MUTATION BREEDING TO INCREASE GENETIC DIVERSITY IN MANGOSTEEN

Valerie Suwanseree[1,*] Salak Phansiri[2], Chalermsri Nontaswatsri[3], & Chinawat Yapwattanaphun[4]

[1]Graduate School, Kasetsart University
[2]Scientific Equipment and Research Division, KURDI, Kasetsart University
[3]Floriculture Breeding Lab, Faculty of Agricultural Production, Maejo University
[4]Department of Horticulture, Faculty of Agriculture, Kasetsart University

*Corresponding author: vbdfadan@yahoo.com; Tel.: +66-086-781-0388

ABSTRACT

Mangosteen, a popular tropical fruit crop, is unusual in that the flowers do not produce viable pollen. There is only one cultivar of mangosteen available in Thailand, and increased genetic diversity is desirable. Traditional hybridization breeding through cross pollination is not possible so this research used mutation breeding. Mangosteen seeds were treated with 8 concentrations of the chemical mutagen ethyl methane sulfonate (EMS) and 4 concentrations of the spindle fiber inhibitor colchicine. Surviving seedlings that sprouted were grown in pots filled with planting mixture under shade. Morphological abnormalities observed included irregularly shaped leaves, asymmetrical leaves, new branches arising from the base of stem or near the top, multiple branches from one part of the stem rather than pairs, asymmetrical branching, curved stem, and recumbent growth habit. Some of the abnormalities were transient and did not persist as the plants grew new sets of leaves or branches. The maximum percentage of leaf abnormalities recorded was 50% in the EMS 0.75% group, compared to 20% in the control. The maximum percentage of unusual branching patterns recorded was 50% in the EMS 0.5% group, compared to 20% in the control. In many cases these abnormalities may not have been mutations but may have been caused by environmental conditions such as insect damage, sunburn, and crowding. For the colchicine treatment groups, 1.8% of the 0.1% colchicine group, 4.9% of the 0.25% colchicine group, 22.9% of the 0.5% colchicine group, and 15.2% of the colchicine 75% group had thick, misshapen leaves with prominent midribs, compared to 0% of the control. Ten of the thick-leaved plants had stomata guard cells that were larger than the control. Six were shown by flow cytometry to have a greater amount of DNA per cell than the control. They will be grown to maturity to observe their horticultural traits.

Keywords: apomixis, colchicine, ethyl methane sulphonate, *Garcinia mangostana*, genetic diversity, morphology, mutagen, polyploid

1. INTRODUCTION

Mangosteen is an important fruit crop in Southeast Asia. Thailand is the world's largest exporter of mangosteen, followed by Indonesia, Vietnam, Malaysia, and the Philippines (Food and Agriculture Organization of the United Nations, 2011). In Thailand, mangosteen orchards cover approximately 66,403 hectares (Office of Agricultural Economics, 2016). Mangosteen is valued for its delicious fruit and the juice is promoted as a healthy beverage. Mangosteen rind also contains antibiotic and antioxidant substances and can be used to make a wide variety of cosmetic products. It is very popular as an ingredient for soap, lotion, facial masques, scrubs, and anti-acne cream. Some manufacturers also put mangosteen rind in deodorant, feminine hygiene products and toothpaste.

Mangosteen is adapted to the climactic conditions of the humid tropics where it originated, so it is suited to a temperature range of 25-35°C. Mangosteen trees need high humidity and moist conditions most of the year but require a dry period to stimulate flowering (Chaisrichonlathan & Noomhorm, 2011; Suwanseree, 2017). The trees are very slow-growing compared to many tropical crops. The seedlings and even mature trees require shade to protect them from sunburn (Verheij & Coronel, 1992). All these constraints have limited the amount of land area used for growing mangosteens.

The main physiological disorders of mangosteen fruit that affect fruit quality are gummosis (also called Gamboge disorder), translucent flesh disorder and hardened rind. Gummosis is when yellow latex seeps from the stem or rind on to the edible aril in the fruit, giving it a yellow color and very bitter flavor. Gummosis is suspected to be related to damage from thrips or other insects (Yapwattanaphun, 2008). Most export markets (for example, Taiwan, Korea, Saudi Arabia, and United Arab Emirates) will not accept mangosteens with spots of yellow latex on the outer rind (Dorly *et al.*, 2011). Gummosis normally affects between 9-50% of fruits in Indonesia (Kurniadinata *et al.*, 2016).

