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# Experiments on Effect of Mitotic Poisoning by Colchicine in *Jatropha curcas* L.

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## Abstract

The objective of this study aimed to evaluate the effect of mitotic poisoning in *Jatropha curcas* L. by colchicine induction in two experiments. In Experiment 1, inflorescences of physic nut were treated under different colchicine concentrations by dropping the solution directly at the stage that the frequency of male flower at microspore stage. In Experiment 2, seeds were soaked in different concentrations of colchicine at various durations.

In Experiment 1 and 2, the results showed that the increasing of colchicine concentration likely to decreased size of guard cells, plant height and stomatal density. However, significant different among those parameters was not observed. The ploidy level determined from leaf cells of plants by flow cytometry in both experiments showed almost of plants were disomic (2x). However, in experiment 1, incomplete aneuploidy or mixoploid plants that mixing between trisomic (2x + 1) (at 0 mM colchicine) and tetrasomic (2x + 2) (at 2.5 mM colchicine) with disomic were also found. In experiment 2, incomplete aneuploid plants were composed of trisomic, tetrasomic and pentasomic (2x + 3) with disomic. The mixoploid plant of disomic, trisomic and hexasomic (2x + 4) was also detected when concentrations of colchicine and times for soaking duration were increased.

Key Words: Mitotic Poisoning, *Jatropha curcas* L., Colchicine , Mixoploidy; Aneuploids

## Introduction

Jatropha *(Jatropha curcas* L.) known as purging-nut or physic nut, is a large shrub or small tree originated from tropical America, but commonly found and utilized throughout most of the tropical and subtropical regions of the world . It is still uncertain where the centre of origin is, but it is believed to be Mexico and Central America. It was introduced to Africa and Asia and is now cultivated worldwide, especially in dry land. Jatropha is resistant to drought and can be planted even in the desert climates; and it thrives on any type of soil; in sandy, gravelly and saline soils . In Indonesia (Java island), people grow Jatropha as fence along the side of road or as border perimeter plant, so it is called *Jarak Pagar* which mean fence of Jatropha .Traditionally, the cultivation of Jatropha has been undertaken primarily for protection of crops or pasture land by serving as a fence to confine livestock or as hedge for erosion control or as wind break. The ability of Jatropha to establish and be productive across a wide range of growing conditions, even on sites with poor quality soils and long period of drought, makes it a desirable species for degraded lands and preventing erosion. In equatorial regions, in which moisture is not a limiting factor, Jatropha is able to bloom and produce fruits around the year .The issue of looming energy crisis around the world (Kung and Tam, 2005), including India, has prompted researchers to pay more attention to research in an alternative energy resources (Paepatung et al., 2009; Peerapong and Limmeechokchai, 2009). *Jatropha curcas* L., an energy plant that has high oil content in seed, is one of the prime target under investigation by various researchers (Heller, 1996; Trabi et al1997; Winkler et al., 1997). Apart from its high oil content, this plant also capable of growing in infertile soil (Heller, 1996; Ginting and Maryono, 2009 ;Bandyopadhyay and Ghosh,2015), making it one of the ideal trees which should be promoted for planting to provide oil for domestic use.

However, one of its shortcoming trait is that these seeds with high oil yield do not reach maturity synchronously, contributing to a low yield of fresh nut per plant during harvesting and requiring many trees to obtain enough matured seeds in a single harvest for oil extraction (Jongschaap et al., 2007; Sharma et al., 2013). Improving Jatropha plant using conventional breeding may encounter difficulty as this plant has narrow genetic diversity (Basha and Sujatha, 2007; Ambrosi et al., 2010; Songsri et al., 2011).

