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# Some Chemical Treatments on Kluai Khai Through Tissue Culture for Mutation Breeding

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## ABSTRACT

Polyploidy of *in vitro* Kluai Khai plantlets were induced by different concentrations of the two mutagens : 0, 0.5, 0.75 and 1% colchicine and 0,15, 30, 45mM oryzalin containing 2% DMSO for 2.5, 5.0, 7.5 hours. It was found that the higher the mutagen doses and the longer the treatment duration, the less the survival rates. After treating plantlets with colchicine and oryzalin at 0.4, 0.5, 0.75% and 13, 15, 22mM, respectively the survival rate of each one was found to be 50%. In $MV_1$, adventitious bud initiation of mutagenic treatment yielded 0.2-1.2 shoots which was lower than those in the control while those of $MV_2$ and $MV_3$ were not significantly different from each other. Treated plantlets were revealed to be abnormal in chimera and stomata sizes were larger than those of the controls. The plantlets with stomata over 28mm were selected for chomosome count. The controls were diploid (2n = 22) whereas three selected clones were tetraploid (2n = 44) derived from 1% colchicine at 7.5 hours and 45mM oryzalin at 2.5 hours. The mutants were then subcultured. The number of suckers of mutants was less than those of the control. MS medium without plant growth regulator was observed to give the best rooting of all treatments. After transfered to the greenhouse, the height of the controls and the colchicine treated plants were revealed to be significantly different from the oryzalin treated ones which were shorter with low survival rate. The results obtained in the field were similar to those in the greenhouse. The foliage of all mutants appeared more drooping with larger stomata cell, lower in stomata number per area and thicker than those of the controls. The numbers of leaf and sucker of each mutant were not significantly different from the controls.

**Key words**: Kluai Khai, tissue culture, mutation breeding, colchicine, oryzalin, Pisang Mas

## INTRODUCTION

Kluai Khai (*Musa acuminata* 'Kluai Khai') or Pisang Mas belongs to AA Group with plant height under 2.5 m.and fruit size quite small of 3.5 width and 10.5 cm. long. Flesh color is yellow-orange and seedless ( Silayoi and Babpraserth, 1983). K.Khai can grow well all year round. The most production occurs in the northern provinces, such as, Kamphangphet, Tak, Sukhothai, Pichit and Nakhonsawan. The quality of flesh is quite excellent, fragrant and sweet but the fruit is quite small with thin peel. According to these problems, the improvement of K.Khai, should therefore, be brought into consideration.

The genetic system of *Musa* is very much complicated owing to the inherent problems of sterility, heterozygosity and polyploidy in most of the clones. Parthenocarpy, sterility are very difficult to obtain viable seeds. These are limiting factors for conventional breeding of K. Khai. Alternative technique to select conventional recombinant

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phenotypic is to explore plant tissue to chemical mutagens and *in vitro* culture, to induce mutation. The degree of mutation depends on the level and duration of the treated explant and potential increasing variation as the size of the exposed explant is reduced (Krikorian, 1987). Many chemicals can be used as mutagens. Colchicine is remarkable in the sense that it will arrest metaphase. All plant organs respond to its treatment. In plants, the somatic tissue responds more readily to colchicine action than the meiotic cells. For the study of haploid mitosis, pollen tube culture, with colchicine added in the medium, is considered to be most satisfactory to dihaploid (Sharma and Sharma, 1980). Chen and Coedem-Kallemeyn (1979) induced tetraploid Day lily by using 20 mg/l of colchicine to their callus. It has been reported that, colchicine at 0.5% could produce hexaploid banana from triploid one (Nukulkarn, 1983)

Another interesting chemical is oryzalin whose trade names include Dirimal, EL-119, Rycelan, Ryzelan, Ryzelon and Surfran. Oryzalin is a selective pre-emergence surface applied herbicide, which is used for control of annual grasses and broadleaf weed seeds by blocking cell division in the meristem. It is also an antimitotic agent the same as colchicine (Hassawi and Ling,1991; Verhoeven *et al*., 1990). It loses centrosomal function as well as spindle fiber function. The mode of loss of the spindle function and spindle fiber is more effective than colchicine in *Nicotiana plembaginifolia* (Verhoeven *et al.*, 1990). Ramulu *et al.* (1991), reported that, 15-35 mM oryzalin could break the cell division at metaphase and made 4X cell of potato. Double haploid could also be done in wheat and corn by using oryzalin (Wan *et al.*, 1991; Hassawi and Ling, 1991).