Botanically, mangosteen is unusual because it is an obligate apomict, producing viable seed without pollination. Research showed that pollen does begin to develop in the staminodes of mangosteen flowers, but it degenerates either before or soon after it is completely formed (Yapwattanaphun *et al.*, 2008). New plants arise from unfertilized seeds through adventitious embryony. Because there is no pollination, all mangosteen trees should theoretically be genetically identical clones of the same mother plant. However, some genetic variation could occur through natural mutations due to UV radiation or mistakes in DNA replication. Several genetic marker studies have been done to assess the genetic uniformity among mangosteen trees from different geographical areas or parentages, and they all reported a certain degree of genetic variation (Mansyah *et al.*, 2013; Ramage *et al.*, 2014; Matra *et al.*, 2014; Mansyah *et al.*, 2010).

There are 2 cultivated varieties of mangosteen recognized by the National Seed Industry Council of the Philippines – 'UPLB Sweet' registered in 2006 and 'Roxas Purple' registered in 2007 (Namuco, 2008). Namuco (2007) states, "There is no available record regarding the origin and parentage of 'UPLB Sweet' mangosteen." It was developed from a tree growing at the University of the Philippines Los Baños. Similarly, "there is no recorded account on the origin and parentage of 'Roxas Purple' mangosteen." It was developed from a tree growing in the orchard of Mr. Marino Roxas in Laguna (Namuco, 2008). The listed fruit characteristics of these cultivars appear to be quite similar to ordinary mangosteen, so it is debatable that they should be considered cultivars.

In Malaysia and Singapore some vendors sell another variety of mangosteen known as 'Masta', 'Mesta', or 'Japanese mangosteen'. 'Masta' is also used instead of *manggis* for the common name of mangosteen in some areas. 'Masta' has fruit that is more ovoid than globose in shape, with more pointed ends. It is possible that it is the fruit of a closely related species, *Garcinia malaccensis*. *G. malaccensis* may soon be considered a sub-species, based on genetic and morphological data that *G. malaccensis* and mangosteen are actually so closely related that they should be combined together and considered as one species (Nazre, 2017).

Because by nature mangosteen trees do not produce viable pollen, crossing to create new hybrids is not possible. In light of this limitation, alternative methods should be used to create

greater genetic variation in mangosteen for the selection of superior cultivars. One approach is to induce random mutations using radiation or chemical treatment. This method is much less precise than genetic modification, it is not banned by governments, feared by consumers, nor criticized activists. In this research we used ethyl methanesulfonate (EMS) and colchicine as chemical mutagens to induce mutations in mangosteen seeds.

EMS is an alkylating agent. It reacts with DNA, converting guanine to alkylated guanine. This can result in base substitutions, depurination or single-strand breaks (Wongpiyasatid, 1997). EMS has successfully been used to develop new cultivars of several agronomic and horticultural crops including beans, oats (Arias & Frey, 1973; Rines, 1985), barley (Arian, 1974), rice (Augustine *et al.*, 1975), black gram (Rao & Jana, 1976), and banana (Omar *et al.*, 1989; Bhagwat & Duncan, 1998)

Colchicine is an alkaloid obtained from the root of *Colchium autumnale* L. or *Iphigenia indica* Kunth et Benth that can be used to induce mutations, especially chromosome doubling, because it binds with tubulin and thus interferes with microtubule formation during mitosis (Kingsbury, 2009). Because polyploid plants are often larger and more robust than plants with the normal chromosome number, scientists have used colchicine to induce tetraploidy in many crops, especially ornamentals. The extra set of chromosomes can stimulate the expression of a greater range of genetic variation, such as changes in leaf size, flower size or flower color (Osborne, *et al.*, 2003). Colchicine has been successfully used to induce polyploidy in fruit crops such as citrus, banana, pear, pomegranate, grape and persimmon (Zeng *et al.*, 2006).

2. MATERIALS AND METHODS

Mangosteen seeds were removed by hand from the flesh of ripe fruit obtained from a wholesale market in Bangkok. They were washed in detergent, rinsed with tap water and then agitated for 2 h (shaken on an Innova 2300 orbital shaker at 105 rpm) in an anti-bacterial and anti-fungal solution containing 0.1% Kanker-X and 0.1% Captan orthocide. Next, the seeds were removed from the anti-bacterial and anti-fungal solution in a laminar flow hood and surface sterilized in 20% Clorox solution for 20 minutes, followed by 10% Clorox solution for 10 minutes, then rinsed with autoclaved water three times. Lastly, the seeds were randomly dispensed into jars containing the different chemical mutagen treatments: aqueous solutions of EMS at the concentrations of 0 (control), 0.1%, 0.25%, 0.5%, and 0.75%; and colchicine at the concentrations of 0 (control), 0.1%, 0.25%, 0.5%, and 0.75% for 16 hours in closed jars on an orbital shaker.

For the first, second, and third EMS replications, 25 seeds were used for each treatment group, while in the fourth and fifth replications the samples sizes were 38 and 32 seeds per treatment group. For both times colchicine was used, the sample size was 34 seeds per treatment group.