To solve these problems, the genetic improvement by inducing mutation through the callus of physic nut has been investigated by various researchers (Sujatha and Mukta, 1996; Sardana et al., 2000; Rajore and Batra, 2005; Sujatha et al., 2006;

Wei et al., 2004). Tetraploid was successfully induced from diploid chromosome set of physic nut using colchicine (Sujatha and Prabhakaran, 2003; Piromya and Kermanee, 2013). However, proper protocol in mass propagation after obtaining a mutant was required and tissue culture, as one of the propagation technique, had been employed to regenerate this mutant tissue into new plants (Sujatha and Sailaja, 2005; Li et al., 2006;Bandyopadhyay and Ghosh,2015) . The dominant method for mutant induction in plant to be polyploidy was established eithers *in vitro* only or combined using between *in vitro* and *ex vitro* (Carretero et al., 2007; Dasgupta et al., 2008; Oates et al., 2013). This study aimed to investigate the effect of colchicines on inducing mutant in both inflorescences at microspore stage and seed embryos of *Jarak Pagar* by soaking. Jatropha oil hold the promise of alternative fuel for diesel engine, because of its potential in substituting the depleting primary fuels, its agriculture oriented, reduces serious air pollutant (such as particulates, carbon monoxides, hydrocarbons and air toxic), non toxic, bio degradable and renewable fuel. It is important to consider the use of mutation breeding for the improvement of Jatropha as a fuel crop, in term of its production and oil content in the seeds. Untill now, there is no report about Jatropha commercial cultivation. However a successful pilot scale commercial production has been made by the Research and development wing B.B.College, Asansol,West Bengal, India.

## Materials and Methods

### Plant genetic and site of plantation

In experiment 1, the effect of treating inflorescences of a 2-year-old physic nut trees; *Jatropha curcas* with colchicines was investigated. The trees were planted using the spacing 2 x 2 $m^2$. This experiment was carried out from July to October, 2019,at Asansol,West Bengal,India (Fig: 1,Fig:2 and Fig:3).

In experiment 2, the effect of soaking seeds of physic nut trees with colchicines on 0, 0.25,1.25 and 2.50 mM was studied. Seeds of *J. curcas* harvested from the physic trees (in the plantation as described above) were used in this experiment. This experiment was carried out from August to October, 2020 in the green house. The seeds from either treated inflorescences (from Experiment 1) or treated seeds (from Experiment 2) were planted in the green house and many characteristics were determined. These included the ratio of female and male flowers after treating by different colchicine concentrations in the inflorescences in Experiment 1. The characteristics of the germinated seedlings (both from treated inflorescences and treated seeds) including plant height, stomatal density and the sizes of guard cell were determined in the laboratory at Burdwan Science Centre Laboratory . Ploidy level was determined by flow cytometry at Plant Biotechnology Laboratory, Department of Botany, Burdwan Raj College,West Bengal ,India.

### Induce mutation

In experiment 1, five inflorescences of physic nut tree were collected from each tree and they were treated with colchicines at different concentrations (at 0, 2.5, 5, 7.5 and 10 mM). This was carried out by dropping this chemical agent on the cotton sheet holding an individual inflorescence, with seven replicates for each colchicine concentration. The colchicine was applied at 6.30 am for 7 days consecutively.

In experiment 2, one hundred and sixty seeds of physic nut, Indonesia variety were used in this experiment. Seeds were soaked with colchicines at different concentrations (0, 0.25, 1.25, 2.50 mM colchicine). These seeds were soaked for 6, 12, 24 and 48 hours for each concentration with 10 replications.

### Characteristic determination and ploidy level determination

In the green house, height of the plantlet (obtained from either treated inflorescence or seed with different concentrations of colchicine at various durations) was measured 14 days after transplanting to the pot (in the Experiment 1 and 2, respectively). At 60 days after planting, young leaves (leaf no. 2-3 from apical shoot) in each plant were determined with respect to their stomatal density, width and length of guard cells, (with microscope). The ploidy level determined with flow cytometry (Partec ploidy Analyse PA II). The assay procedure for preparing material was carried out using 50 mg of plant tissue. The tissue was placed a plastic petri dish, added 0.5 ml of CyStain UV ploidy and chopped the tissue with a sharp razor blade for releasing the nuclei from the cells. Then, added 1.5 ml of CyStain UV ploidy and incubate at room temperature for 5 minutes. The sample was filtered through a Partec 50 □m CellTrics disposable filter and to analyzed in flow cytometer using UV excitation (HBO-lamp, UV- laser) and measure blue emission.