The objective of this study was to produce the 4X Kluai Khai mutants by using colchicine and oryzalin solution at different concentrations.

## MATERIALS AND METHODS

The experiment was carried out at the Department of Horticulture, Kasetsart University. Tissue culture plants of K.Khai were cultured on MS media supplementing with 4 mg/l BA for each treatment. One hundred of K.Khai plantlets were soaked in 0.5, 0.75, 1% colchicine and 15 mM, 30 mM, 4 mM of oryzaline containing DMSO for 2.5, 5.0 and 7.5 hours while the control was soaked in distilled water. The explants were washed after soaked in distilled water and then cultured in modified MS medium with 4 mg/l BA. Subcultures of selected plants were prepared every month.

Observations were made on survival rate in $MV_1$, and stomata size measuring with ocular micrometer in $MV_2$. The plantlets with stomata about 2 times the normal size were selected for chromosome number counting. The plantlets that possessed the tetraploid chromosome were later selected for further study. 100 plantlets from each treatment of tetraploid plant were multiplied for 4 generations. They were rooted on MS media without hormone (Silayoi, 1985). The rooted plantlets were then transplanted to pots and to field respectively. In the field, the height, circumference of pseudostem, number of leaf and number of sucker were observed.

## RESULTS AND DISCUSSION

### Survival percentage

After one month, the survival percentage of the control was noticed to be 100 per cent but at higher concentrations of either colchicine or oryzalin, they decreased as shown in Table 1. It was also found that $LD_{50}$ of colchicine treatments at 2.5, 5.0 and 7.5 hrs. were 0.4, 0.5, 0.75 per cent while those of oryzalin at 2.5, 5.0 and 7.5 hrs were 13, 15 and 20 mM respectively (Figure 1 and 2).

The growth of treated plantlets in $MV_1$-$MV_3$ was noticed to be not so good as in the control. The treated plants appeared albino, dwarf with leaf thickness (Figure 3). The control of $MV_1$ gave

**Table 1** Survival plantlets ( as % of control) after 1 month treatment.

| Chemical | Doses | Treated time (hrs) | | | Average |
|---|---|---|---|---|---|
| | | 2.5 | 5 | 7.5 | |
| Control | 0 | 100.00 | 100.00 | 100.00 | 100.00 |
| Colchicine | 0.50 % | 61.11 | 50.00 | 39.89 | 50.33 |
| | 0.75% | 50.00 | 44.44 | 33.33 | 42.59 |
| | 1.00% | 38.89 | 27.78 | 16.67 | 27.78 |
| | Average | 50.00 | 40.74 | 29.96 | |
| Oryzalin | 15mM | 66.67 | 38.89 | 50.00 | 51.85 |
| | 30mM | 27.78 | 33.33 | 16.67 | 25.93 |
| | 45mM | 38.89 | 16.67 | 11.11 | 22.22 |
| | Average | 44.45 | 29.63 | 25.93 | |

significantly more shoots than the other treated explants. At $MV_2$, the 45 mM, 7.5 hrs. oryzalin treated plants died because of high dose and too long time treating (Table 2). The results agreed with Keawsompong (1993) who reported that 20% Kluai Khai died after soaked in 500-1500ppm colchicine at 24-72 hrs.

Stomata sizes were measured in $MV_3$. The stomata of 9 colchicine and 7 oryzalin treated plants were longer than 28 mm while the control one was 19 mm (Table 3, Figure 4). They were then selected for chromosome counting. The polyploid were selected for further study in the field. Only 2 plants of C3 (explant treated with 1% colchicine) or plant no. 5 and 6 (C3-5 and C3-6) and 1 plant of Z1 (explant treated with 45mM oryzalin) or plant no.6 (Z1-6) were selected as tetraploid (Figure 5). The plants are shown in Figure 6. These should imply that both chemicals, colchicine and oryzalin, could double chromosome of K. Khai. The results agreed with Chen and Coeden-Kallemeyn (1979) who reported that colchicine could induce tetraploid Day lily and with Wan *et al.* (1991) on doubled haploid of wheat and corn by using oryzalin.