After the mutagen treatment, the seeds were rinsed in autoclaved dH2O 5 times and placed, one to a jar, in tissue culture vessels containing 25-30 ml MS medium supplemented with 0.5 g l^{-1} PVP, 3% sucrose and 7 g l^{-1} agar (pH 5.7). They were maintained at 25±5°C with an 8:16 h photoperiod until leaves had emerged.

After approximately 50 days in tissue culture, surviving seedlings were planted out in seed trays filled with peat moss (Kekkilä), sprayed with benomyl fungicide and the seed trays were covered with a plastic bag and placed in the shade. After approximately 36 days the seedlings were transferred to 25 cm plastic pots filled with KU potting mixture (compost : rice husk charcoal : coconut husk chips : fine ground coconut husk, 1:2:4:4 by volume) and kept in partial shade. They were fertilized with 15-15-15 formula slow-release fertilizer every 3 months and watered

daily. Plant height and the width and length of the largest pair of leaves and the newest pair of leaves were recorded every 3 months.

Stomata guard cells of 12 month old colchicine-treated plants were compared by microscopic analysis. Impressions were made of the underside of mature leaves by applying a coat of clear nail polish over an area of about 2-3 square centimeters on one side of the leaf (around the middle of the leaf from midvein out to the margin) and waiting approximately 15 minutes for it to dry, then carefully peeling it off. The leaf impressions were viewed at 40x magnification with a Xenon light microscope and photographed with Future Winjoe software.

Selected specimens from the colchicine treatments that had thick, misshapen leaves and were presumed to be polypoid were analyzed by flow cytometry at the floriculture breeding lab of Dr. Chalermsri Nontaswatsri at the Faculty of Agricultural Production, Maejo University, in Chiang Mai, Thailand. A piece of leaf lamina from a newly expanded leaf measuring about 1.5 cm square was finely macerated with a razor blade in 1% PVP aqueous solution with Tris MgCl buffer, then filtered through a 41 µ nylon net and chilled at approximately 5° C for 15 minutes, then vortexed and Guava® Cell Cycle Reagant dye was added. The samples were analyzed in a Guava EasyCyteTM flow cytometer (EMD Millipore Corp.).

3. RESULTS

For the seed experiments, 8 batches of surface-sterilized seeds from ripe fruit were soaked in EMS or colchicine solution of varying concentrations, or in plain water for the control, for 16 hours and then after rinsing were left to sprout in vitro.

In the initial test, (sample size 25 seeds per treatment) the survival rate was 96% for control and 48% for 0.5% EMS but dropped to zero for the 1%, 1.5%, and 2% EMS treatment groups. As a result, lower concentrations were used for the subsequent experiments – 0.75%, 0.5%, 0.25%, 0.1%, and 0% EMS, and the same concentrations were used for colchicine.

A total of 130 seeds per treatment group were treated with EMS, divided into 4 replications spanning August 2016 to June 2017. As expected, the survival rate dropped with increasing concentration of EMS. The lethal dose or LD50 for EMS (the concentration at which half the seeds died) was calculated to be 0.43% (Figure 1).

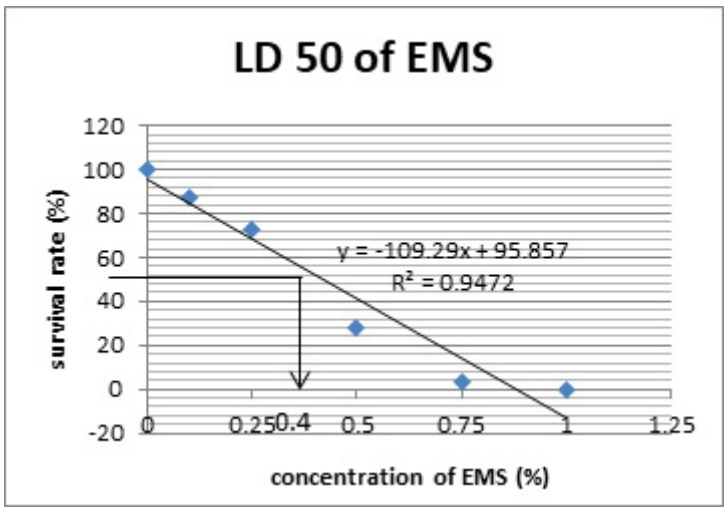

Figure 1. LD50 of EMS for mangosteen seeds exposed to aqueous solutions of EMS for 16 hours

Two batches of seeds were treated with colchicine at the same concentrations and duration as that was used for EMS. The sample size was 34 seeds per treatment. The survival rate was high for all concentrations of colchicine, but dropped slightly with increasing concentration, from 97% in the control down to 94% in the 0.1% and 0.25% colchicine groups, 92% in the 0.5% colchicine group and 85% in the 0.75% colchicine treatment group. It was not possible to determine the LD50 at the concentrations of colchicine tested because the survival rate was high for all concentrations tested.