**Figure:1 Preparation of Seedlings in the Nursery Bed (Left) and Two Years old plant (Right)**

**Experimental design and statistical analysis**

Randomized completed block design (RCBD) and completely randomized design (CRD) were conducted in the experiment 1 and 2, respectively. Analysis of variance (ANOVA) was conducted and means among treatment were compared with the Duncan's multiple range test (DMRT).

## Results and Discussion

**Experiment 1: Effect of colchicine on morphological and physiological characters of *Jatropha curcas* under field condition.**

The results were shown in Table 1 and 2. Non-significant different was observed o ratio of female and male flowers of physic nut (ranged from 0.043-0.117) after the inflorescences were treated with different colchicine concentrations (Table 1).Different concentration of colchicine did not affect plant height at 14 days after planting the seedling (from treated inflorescences), with the values ranging from 9.50-11.37 cm (Table 1).

At 60 days after planting of Jatropha plant , the density of guard cells from young leaf was not significantly affected by concentrations of colchicine, in which the density of guard cells ranged between 9.36-10.42 guard cells $mm^{-2}$. The highest stomata density was found in the nil control treatment (0 mM) at 10.42 guard cells $mm^{-2}$ (Table 1).

**Table 1** Characteristics of female/male flowers ratio, plant height, stomatal density and guard cell size from leaf of physic nut seedling (mean ± SE) plants obtained from fertilized ovule with colchicine-treated inflorescences (Experiment 1)

| Colchicine concentrations (mM) | Female/male flowers ratio | Plant height (cm) (at 14 DAS[†]) | Stomatal density (per $mm^2$) (at 60 DAS) | Width of guard cell (mm) (at 60 DAS) | Length of guard cell (mm) (at 60 DAS) |
|---|---|---|---|---|---|
| 0 | 0.081 ± 5.70 | 10.68 ± 3.58 | 10.42 ± 0.28 | $0.410 \pm 8x10^{-5}$ a | $0.039 \pm 4.3x10^{-3}$ ab |
| 2.5 | 0.058 ± 6.64 | 10.43 ± 1.54 | 9.95 ± 0.38 | $0.034 \pm 1.2x10^{-4}$ ab | $0.034 \pm 1.6x10^{-3}$ c |
| 5 | 0.177 ± 0.08 | 9.85 ± 1.20 | 9.36 ± 0.27 | $0.038 \pm 2.0x10^{-4}$ ab | 0.040 ± 0.00 a |
| 7.5 | 0.043 ± 6.01 | 11.37 ± 0.37 | 9.48 ± 0.12 | 0.030 ± 0.00 c | $0.033 \pm 7x10^{-4}$ c |
| 10 | 0.049 ± 5.70 | 9.50 ± 2.12 | 9.71 ± 0.07 | $0.036 \pm 2.0x10^{-4}$ ab | $0.035 \pm 6.0x10^{-4}$ bc |
| F-test | Ns[‡] | Ns | Ns | ** | * |

[†] DAS, days after seeding.

[‡] Ns, not significant difference at the 0.05 level of probability.

* significant difference at the 0.05 level of probability.

** significant difference at the 0.01 level of probability.

Both width and length of guard cells of the young leaf were significantly affected by colchicine concentrations (Table 1). The width of guard cell was the highest at 0 mM colchicine (0.410 mm) and decreased when higher concentrations of colchicine were applied. The lowest width at 0.030 mm was obtained from treating with colchicines at concentration of 7.5 mM. For the length of guard cell, the longest of them was found in seedling from treating inflorescence with 5 mM colchicine at 0.040 mm while the lowest length was detected at 2.5 mM (0.034 mm) and 7.5 mM (0.033 mm) colchicine.
The result of ploidy level from leaf was detected by flow cytometry was shown in Table 2.

