Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

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OPEN ACCESS Asian Journal of Plant Sciences

ISSN 1682-3974
DOI: 10.3923/ajps.2022.111.118

Research Article
In vitro Tissue Culture Techniques and Colchicine-Induced Polyploidy in Banana (*Musa*, AA Group) 'Kluai Khai'

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Abstract

Background and Objective: *In vitro* tissue culture techniques for both shoot and root induction and the colchicine-induced approach on the banana (*Musa*, AA group) 'Kluai Khai' cultivar have been very limited. The purpose of this research was to determine the optimal conditions for plantlet regeneration and polyploidy induction in Kluai Khai. **Materials and Methods:** Shoot tips of banana were cultured on Murashige and Skoog (MS) solidified medium amended with 6-benzylaminopurine (BAP) at different concentrations for shoot induction and the obtained shoots were transferred to MS medium containing Naphthalene Acetic Acid (NAA) at different concentrations for root induction. For polyploidy induction, banana shoots were transferred to liquid MS medium supplemented with various concentrations of colchicine (0, 0.05, 0.10, 0.15 and 0.20%) for 1, 2, 3, 4 and 5 days. **Results:** MS medium supplemented with 2.5 mg L^{-1} BAP was most effective in inducing shoot multiplication, with maximum shoots per explant and height of shoot. MS medium amended with 0.5 mg L^{-1} NAA was most appropriate for rooting (100%) with an average of 9.30 roots per shoot and a length of 5.48 cm. Moreover, the treatment with 0.15% colchicine for 3 days appeared to be the most effective combination for generating tetraploids, with the highest viability of explants. Moreover, in terms of leaf anatomy, the tetraploid plants had larger stomata than the diploid of Kluai Khai. **Conclusion:** MS medium supplemented with 2.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA were optimal conditions for the shoot and root induction and 0.15% colchicine for 3 days was most effective for tetraploids generation of Kluai Khai banana.

Key words: Multiplication, root induction, chromosome doubling, tetraploid, Colchicine, tetraploids, stomata, leaf spot disease

Citation: Khamrit, R. and N. Jongrungklang, 2022. *In vitro* tissue culture techniques and colchicine-induced polyploidy in banana (*Musa*, AA group) 'Kluai Khai'. Asian J. Plant Sci., 21: 111-118.

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

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

INTRODUCTION

Banana is an important fruit crop generally grown in tropical and subtropical regions[1-3]. Banana is usually used as a consumption fruit and provides several by-products[4]. It plays an important role as a good source of carbohydrates, proteins and minerals[5,6]. However, there are variations in organic compounds with different banana genotypes. A banana genotype that is widely grown in tropical regions, including Thailand, is *Musa* AA group 'Kluai Khai', a tiny banana with an intense aroma, soft texture and sweet taste. Kluai Khai is a diploid cultivar, which like all bananas, belongs to the AA cultivar group. Despite that this genotype is appropriate for consumption, low productivity is a crucial problem in the commercial aspect. In addition, Kluai Khai is susceptible to leaf spot disease and fruit yield is strictly reduced[7].

In this regard, the cultivated area of this banana cultivar is continuously decreasing. A strategy to eliminate these constraints may improve the Kluai Khai cultivar, which provides a bigger fruit. A feasibly rapid approach uses colchicine to double the number of chromosomes. Colchicine is usually used to induce chromosome duplication in breeding aspects proposed for the improvement of crops, forest species and ornamental plants. It disrupts the binding tubulin dimer, causing a conformational change that inhibits the polymerization of the mitotic spindle, thus being anti-mitotic. Colchicine applied at low concentrations promotes the depolymerization of the mitotic spindle, leading to the induction of polyploidy in metaphase[8].

Edible bananas are almost always seedless and sterile, therefore, they are propagated vegetatively via sucker[9]. In this context, there are many limitations for reproductive multiplication, for instance, (1) The rate of multiplication of suckers is very slow with low production, (2) Spread of diseases and (3) Poor preservation of genetic resources[10]. An approach to managing these issues is the usage of *in vitro* tissue culture technology, an alternative approach that produces many plants in a short time, in less space and that are disease-free[11].

To date, information on *in vitro* tissue culture techniques for both shoot and root induction and the colchicine-induced approach on the Kluai Khai cultivar has been very limited. Therefore, this study aimed to determine the optimum 6-benzylaminopurine (BAP) and Naphthalene Acetic Acid (NAA) for shoot and root regeneration and to determine the appropriate approach for colchicine-induced polyploidy in the Kluai Khai banana. This information will be useful as an alternative approach to multiply plants in a short time that are disease-free and to improve genetic resources for developing Kluai Khai banana.

MATERIALS AND METHODS

Study area: This study consisted of two experiments: tissue-culture for (1) Shoot and root induction and (2) Colchicine-induced polyploidy (survival rate, chromosome number, ploidy level and stomatal size). Both experiments were separately investigated at the Department of Biology, Faculty of Science and Technology, Loei Rajabhat University, Thailand from April, 2017-January, 2020.

Plant material preparation and sterilization: Stems of the Kluai Khai banana sucker were collected. The older leaves were removed from the plant samples and stems were cut into 1×1-inch sections. The shoot tip of the sucker was used as an explant. The explants were disinfected in 70% ethanol for 5 min, soaked in 30% Clorox solution (sodium hypochlorite 6%) with Tween 20 (1-2 drops) for 30 min and rinsed three times for 5 min in sterile distilled water. The sterilized shoot tip was cut into four pieces (1 cm^2).