Several morphological abnormalities were observed in mutagen-treated seedlings, as well as in some of the control seedlings, both when they were first removed from the tissue culture jars, and later when they were growing in the nursery. Many of the leaf abnormalities were no longer observable after the plants developed 3 or more pairs of leaves. However, many of the abnormalities, such as lack of a main root, almost certainly affected the later survival of the seedlings.

Out of the control group, about 6% of sprouting seeds had no root at 8-10 weeks after treatment time. Of the EMS group, the percentage of seedlings with no root increased with increasing concentration of EMS (6%, 19%, 25%, and 29% had no root in the EMS 0.1%, 0.25%, 0.5%, and 0.75% groups, respectively). For colchicine, the percentage of seedlings with no root was rather high in every treatment group at 25%, 18%, 17%, and 24% in the colchicine 0.1%, 0.25%. 0.5% and 0.75% groups, respectively (Figure 2).

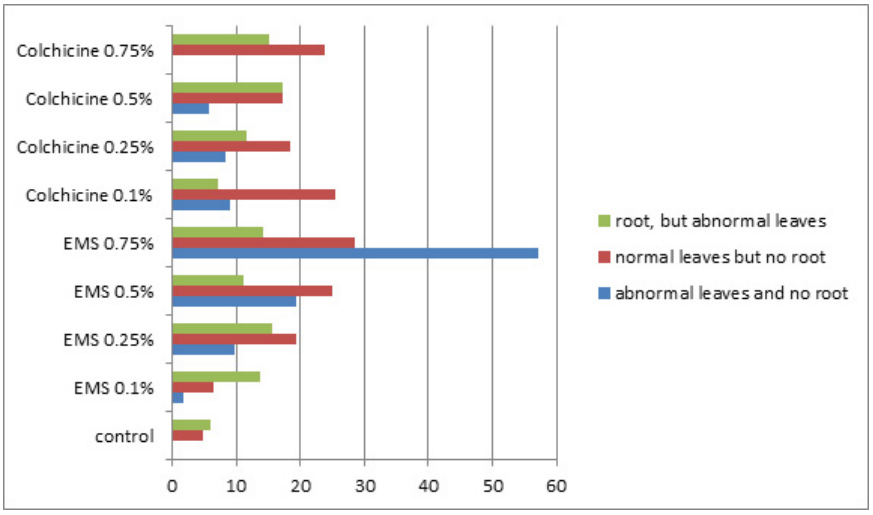

Figure 2. Percentage of abnormal seedlings 9 weeks after treatment. Note: "normal leaves, no root" means 1 or more pairs of approximately symmetrical leaves and no visible root, or root less than 3 mm in length; "root but abnormal leaves" means normal primary or main root or both with a single leaf per node, 3 leaves per node, a pair of leaves of unequal size, misshapen leaves, or leaves arising almost directly from the seed with almost no stem; "abnormal leaves and no root" means no visible root or root less than 3 mm in length and a single leaf per node, 3 leaves per node, a pair of leaves of unequal size, misshapen leaves, or leaves arising almost directly from the seed with almost no stem.

Approximately 6% of control seedlings had leaf abnormalities (one leaf larger than its pair, or misshapen, or an odd number of leaves). In the EMS group, the number of seedlings with abnormal leaves at planting out time was about double that in the control for every concentration of EMS (14%, 16%, 11%, and 14% for the EMS 0.1%, 0.25%, 0.5%, and 0.75% groups, respectively). For the colchicine group, the percentage of seedlings with leaf abnormalities was only slightly higher than in the control for the lowest concentration (7% in the 0.1% colchicine group), but

quite higher than in the control in the other groups (12%, 17%, and 15% in the 0.25%, 0.5% and 0.75% colchicine groups, respectively). This high occurrence of abnormalities in the treatment groups definitely suggests that mutations were induced by the treatments. However, because there was a 6% occurrence of root and leaf abnormalities in the control seedlings, it is possible that some of the abnormalities observed at the planting out stage may have been a result of the tissue culture process rather than the mutagens, or simply natural variation (Figure 2).

Most striking was the number of seedlings with both no root and with abnormal leaves among the EMS-treated seedlings, which was much higher than control and the percentage increased with increasing concentration of EMS. None (0%) of the control seedlings fell in this category, but 2%, 10%, 19%, and 57% of the seedlings in the EMS 0.1%, 0.25%, 0.5%, and 0.75% groups, respectively, had no root and abnormal leaves. The number of colchicine-treated seedlings that had both no root and abnormal leaves was 9%, 8%, 6%, and 0% in the 0.1%, 0.25%, 0.5% and 0.75% colchicine groups, respectively (Figure 2).