For the length of guard cell, the longest of them was found in seedling from treating inflorescence with 5 mM colchicine at 0.040 mm while the lowest length was detected at 2.5 mM (0.034 mm) and 7.5 mM (0.033 mm) colchicine.The result of ploidy level from leaf of physic nut detected by flow cytometry was shown in Table. From 22 plants (plant no. 40 to no. 61), disomic (2x) was found in 19 plants (86.36%). Three (13.64%) possessed mixoploid which mixed DNA concentrations (2 peaks), trisomic (2x + 1)(0 mM colchicine) (plant no. 40), tetrasomic (2x + 2)(0 and 2.5 mM colchicine) (plant no. 41 and 42) and disomic (Table 2). The ratio of female and male flowers of physic nut (0.043-0.177) was not affected by colchicine concentrations. However, the ratio was decreased at high concentration (Table 1).

The decrement of female and male flowers in the same inflorescence occurred after treatment with different concentrations of colchicine. Increase in concentrations of colchicine reduced male flowers greater than female flowers. Plant height was used as an indicator to predict the ploidy level in plant (Miller et al., 2012). Other characteristics such as stomatal size, flower size, seed weight, plant dry matter, chlorophyll and starch contents, and strength of growth were also important characteristics to evaluate the ploidy level in plant (Miller et al., 2012). Nevertheless, in this study plant height was not significant different (at 14 days after planting) after inflorescences were treated with different concentrations of colchicine. In this study, guard cell sizes were significant different but the stomatal density was not affected by colchicine treatments (Table 1). The frequency of stomatal density and epidermal cell was reported to decrease and the size of guard cell increased in brome grass (Tan and Dunn, 1973), triticale (Sapra et al., 1975), *Coffea canephora* (cultivar S. 274) (Mishra, 1997) and *Arabidopsis thaliana* (Miller et al., 2012).

**Table 2** Ploidy level of physic nut prepared from young leaf of plant obtained from fertilized ovule with colchicine-treated inflorescences (Experiment 1)

| Plant No. | Peak | Index | Mean | Area | Area (%) | C.V. (%) | Chi-square |
|---|---|---|---|---|---|---|---|
| 40 | 1 | 1.000 | 210.30 | 1135 | 69.51 | 5.81 | 0.56 |
| | 2 | 1.406 | 295.72 | 498 | 30.49 | 43.17 | 0.56 |
| 41 | 1 | 1.000 | 197.18 | 1325 | 82.37 | 5.12 | 0.51 |
| | 2 | 1.981 | 390.56 | 284 | 17.63 | 4.27 | 0.51 |
| 42 | 1 | 1.000 | 178.84 | 1387 | 98.01 | 6.88 | 0.53 |
| | 2 | 2.281 | 407.88 | 28 | 1.99 | 0.49 | 0.53 |
| 43 | 1 | 1.000 | 180.39 | 1378 | 100.00 | 15.84 | 0.62 |
| 44 | 1 | 1.000 | 187.37 | 1349 | 100.00 | 6.40 | 0.61 |
| 45 | 1 | 1.000 | 188.69 | 1208 | 100.00 | 9.61 | 0.66 |
| 46 | 1 | 1.000 | 176.43 | 1438 | 100.00 | 15.47 | 0.63 |
| 47 | 1 | 1.000 | 172.03 | 1551 | 100.00 | 11.62 | 0.53 |
| 48 | 1 | 1.000 | 182.72 | 1324 | 100.00 | 11.20 | 0.60 |
| 49 | 1 | 1.000 | 175.80 | 1473 | 100.00 | 11.85 | 0.61 |
| 50 | 1 | 1.000 | 177.24 | 1477 | 100.00 | 13.70 | 0.59 |
| 51 | 1 | 1.000 | 176.95 | 16.73 | 100.00 | 8.57 | 0.45 |
| 52 | 1 | 1.000 | 169.72 | 1527 | 100.00 | 13.73 | 0.53 |
| 53 | 1 | 1.000 | 175.45 | 1461 | 100.00 | 13.76 | 0.57 |
| 54 | 1 | 1.000 | 168.77 | 1688 | 100.00 | 12.35 | 0.50 |
| 55 | 1 | 1.000 | 153.37 | 1414 | 100.00 | 9.88 | 0.73 |
| 56 | 1 | 1.000 | 177.06 | 1587 | 100.00 | 11.94 | 0.53 |
| 57 | 1 | 1.000 | 180.88 | 1771 | 100.00 | 8.19 | 0.45 |
| 58 | 1 | 1.000 | 173.27 | 1586 | 100.00 | 13.53 | 0.57 |
| 59 | 1 | 1.000 | 183.85 | 1662 | 100.00 | 14.93 | 0.78 |
| 60 | 1 | 1.000 | 180.18 | 1783 | 100.00 | 14.01 | 0.55 |
| 61 | 1 | 1.000 | 185.54 | 1527 | 100.00 | 17.44 | 0.82 |