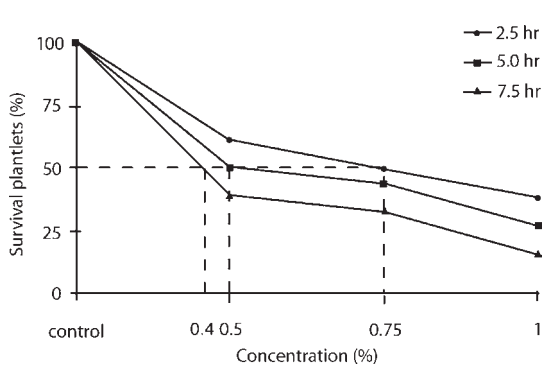


**Figure 1** Survival percentages after one month colchicine treatment.

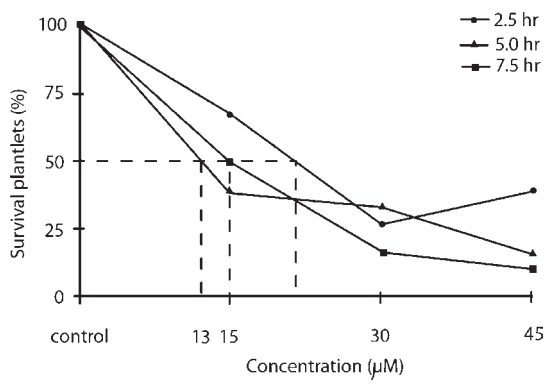


**Figure 2** Survival percentages after one month oryzalin treatment.

The selected plants were subcultured with the number of shoots produced shown in Table 4. Numbers of shoot of mutants were found to be significantly different from those of the control. For Z1 or oryzalin treated plants, they were noticed to produce significantly more shoots than the colchicine treated plants in the 5th week (Table 4).

After 5 weeks in MS with 5 ppm of BA, the plantlets were rooted in MS without hormone. 1 point was given to 0-1 root, 5 points for the ones with more than 6 roots. The score above 3 points was good for planting. The oryzalin treated plants obtained the highest score of 4.6 points, which was significantly different to the control and colchicine treated plants (Table 5). Leaves of oryzalin treated plants were also revealed to be bigger than those of the other treatments, resulting in stronger roots produced. The results were quite similar to those of Phoengchan (1995).

One hundred rooted plantlets of each treatment (control, Z1-6,C3-5,C3-6) were transplanted to nursery with 98% found to survive.

**Table 2** Average numbers of shoot produced from $MV_1$-$MV_3$.

| Chemical | Symbol | Concentration | Time(hrs) | $MV_1$ 1/ | $MV_2$ | $MV_3$ |
|---|---|---|---|---|---|---|
| Control | | | | $2.1^{e}$ | 1.4 | 1.9 |
| Colchicine | A1 | 0.5% | 2.5 | $0.8^{a-d}$ | 1.4 | 1.3 |
| | A2 | | 5.0 | $0.9^{bcd}$ | 1.1 | 1.1 |
| | A3 | | 7.5 | $1.0^{bcd}$ | 1.3 | 1.3 |
| | B1 | 0.75% | 2.5 | $1.1^{cd}$ | 0.9 | 1.0 |
| | B2 | | 5.0 | $0.9^{bcd}$ | 0.9 | 1.3 |
| | B3 | | 7.5 | $1.1^{cd}$ | 1.1 | 1.6 |
| | C1 | 1.0% | 2.5 | $1.2^{d}$ | 1.3 | 1.3 |
| | C2 | | 5.0 | $1.0^{bcd}$ | 0.4 | 1.0 |
| | C3 | | 7.5 | $0.8^{a-d}$ | 1.4 | 1.0 |
| Oryzalin | X1 | 15mM | 2.5 | $0.8^{a-d}$ | 1.6 | 1.1 |
| | X2 | | 5.0 | $0.7^{a-d}$ | 0.7 | 0.9 |
| | X3 | | 7.5 | $0.7^{a-d}$ | 0.9 | 1.1 |
| | Y1 | 30mM | 2.5 | $0.5^{abc}$ | 1.0 | 1.1 |
| | Y2 | | 5.0 | $0.4^{ab}$ | 1.3 | 1.1 |
| | Y3 | | 7.5 | $0.4^{ab}$ | 1.0 | 1.0 |
| | Z1 | 45mM | 2.5 | $0.5^{abc}$ | 1.4 | 1.1 |
| | Z2 | | 5.0 | $0.5^{abc}$ | 1.1 | 0.7 |
| | Z3 | | 7.5 | $0.2^{a}$ | 0.0 | - |
| F-test | | | | ** | ns | ns |
| CV(%) | | | | 74.9 | 91.4 | 64.8 |