In the nursery, the main abnormalities observed over the first 2 years were in the following categories: leaf abnormalities (irregularly shaped leaves, asymmetrical leaves, missing or extra leaves, or wavy or ridged leaves), unusual branching pattern (new branches arising from the base of stem or near the top, early development of lateral branches before 27 months, multiple branches from one part of the stem rather than pairs, or asymmetrical branching), curved stem, and recumbent growth habit.

Some of the morphological abnormalities were transient in nature and did not persist as the plants grew new sets of leaves or branches. The number of samples in each treatment group also changed over time as some of the plants died for various reasons (primarily sunburn). The following data are therefore approximate percentages. The maximum percentage of leaf abnormalities recorded was 50% in the EMS 0.75% group (a group with a very small sample size because of the lethal effect of EMS), followed by approximately 31% in the EMS 0.5% group, 21% in the EMS 0.1% group, 20% in the control, and 12% in the EMS 0.25% group. The maximum percentage of unusual branching patterns recorded was 50% in the EMS 0.5% group, followed by 24% in the EMS 0.1% group, 20% in the control, and 19% in the EMS 0.25% group. The maximum percentage of curved stems was 29%, recorded in the control group, followed by 19% in the EMS 0.25% group, and 10% in the EMS 0.1% group. The occurrence of recumbent habit was 9% in both the EMS 0.1% and 0.25% groups, followed by 8% in the control. In many cases these abnormalities may not have been mutations but may have been caused by environmental conditions such as insect damage, sunburn, lack of sufficient water and lack of space, because the seedlings were spaced very closely together in plastic pots.

Morphological variations were more easily observable in the colchicine-treated groups compared to the EMS-treated groups, as several plants from the colchicine groups displayed a thick leaf phenotype that may be indicative of induced polyploidy (Figure 3). Many of the colchicine-treated plants also exhibited early or unusual branching patterns, short internodes (stunted appearance), or curved stems (Table 1).

Table 1. Percent morphological abnormalities in 12-15 month old mangosteen seedlings from colchicine-treated treatment groups and control.

Phenotype	Control (n=15)	0.1% colchicine (n=55)	0.25% colchicine (n=61)	0.5% colchicine (n=48)	0.75% colchicine (n=46)
Thick, misshapen leaves with prominent midrib	0%	1.8%	4.9%	22.9%	15.2%
Short internodes	6.7%	10.9%	13.1%	25%	17.4%
Unusual branching	20%	25.5%	26.2%	12.5%	30.4%
Curved stem	0%	3.6%	3.3%	6.3%	4.3%

Leaf impressions were taken of 12-month-old seedlings from the colchicine trial. Ten of the plants with thicker leaves and midribs as pictured in Figure 3 had stomata guard cells that were noticeably larger than the control – 2 from the 0.25% treatment group, 3 from the 0.5% treatment group and 5 from the 0.75% treatment group (Appendix A).

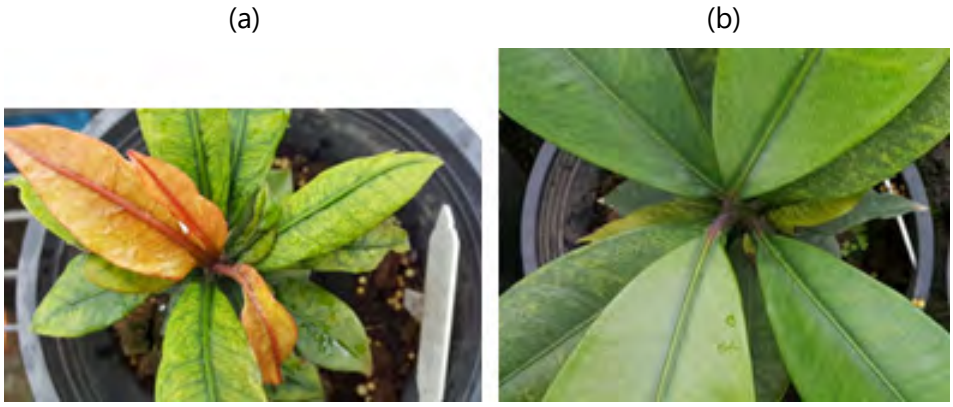

Figure 3. Example of a mangosteen seedling grown from colchicine-treated seed with thick, misshapen leaves and prominent midribs: (a) 0.75% colchicine group, 12 months; (b) control, 12 months.