These results may be due to both larger epidermal cells and lower density of guard cells indicating ploidy level of plants in comparison with diploid plant (Mishra, 1997). However, in this study, the stomatal sizes tended to decrease with the increase in colchicine concentration (Table 1). There are probably at least two important factors affecting both stomatal density and size of guard cells. Those are genotype and environment (such as light, temperature, moisture affected to epidermal cell size) (Fernandez and Muzica, 1973; Mishra, 1997). Thus, the determination of ploidy level using the flow cytometry was employed to enumerate ploidy in physic nut (Heslop-Harrison, 1995; Inagaki, 2003). Flow cytometry was used for estimating DNA quantity in cell nuclei, screening for ploidy, and detecting aneuploidy and mixoploid or the imbalance of chromosome number in cell (not complete aneuploidy). This multiple copies of chromosomes and genes located on that chromosome may affect gene expression or phenotype, in which the magnitude of an effect depended upon plant species, characteristics, and type of aneuploidy (Lee et al., 1996; Birchler et al., 2001; Birchler, 2010; Henry et al., 2010). Mixoploidy could occur by treating many plant tissues (such as seed, buds and shoots) with colchicine in different plant species (such as ryegrass, fenugreek (*Trigonella foenum- graecum* L.) and *Dioscorea zingiberensis*) (Hill and Myers, 1944; Huang et al., 2010; Omezzine et al., 2012).

Many researchers reported about the type of ploidy; trisomic (Koh et al., 1993; Henry et al., 2010) or tetrasomic (Moody et al., 1993; Jenczewski et al., 2002) in plant tissues after they were treated with chemical agents. The complex of mixoploid (the combination of disomic, trisomic and tetrasomic) was observed in the physic nut cells in this study. Tetrasomic may occur as a

result of spontaneous or induced mutation (Mayo, 1971; Bever and Felber, 1991; Moody et al., 1993). The combination of many types of ploidy level may cause the complex of phenotype expression in many traits. Trisomic was reported to alter the expression of many genes, including dosage-sensitive, in phenophytes (Henry et al., 2010).

The results in this study showed mixoploidy of trisomic (No. 40) and tetrasomic (No. 41) with disomic in leaf cells from non-treated control seeds (Table 2). However, trisomic and tetrasomic had less areas than that of disomic areas in plant cells after determination with a flow cytometry.

It was possible that the progeny seeds were from the fertilization between female flower with normal monoploid chromosome (receiving 0 mM colchicine treatment) and male flower with imbalance chromosome number (extra 1; x + 1 or extra 2; x + 2 in some chromosomes) (receiving colchicine treatment) from different inflorescences. In this study, the treated inflorescences were not covered with the bag, leading to a possibility of cross-fertilization between monoploid chromosome number of female gamete and imbalance chromosome number (extra 1; x + 1 or extra 2; x + 2 in some chromosomes) of male gamete.