ns = non significance

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

**Table 3** The selected plants with stomata longer than 28 mm.

| Chemical | Symbol | Concentration | Time(hrs) | Total plantlet | Selected plantlet (no.) |
|---|---|---|---|---|---|
| Control | | | 25 | 25 | - |
| Colchicine | A1 | 0.5% | 2.5 | 22 | 5 |
| | A2 | | 5.0 | 14 | 4 |
| | A3 | | 7.5 | 13 | 1 |
| | B1 | 0.75% | 2.5 | 13 | 1 |
| | B2 | | 5.0 | 21 | 2 |
| | B3 | | 7.5 | 15 | 1 |
| | C1 | 1.0% | 2.5 | 30 | 5 |
| | C2 | | 5.0 | 8 | 2 |
| | C3 | | 7.5 | 9 | 2 |
| Oryzalin | X1 | 15mM | 2.5 | 18 | 1 |
| | X2 | | 5.0 | 5 | 1 |
| | X3 | | 7.5 | 17 | 5 |
| | Y1 | 30mM | 2.5 | 7 | 1 |
| | Y2 | | 5.0 | 16 | 3 |
| | Y3 | | 7.5 | 7 | 0 |
| | Z1 | 45mM | 2.5 | 27 | 6 |
| | Z2 | | 5.0 | 14 | 2 |
| | Z3 | | 7.5 | - | - |

**Table 4** Numbers of shoot of mutant in tissue culture compared to those of the control.

| Treatment | Number of shoot[1/] | | | | |
|---|---|---|---|---|---|
| | 1st week | 2nd week | 3rd week | 4th week | 5th week |
| Control | 1.0 | 2.1[b] | 3.0[b] | 4.1[b] | 4.9[c] |
| Z1-6 | 0.8 | 1.3[a] | 2.2[a] | 2.5[a] | 3.3[b] |
| C3-5 | 0.8 | 1.1[a] | 1.5[a] | 2.1[a] | 2.2[a] |
| C3-6 | 0.9 | 1.1[a] | 1.5[a] | 2.2[a] | 2.5[a] |
| F-test | ns | ** | ** | ** | ** |
| CV(%) | 70.7 | 54.8 | 43.6 | 44.0 | 37.7 |

ns = non significance

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

**Table 5** Score of healthy root of mutant plantlets compared to those of the control *in vitro*.

| Treatment | Score[1/] |
|---|---|
| Control | 3.8[a] |
| Z1-6 | 4.6[b] |
| C3-5 | 3.7[a] |
| C3-6 | 3.8[a] |
| F-test | ** |
| CV(%) | 25.6 |

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

**Table 6** The height of mutant plants compared to the control in nursery.

| Treatment | Height (cm) | |
|---|---|---|
| | 1st month [1/] | 2nd month [1/] |
| Control | 6.12[b] | 8.11[b] |
| Z1-6 | 3.63[a] | 4.13[a] |
| C3-5 | 6.98[c] | 7.53[b] |
| C3-6 | 6.47[bc] | 8.10[b] |
| F-test | ** | ** |
| CV(%) | 19.4 | 19.9 |

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

After 2 months in nursery, the heights of those were measured (Figure 7 and Table 6). Oryzalin could inhibit growth of treated plants to nearly 4.13 cm. high which were significantly shorter than the other treatments. This is due to the fact that oryzalin is herbicide which can retard the growth of plants as indicated by Hassawi and Ling (1991).