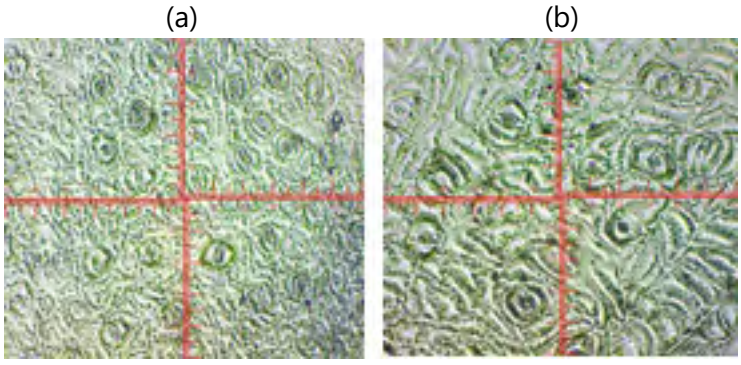

Figure 4. Example of stomata guard cells viewed under light microscope at 40x magnification; (a) control, (b) specimen #7 from the 0.5% colchicine treatment group. For additional stomata comparison photos, see Appendix A.

Flow cytometry analysis was used to determine the amount of DNA in the cells of experimental plants that were selected as putative polyploids based on morphological characteristics and examination of stomata guard cell size. Five specimens were confirmed to be polyploid based on the amount of DNA – 2 from the 0.5% colchicine treatment group and 3 from the 0.75% colchicine treatment group (for dendrograms, see Appendix B). This data affirms that treatment

with colchicine at the rate of 0.5-0.75% applied in aqueous solution on mangosteen seeds for 16 hours can induce polyploidy.

4. DISCUSSION

The LD50 for EMS was 0.43% (Figure 1). This is similar to the findings of Te-chato, who reported that the LD50 was 0.5% EMS when young leaves of mangosteen were exposed to varying concentrations of EMS in aqueous solutions for 2 hours (Te-chato, 1998). Te-chato and colleagues also reported a growth inhibition rate of 50% at the concentration of 0.5% EMS for in vitro mangosteen callus tissue when it was exposed to EMS in aqueous solutions for 2 hours (Te-chato & Phrommee, 1999).

Some of the seedlings from both the control and the mutagen-treated treatment groups were very small or abnormal at the planting out stage, and most of those tended to die off during the first few months. Some of the surviving mutagen-treated plants seemed to grow somewhat faster than the control, and this could be because they were hardier individuals that were able to withstand the mutagen treatment. Another important factor explaining much of the variation in our results is that it was not possible to make the conditions in the nursery absolutely identical for all the experimental plants. For example, some that were in pots that were at the end of a row or in the side-most rows were exposed to more sunlight than the others and may have suffered more sunburn or insect damage.

However, some of the abnormalities observed were probably due to EMS and colchicine treatment. EMS-induced mutations reported in the literature include several changes in leaf shape such as narrow, crinkled leaf phenotype in black gram beans (Rao & Jana, 1976), unspecified leaf morphology changes in oat (Chawade *et al.*, 2010), thin, elongated leaves in *Silene latifolia* (white campion flower) (Jenkins *et al.*, 2005), curly leaf phenotypes in rapeseed (Wang *et al.*, 2008), brown midribs, erect leaves and multiple tillers in sorghum (Xin *et al.*, 2008), and taller plants with more branches and more and larger leaves in mulberry (Kumar *et al.*, 2013). In the present research the occurrence of curved leaves, unequal sizes for leaf pairs, notched leaves and heart-shaped leaves were noted in the EMS-treated seedlings and also some of the control group, but none of these changes in morphology were permanent and usually the next pair of leaves that developed was normal, so they were probably not due to permanent genetic mutations.

Other EMS-induced mutations reported in the literature include changes to plant size. For instance, both dwarf and giant mutants were identified from among oat plants grown from seeds exposed to EMS (Chawade *et al.*, 2010). Other researchers reported some dwarf and also some oversized plants and decreased fruit size in tomato (Gady *et al.*, 2009), increased plant height in papaya (Santosh *et al.*, 2010), shorter internodes in asparagus (Sonoda *et al.*, 2008), dwarf plants in Indian mustard (Prem *et al.*, 2012), and short internodes in mulberry (Kumar *et al.*, 2013). In this research we did note short internodes on many plants in both the EMS-treated groups and the control and we suspect this may be due mainly to environmental factors.