**Experiment 2: Effect of colchicine on growth of physic nut by seeds soaking.**

The plant height at 14 days after planting was not significantly affected by the combination of colchicine concentrations and soaking durations (ranging from 7.50-11 cm) (Table 3). At 60 days after planting, stomatal density was not significant different among treatments, which the values ranged from 6.23 to 13.48 stomata $mm^{-2}$ (Table 3). However, size of guard cells (width and length) was significant affected by treatments, in which the width values ranged from 0.027 to 0.046 mm and the length values ranged from

**Table 3** Characteristics of physic nut (mean ± SE) seedlings obtained from soaking seeds with differe ttimes and concentrations of colchicine (Experiment 2)

| Colchicine concentration-Soaking duration | Plant height (cm) (at 14 DAS[ł]) | Stomatal density (no. $mm^{-2}$) (at 60 DAS) | Width of guard cell (mm) (at 60 DAS) | Length of guard cell (mm) (at 60 DAS) |
|---|---|---|---|---|
| 0 mM - 6 hrs. (T1) | 10.42 ± 1.80 | 10.79 ± 0.45 | 0.027 ± 1x10$^{-4}$ c | 0.038 ± 1x10$^{-5}$ ab |
| 0 mM - 12 hrs. (T2) | 8.00 ± 0.00 | 8.22 ± 0.00 | 0.042 ± 0.00 ab | 0.041 ± 0.00 ab |
| 0 mM - 24 hrs. (T3) | 8.00 ± 0.00 | 6.23 ± 0.00 | 0.046 ± 0.00 a | 0.044 ± 0.00 ab |
| 0.25 mM - 6 hrs. (T4) | 10.13 ± 0.85 | 11.14 ± 0.40 | 0.040 ± 6x10$^{-5}$ ab | 0.042 ± 8x10$^{-5}$ ab |
| 0.25 mM - 12 hrs. (T5) | 9.50 ± 0.00 | 10.15 ± 0.00 | 0.042 ± 0.00 ab | 0.047 ± 0.00 a |
| 0.25 mM - 48 hrs. (T6) | 9.75 ± 1.06 | 13.19 ± 0.76 | 0.038 ± 1.5x10$^{-4}$ ab | 0.039 ± 2.2x10$^{-4}$ ab |
| 1.25 mM - 6 hrs. (T7) | 9.00 ± 1.56 | 13.28 ± 5.51 | 0.037 ± 5x10$^{-5}$ b | 0.037 ± 4x10$^{-5}$ b |
| 1.25 mM - 12 hrs. (T8) | 8.33 ± 1.53 | 13.48 ± 0.63 | 0.036 ± 2x10$^{-5}$ bc | 0.042 ± 1x10$^{-4}$ ab |
| 1.25 mM - 24 hrs. (T9) | 7.50 ± 3.28 | 13.06 ± 0.18 | 0.036 ± 2x10$^{-5}$ bc | 0.037 ± 4x10$^{-5}$ b |
| 2.50 mM - 6 hrs. (T10) | 11.00 ± 1.00 | 11.69 ± 0.77 | 0.040 ± 1.5x10$^{-4}$ ab | 0.039 ± 6x10$^{-5}$ ab |
| 2.50 mM - 12 hrs. (T11) | 8.25 ± 1.92 | 11.09 ± 0.48 | 0.038 ± 9x10$^{-5}$ ab | 0.043 ± 5x10$^{-5}$ ab |
| 2.50 mM - 24 hrs. (T12) | 10.50 ± 2.12 | 12.73 ± 0.25 | 0.039 ± 3x10$^{-5}$ ab | 0.037 ± 1x10$^{-5}$ b |
| 2.50 mM - 48 hrs. (T13) | 9.25 ± 0.35 | 7.46 ± 0.24 | 0.042 ± 1.2x10$^{-4}$ ab | 0.041 ± 8x10$^{-5}$ ab |
| F-test | Ns[‡] | Ns | ** | * |

[ł] DAS, days after seeding.