**In the field**

After 3 months in nursery, the plants were transplanted to be cultivated in the field at Kasetsart University, Kamphangsaen campus. The survival plants were recorded after 1 month. Only 43.8% oryzalin treated plants were found to survive whereas the control and colchicine treated ones survived 100, 87.5 and 87.5 % respectively.

**Table 7** Height of mutants compared to the control in the field.

| Treatment | Height (cm.)[1/] | | | | | |
|---|---|---|---|---|---|---|
| | 1st month | 2nd month | 3rd month | 4th month | 5th month | 6th month |
| Control | 8.43[b] | 15.23[b] | 25.58[b] | 36.88[b] | 57.20[b] | 81.90[b] |
| Z1-6 | 3.40[a] | 4.95[a] | 7.57[a] | 10.05[a] | 14.30[a] | 19.20[a] |
| C3-5 | 7.78[b] | 14.48[b] | 25.58[b] | 38.50[b] | 61.13[b] | 79.53[b] |
| C3-6 | 8.15[b] | 14.55[b] | 27.35[b] | 39.45[b] | 62.33[b] | 85.05[b] |
| F-test | ** | ** | ** | ** | ** | ** |
| CV(%) | 14.3 | 16.3 | 15.9 | 14.1 | 14.7 | 13.8 |