Another category of EMS-induced mutations often reported in the literature is chlorophyll variations, such as albino, viridis, xantha, striata and intermediate leaves in barley seeds (Aram, 1974); albino, yellow, and variegated leaves in *Tillandsia fasciculata* (Koh & Davies, 2001); increased chlorophyll content and increased yield in yam (Sahoo *et al.*, 1986); chlorophyll deficiency and decreased yield in diploid oat (Rines, 1985); orange pericarp and short plant height in capsicum (Bhargava & Umalkar, 1989); chlorosis in oat (Chawada *et al.*, 2010); chlorophyll deficiency in

tomato (Gady *et al.*, 2009); and variegated leaves in *Silene latifolia* (Jenkins *et al.*, 2015). In the present research chlorophyll content was not tested. Some plants did have more pale green or yellowish leaves at some times, but again, this characteristic was observed in the control group as well, and the change was not a permanent one. When newer pairs of leaves developed (especially in the rainy season) they usually reverted to the normal color.

As for cases in which EMS has successfully been used to generate commercially useful mutations in crop plants, some examples include increased gum content in senna (Bhargava & Umar, 1989), abscisic acid insensitivity leading to reduced grain dormancy in wheat (Schramm *et al.*, 2012), auxin resistance in rice (Meng *et al.*, 2009), tolerance to *Fusarium oxysporum* fungus in banana (Bhagwat & Duncan, 1998), and tolerance to *Rhizoctonia solani* (damping off fungi) in wheat (Okubaka *et al.*, 2009). It was beyond the scope of the present research to test pathogen resistance and xanthone content in the mutagen-treated mangosteen plants, but it would be very lucky indeed if any of the specimens had such qualities.

Some preliminary work has been done in the past using colchicine as a mutagen on mangosteen. Te-chato and Sujaree (1999) treated shoot buds of mangosteen with colchicine at concentrations ranging from 500 up to 10,000 mg l^{-1} and durations ranging from 2 hours to 30 days. At the concentration of 10,000 mg l^{-1} and exposure time of 10 hours the survival rate of shoot buds treated with colchicine dropped to 58%, and after exposure of 30 hours the survival rate dropped to 12% (Te-chato & Sujaree, 1999). In another study, 20-day-old mangosteen callus tissue was cultured on media containing colchicine for 30 days. Morphological abnormalities such as 5 roots growing from one shoot and 3 leaves from one node were observed, but the shoots did not survive to the stage of transfer to the greenhouse (Te-chato & Sujaree, 2000). Te-chato and Sujaree (1999) also observed enlarged stomata guard cells on the leaves of mangosteen plants grown from shoot buds that were treated with colchicine at concentrations of 750 mg l^{-1}, 3,000 mg l^{-1}, and 10,000 mg l^{-1} for 2 hours (Te-chato & Sujaree, 1999). Stomatal guard cell size has been used as an indicator of ploidy level in coffee (Mishra, 1997) and rye grass (Speckman *et al.*, 1965).

We recorded 10 colchicine-treated plants with enlarged stomata guard cells, and 5 of these were confirmed by flow cytometry to contain more DNA than the control, indicating polyploidy. Our findings are consistent with the work of Blasco *et al.* (2015), who obtained polyploid loquat plants after treating the seeds with 0.5% colchicine for 24 hours (Blasco *et al.*, 2015). The results are also similar to those of Rey *et al.* (2002), who induced tetraploidy in *Ilex paraguariensis* by exposing zygotic embryos to 0.5% colchicine solution for 48 hours (Rey *et al.*, 2002). The success rate of the present research was greater, however, because Rey et al (2002) found only 2 tetraploid plants in 152 specimens whereas we detected 2 out of 48 in the 0.5% colchicine group for a polyploidy induction rate of 4.2% in that group, and 3 out of 46 in the 0.75% colchicine group, for a polyploidy induction rate of 6.5% in that group. Based on the results, we recommend using colchicine at a concentration of 0.75% to induce polyploidy in mangosteen.

The colchicine-treated plants are less than 2 years old now at the time of writing and are still very small, but if possible they will be raised to maturity to see what characteristics they exhibit. It is possible that having double or quadruple the number of chromosomes will enable the plants to express more genetic diversity.

5. CONCLUSION

The objective of this research was to increase genetic diversity in mangosteen through mutation

breeding. Some of the seedlings from the EMS treatments did exhibit different morphological characteristics from standard mangosteen seedlings but genetic testing has not yet been performed. The colchicine treatments did induce polyploidy, with a polyploidy induction rate of 4.2% - 6.5%. Hopefully, the experimental plants from both the EMS and colchicine treatment groups will be able to be grown to full size and will produce fruit in the future. Then they can be assessed to discover if their characteristics are valuable for commercial production or not. If any of them prove to be of use in the future, then they can be propagated through cloning and also used for a breeding program.

AUTHOR CONTRIBUTIONS

Conceptualization, C.Y., V.S. and S.P.; Methodology, C.Y., S.P. and V.S.; Validation, C.N., C.Y. and S.P.; Formal Analysis, V.S. and C.Y.; Investigation, V.S.; Resources, S.P., C.N. and C.Y.; Data Curation, V.S.; Writing-Original Draft Preparation, V.S.; Writing-Review & Editing, S.P. and C.Y.; Visualization, V.S. and S.P.; Supervision, S.P. and C.Y.; Project Administration, C.Y.