[‡] Ns, not significant difference at the 0.05 level of probability.

* significant difference at the 0.05 level of probability.

** significant difference at the 0.01 level of probability.

0.037 to 0.047 mm (Table 3). Flow cytometry study showed that the plantlets possessed both disomic and mixoploid plants (Table 4). Mixoploid of trisomic (plant no. 16), tetrasomic (plant no. 21, 23, 26, 31 and 34) and pentasomic (2x + 3) (plant no. 2) were observed. Moreover, the mixoploid plant of disomic, trisomic and hexasomic (2x + 4) also was found at 2.5 mM colchicine after soaking the seeds for 6 hrs (plant no. 3). In this experiment, the result on plant height was similar to that observed in Experiment 1. This trait was not significantly affected at 14 days after planting (Table 3). However, size of guard cells was affected by the concentrations of colchicine and soaking duration (Table 3). The values of width and length of the guard cells decreased when the leaves were treated with the increased concentrations of colchicines, the result similar to that in Experiment 1DNA content quantification by flow cytometry revealed that most of the non-treated seeds had normal diploidy

(Table 4), in which many types of mixoploidy were also observed in different combinations of colchicine concentration and soaking duration. The mixoploid of plant came from treated seed may cause by chimera mutant of cells composed within embryo. Using seeds as material for induction of mutagenesis also frequently produces chimera which single cells may be induced mutation, then divide and differentiate into parts of the plant (Acquaah, 2007). In chimeric tissues, the mutated cells are present along with normal cells which are the main bottleneck for mutagenesis induction in plant (Datta and Chakrabarty, 2009).

**Figure 2 and 3. Fruits and flower of *Jatropha curcas.* in the experimental field**

**Table 4** Ploidy level of physic nut prepared from young leaf of plant grew form seeds soaking by colchicines treatments (Experiment 2)