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

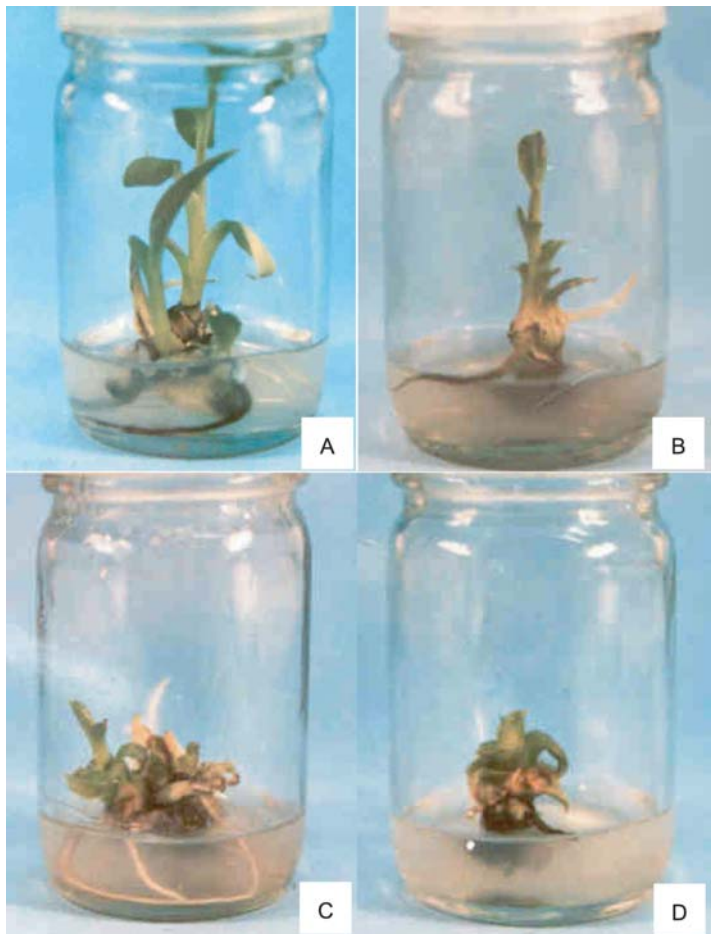


**Figure 3** Plantlets in tissue culture.
A. normal B-D. abnormal

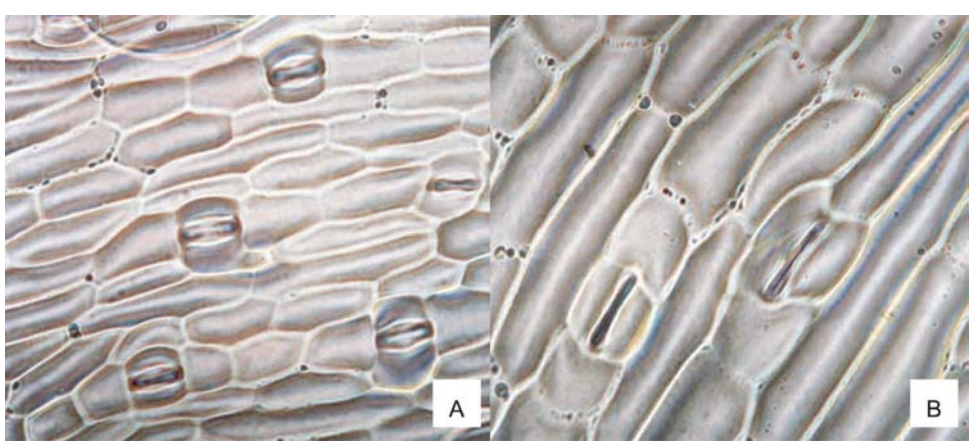


**Figure 4** Stomata size (400X).
A. normal plant B. abnormal plant

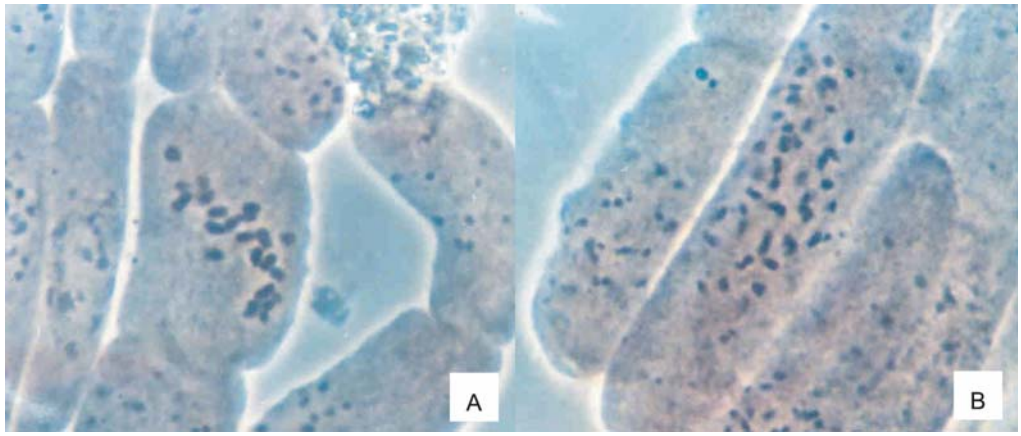


**Figure 5** Chromosome number (1850X).
A. 2n=22 B. 2n=44


**Figure 6** Control and mutant plantlets in tissue culture.


**Figure 7** Control and mutant plants after 2 months in nursery.

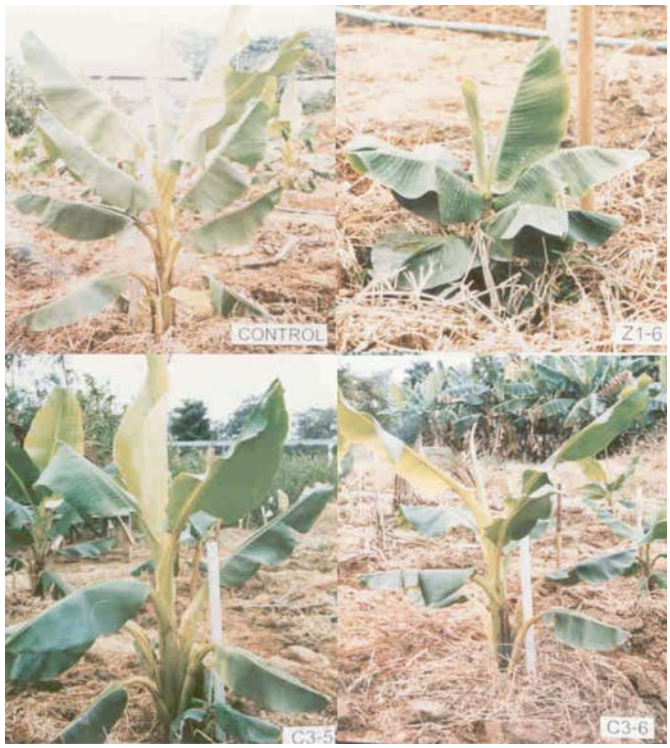


**Figure 8** Control and mutant plants in the field.

**Figure 9** Bunch of 4X Kluai Khai.

**Figure 10** Kluai BEP in 4 inches pot (1 year old).

The heights of plant were measured every month. The oryzalin treated plants were noticed to be at the shortest in every month and were highly significantly different from the other treatments, the same as in nursery. This result was due to the effect of herbicide that retarded growth of broadleaf weeds. The growth of oryzalin treated plants were 3.40, 4.95, 7.57, 10.05, 14.30 and 19.20 cm. at the 1-6$^{th}$ month respectively. On the 6$^{th}$ month, the control was 81.90 cm. while the colchicine treated plants were 79.53 and 85.05 cm. which were not significantly different from one another (Figure 8 and Table 7). The results were similar to the work of Vakili (1967) who studied the growth of 2X and 4X of *Musa balbisiana*.

When the plants were 6 months old in the field, number of leaf, number of sucker and circumference of pseudostem at 10 cm. above the ground were recorded. The circumferences of the oryzalin treated plants were found to be significantly smaller than those of the other treatments (Table 8). Numbers of leaf and sucker were also noticed not to be different.

At six months old, the control was revealed to flower whereas the treated plants had not yet. That could mean the shooting time of treated plants took longer time than the normal ones, which was not good characteristic of banana. It should therefore be discarded.