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APPENDIX A. STOMATA GUARD CELL COMPARISON PHOTOS

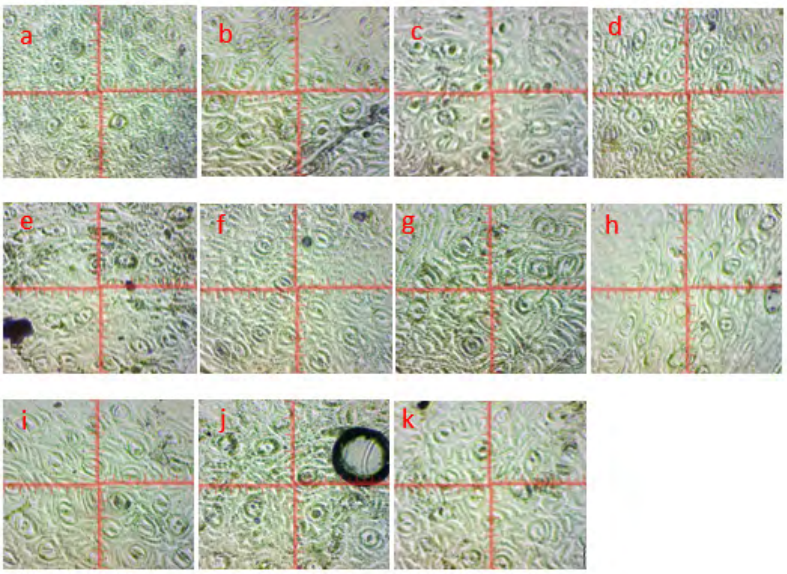

Figure A1. Stomata guard cells viewed under light microscope at 40x magnification; (a) control, (b) specimen # 19 from the 0.75% colchicine treatment group, (c) specimen # 3 from the 0.75% colchicine treatment group, (d) specimen # 9 from the 0.75% colchicine treatment group, (e) specimen # 17 from the 0.75% colchicine treatment group, (f) specimen # 4 from the 0.75% colchicine treatment group, (g) specimen #7 from the 0.5% colchicine treatment group, (h) specimen #19 from the 0.5% colchicine treatment group, (i) specimen # 20 from the 0.5% colchicine treatment group, (j) specimen #16 from the 0.25% colchicine treatment group, (k) specimen # 22 from the 0.25% colchicine treatment group.

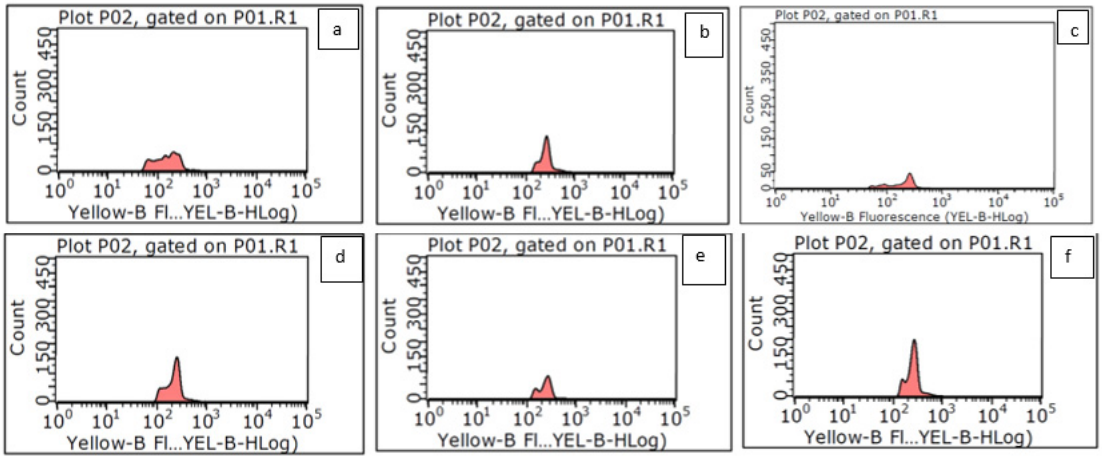

Figure B1. Flow cytometry histogram showing the frequency of cells counted with intensity of fluorescence, indicating amount of DNA detected; (a) control, (b) specimen #19 from the 0.5% colchicine treatment group, (c) specimen #20 from the 0.5% colchicine treatment group, (d) specimen #4 from the 0.75% colchicine treatment group, (e) specimen #9 from the 0.75% colchicine treatment group, (f) specimen #19 from the 0.75% colchicine treatment group.

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