| Plant No. | Peak | Index | Mean | Area | Area (%) | C.V. (%) | Chi-square |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 1.000 | 200.79 | 1.422 | 100.00 | 9.60 | 0.56 |
| 2 | 1 | 1.00 | 247.81 | 1285 | 87.78 | 5.19 | 0.54 |
| | 2 | 1.977 | 490.00 | 179 | 12.22 | 4.64 | 0.54 |
| 3 | 1 | 1.000 | 237.29 | 578 | 38.79 | 29.60 | 0.58 |
| | 2 | 1.180 | 279.92 | 731 | 49.06 | 5.08 | 0.58 |
| | 3 | 2.395 | 568.30 | 181 | 12.15 | 4.13 | 0.58 |
| 5 | 1 | 1.000 | 186.30 | 1471 | 100.00 | 11.64 | 0.64 |
| 6 | 1 | 1.000 | 220.12 | 1357 | 100.00 | 22.47 | 0.70 |
| 8 | 1 | 1.000 | 217.87 | 1317 | 88.14 | 24.76 | 0.61 |
| | 2 | 1.129 | 246.02 | 177 | 11.86 | 2.78 | 0.61 |
| 9 | 1 | 1.000 | 226.26 | 1225 | 100.00 | 16.20 | 0.62 |
| 10 | 1 | 1.000 | 264.66 | 1394 | 100.00 | 7.98 | 0.92 |
| 11 | 1 | 1.000 | 248.19 | 1101 | 100.00 | 5.65 | 0.73 |
| 12 | 1 | 1.000 | 206.82 | 1136 | 100.00 | 23.67 | 0.63 |
| 13 | 1 | 1.000 | 242.37 | 1163 | 100.00 | 4.88 | 0.69 |
| 14 | 1 | 1.000 | 189.12 | 1193 | 100.00 | 9.46 | 0.66 |
| 15 | 1 | 1.000 | 184.29 | 1270 | 100.00 | 8.39 | 0.61 |
| 16 | 1 | 1.000 | 181.16 | 1327 | 73.13 | 6.86 | 0.42 |
| | 2 | 1.726 | 312.64 | 488 | 26.87 | 26.33 | 0.42 |
| 17 | 1 | 1.000 | 194.17 | 1563 | 100.00 | 6.52 | 0.46 |
| 18 | 1 | 1.000 | 191.40 | 1460 | 100.00 | 5.74 | 0.54 |
| 19 | 1 | 1.000 | 179.77 | 1393 | 100.00 | 15.22 | 0.57 |
| 20 | 1 | 1.000 | 186.59 | 1372 | 100.00 | 14.24 | 0.63 |
| 21 | 1 | 1.000 | 197.75 | 1479 | 85.81 | 4.70 | 0.41 |
| | 2 | 1.992 | 393.90 | 245 | 14.19 | 3.59 | 0.41 |
| 22 | 1 | 1.000 | 186.77 | 1459 | 100.00 | 8.19 | 0.67 |
| 23 | 1 | 1.000 | 193.56 | 1223 | 73.16 | 6.10 | 0.51 |
| | 2 | 2.017 | 390.37 | 449 | 26.84 | 7.33 | 0.51 |
| 24 | 1 | 1.000 | 171.94 | 1308 | 100.00 | 12.93 | 0.64 |
| 25 | 1 | 1.000 | 175.20 | 1427 | 100.00 | 6.41 | 0.50 |
| 26 | 1 | 1.000 | 195.11 | 1394 | 86.68 | 4.76 | 0.47 |
| | 2 | 1.970 | 384.35 | 214 | 13.32 | 5.97 | 0.47 |
| 27 | 1 | 1.000 | 201.44 | 1454 | 100.00 | 8.89 | 0.63 |
| 28 | 1 | 1.000 | 176.83 | 1692 | 100.00 | 14.93 | 0.91 |
| 29 | 1 | 1.000 | 180.13 | 1405 | 100.00 | 15.82 | 0.58 |
| 30 | 1 | 1.000 | 172.23 | 1374 | 100.00 | 6.32 | 0.57 |
| 31 | 1 | 1.000 | 195.70 | 1367 | 76.73 | 5.76 | 0.40 |
| | 2 | 1.976 | 386.75 | 415 | 23.27 | 4.90 | 0.40 |
| 32 | 1 | 1.000 | 183.10 | 1585 | 100.00 | 8.44 | 0.50 |
| 33 | 1 | 1.000 | 188.4 | 1387 | 100.00 | 6.26 | 0.67 |
| 34 | 1 | 1.000 | 193.69 | 1556 | 84.61 | 8.51 | 0.45 |
| | 2 | 1.942 | 376.14 | 283 | 15.39 | 6.63 | 0.45 |
| 35 | 1 | 1.000 | 195.41 | 924 | 100.00 | 11.83 | 0.64 |
| 36 | 1 | 1.000 | 185.97 | 1179 | 100.00 | 8.24 | 0.64 |
| 37 | 1 | 1.000 | 194.96 | 1217 | 100.00 | 6.50 | 0.63 |
| 39 | 1 | 1.000 | 177.02 | 1476 | 100.00 | 15.11 | 0.58 |

## Conclusion

This study emphasized the importance of colchicine treatment for mutation induction both on directly dropping on inflorescences and seed soaking. Although some characteristics such as stomatal density and guard cell size were introduced for determining the ploidy chromosome set, but in this study, only guard cell size (width and length) showed the value decreasing when colchicine concentration was increased. For the study of DNA content by the flow cytometry, except to normal diploidy (2x), many types of mixoploid occurred in both experiments. Directly treated the inflorescences with colchicine showed mixoploidy of trisomic (2x + 1) and tetrasomic (2x + 2) with disomic. Soaking seeds with colchicine showed many types of mixoploid with disomic (2x) such as trisomic, tetrasomic, pentasomic (2x + 3) and hexasomic (2x + 4). Extra chromosome number of incomplete aneuploidy and the complex of mixoploid increased when the higher concentration of colchicine was treated.

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