Following this experiment, the C3-5, C3-6 and Z1-6 were grown for further observing and found that, both of C3 flowered after 1 year. The bunches and fruits were smaller than those of the normal K.Khai as shown in Figure 9. The discard of those plants should obviously be done as suggested. For Z1-6 plant, the growth were very low. Studying on the characteristic of psuedostem, the leaves were also found to be absolutely changed from those of the normal K.Khai. This was due to the fact that oryzalin was a growth inhibitor and antimitotic agent which could retard the growth of plant (Verhoeven *et al.*, 1990; Hassawi and Liang, 1991; Ramula *et al.*, 1991). Z1-6 was found to be very short which was good as ornamental plant (Figure 10). It was then named BEP and the plant was registered at Ministry of Agriculture, Thailand on the year 2000.

**Table 8** Circumferences at 10 cm height, numbers of leave and numbers of sucker at 6 months old of mutants compared to the control.

| Treatment | Circumference (cm.) 1/ | Number of leave | Number of sucker |
|---|---|---|---|
| Control | 23.95$^{b}$ | 17.58 | 2.12 |
| Z1-6 | 15.33$^{a}$ | 14.08 | 1.25 |
| C3-5 | 23.88$^{b}$ | 15.20 | 1.17 |
| C3-6 | 24.45$^{b}$ | 14.95 | 1.21 |
| F-test | ** | ns | ns |
| CV(%) | 11.4 | 15.6 | 56.1 |

ns = non significance

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

## CONCLUSION

1. $LD_{50}$ at 2.5, 5.0 and 7.5 hrs of colchicine concentration on Kluai Khai are 0.4, 0.5,0.75 per cent and of oryzalin are 13, 15, 20 mM at 2.5, 5 and 7.5 hrs. of soaking respectively.

2. 1% colchicine at 7.5 hrs. and 45 mM oryzalin at 7.5 hrs. could change the chromosome number of Kluai Khai from 2n = 22 to 2n = 44.

3. The growth of colchicine treated plants were similar to the control but fruiting period was too long and the fruits were quite small.

4. The growth of oryzalin treated plants was very slow, both in the culture media and in the field. Morphology of leaf and psuedostem changed from the normal Kluai Khai. The result was good to be furtherly produced as ornamental plant. It was later registered by the name of BEP.

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