *African Journal of Biotechnology*, **2003**, 2, 276-279.
[43] Nagoaka T, Ogihara Y, *Theoretical and Applied Genetics*, **1997**, 93, 133-139.
[44] Nei M,In:Morton NE, (Ed.), *Genetic structure of populations*,University Press of Hawaii, Honolulu, **1973**, pp45-51.
[45] Nyla J, Mirza B, *International Journal of Agriculture and Biology*, **2004**, 6(2), 340-345.
[46] Okagaki RJ, Neuffer MG, Wessler SR, *Genetics*, **1991**, 128, 425-431.
[47] Okon S, Agnieszka SM, Edyta PG, *Acta Scientiarum Polonorum Hortorum Cultus*, **2013**, 12(2), 43-50.
[48] Patamsyte J, Cesniene T, Naugzemys D, Kleizaite V, Vaitkuniene V, Zvingila VD, *Agriculture*, **2011**, 98(30), 293–300.
[49] Patil AN, Meshram LD, Nandanwar RS, *Soils and Crops Journal*, **1997**, 7, 15-8.
[50] Prasad P, Nautiyal AR, *Seed Science and Technology*, **1996**, 24, 305-308.
[51] Ramesh B, Prasad BK, Singh VP, *Mutation Breeding Newsletters*, **2001**, 45, 26-27.
[52] Ramulu, S.,*Mutation research*, **1970**, 10: 197- 205.
[53] Saba N, Mirza B, *International Journal of Agriculture and Biology*, **2002**, 4(1), 89-92.
[54] Sakin MA, Yildirim A, *Journal of Food Agriculture and Environment*, **2004**, 2(1), 285-290.
[55] Sharma S, Naithani R, Varghese B, Keshavkant S, Naithani SC, *J. Trop. For.*, **2008**, 24, 49-53.
[56] Singh S, Richharia AK, Joshi AK, *Indian Journal of Genetics*, **1998**, 58(4), 455-463.
[57] Soerianegara I, LemmensRHMJ, *Plant resources of South-east Asia 5(1): Timber trees: Major commercial timbers*, Pudoc Scientific Publishers, Wageningen, Netherlands, **1993**.
[58] Sunil MS, Mahamune SE, Kharat SN, Kothekar VS, *Bioscience Discovery*, **2011**, 2(1), 2229-3469.
[59] Tadros MJ, Samarah NH, Alqudah AM, *New Forests*, **2011**, 1-11.
[60] Talebi AB, Shahrokhifar B, *American Journal of Plant Sciences*, **2012**, 3, 1661-1665.
[61] Tiong SY, Chew SF, Ho WS, Pang SL, *Advances in Applied Science Research*, **2014**, 5(3), 458-463.
[62] Tsumura Y, Ohba K, Strauss SH, *Theoretical and Applied Genetics*, **1996**, 92, 40-45.

[63] Van Harten AM,*Mutation breeding, theory and practical applications*, Cambridge University Press, Cambridge, United Kingdom, **1998**,pp 127-140.
[64] Wang CL, Shen S, Chen QF, *Acta Agriculture Nucleatae Sinica*, **1995**, 9, 13-19.
[65] Warrag EI,Eltigani M,*Tropical Ecology*,**2005**, 46 (1), 127-132.
[66] Wi SG, Chung BY, Kim JS, *Micron*, **2007**, 38, 553-564.
[67] Wickneswari R, Ho WS, *TROPICS*, **2003**, 13(2), 139-149.
[68] Yeh FC, Yang RC, Boyle T, *POPGENE Version 1.32: Microsoft Windows–based freeware for population genetic analysis, quick user guide*, Center for International Forestry Research, University of Alberta, Edmonton, Alberta, Canada, **1999**.
[69] Zaky ZM, Zaki MA, Fasihuddin BA, Ho WS, Pang SL, *International Journal of Scientific & Technology Research*, **2014**, *in press*.
[70] Zaky ZM, Zaki MA, Fasihuddin BA, Ho WS, Pang SL, *Archives of Applied Science Research*, **2014**, *in press*.