Experiment 1

Tissue-culture medium for the shoot and root induction: Each shoot tip was cultured on Murashige and Skoog[12] (MS) medium with different concentrations of BAP (0, 2.5, 5.0, 7.5 and 10 mg L^{-1}). All cultures were maintained at 25±2°C under white illumination with a 16 hrs photoperiod (1,500 lux) for 8 weeks. The number of shoots, percentage of shoot formation and average shoot height were recorded.

The formative shoots, which were approximately 1.5 cm in height, were cultured on MS medium with NAA at 0, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}. All cultures were maintained at 25±2°C under white illumination with a 16 hrs photoperiod (1,500 lux) for 8 weeks. The number of roots per shoot, percentage of root induction and average root length were determined.

Shoot and root induction experiments were arranged in a Completely Randomized Design (CRD) with 10 replicates. The measurement data were subjected to a one-way analysis of variance. Mean values of all parameters were separated according to Duncan's Multiple Range Test (DMRT) at a confidence level of 0.05.

Experiment 2

Colchicine-induced polyploidy in Kluai Khai banana: A 5x5 factorial in CRD was used for colchicine-induced polyploidy with 3 replications. Each replication included 10 shoots for a total of 30 shoot samples per replicate. Factor A was the colchicine concentration (0, 0.05, 0.10, 0.15 and 0.20%) and factor B was treatment duration (1, 2, 3, 4 and 5 days). The

shoots derived from tissue culture were soaked (120 rounds sec^{-1}) in MS liquid with different colchicine concentrations and soaking durations. The shoots were then washed once with sterile distilled water and moved to a sterilized room. The shoot samples were cultured on MS medium under light conditions with a period of 16/8 hrs (light/dark, 1,500 lux) at 25±2°C for 120 days.

Survival rate and chromosome number: Plantlet survival was recorded at 120 days after culture to calculate the survival rate. Root tip samples were soaked with 0.02% (w/v) 8-hydroxyquinoline for 3-5 hrs at 4°C and then moved to a 3:1 solution of absolute alcohol and glacial acetic acid for 30 min. The roots were kept in 70% alcohol at a low temperature (4-6°C). The chromosome number was recorded in each treatment. The root samples were washed with distilled water 2-3 times, soaked with 1N HCl at 60°C for 5 min, washed with distilled water 2-3 times and soaked in 45% acetic acid for 10 min. Then, chromosomes were stained with 2% aceto-orcein for 20 min. Chromosomes were counted using a light microscope at 1000× magnification.

DNA content: Leaf samples (0.5 g) of each treatment were collected and DNA was extracted with 500 µL CyStain UV Precise P (comprised of nuclei extraction buffer and staining buffer) and 0.1 g PVP (polyvinyl-pyrrolidone). The leaf sample was chopped and filtered with 30 µm celltrics disposable filters and CyStain UV Precise P was added (500 µL). The DNA content of the samples was analyzed using flow cytometry (Partec, PA II flow cytometer).

Determination of stomatal size between diploid and tetraploid of Kluai Khai banana: Kluai Khai banana seedlings were induced with 0.20% colchicine for 3 days and seedlings without colchicine application were used as a control. At 120 days after colchicine application, the peeling method with safranin O was used to determine the width and length of stomata in 3 leaf samples per treatment and 10 stomata per leaf. The width and length of stomata were then observed using a light microscope at 400× magnification. The mean comparison for all parameters was done using an independent t-test.

RESULTS

Tissue-culture medium for the shoot and root induction: The shoot tips were obtained from substantial MS media containing 2.5, 5.0, 7.5 and 10.0 mg L^{-1} BAP 1 week after treatment. The shoot tip was larger and its colour changed from white to green. At 8 weeks after treatment, plantlets that were cultured in MS media with BAP were completely induced (100%) from shoot explants. In the BAP-free treatment, green colour and larger size were observed on proliferating shoot tips 8 weeks after treatment but there was no plantlet formation. MS media with 2.5 mg L^{-1} BAP was the best option with 2.20 shoots per explant and a 4.27 cm shoot height (Table 1). Although three concentrations of BAP (5.0, 7.5 and 10.0 mg L^{-1}) had significantly more shoots (1.00-1.20 shoots) and a higher shoot height (1.31-1.51 cm) when compared to the no BAP treatment.

At 2 weeks after treatment, the basal shoot was initiated in all NAA levels. Different concentrations of NAA (0.0, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}) altered root induction, root number per shoot and root length after 8 weeks of culture. In general, the application of 0.5 mg L^{-1} NAA provided the best conditions for root induction of the Kluai Khai genotype, providing 100% root induction, the highest root number per shoot (9.30) and the longest root length (5.48 cm, Table 2). Although the no NAA treatment-induced root formation from the shoot but the root number per shoot was rather low. Moreover, 1.0 and 2.0 mg L^{-1} NAA treatment were not significantly different from the NAA-free treatment in terms of percentage of root formation and the number of roots and 2.0 mg L^{-1} NAA treatment provided poor root induction. The root length in the 3.0 mg L^{-1} NAA treatment was low but this treatment performed well for root induction percentage and the number of roots.

Colchicine-induced polyploidy in Kluai Khai banana: The results showed that there was an interaction between colchicine concentration and application duration and both factors significantly contributed to the survival rate. At 120 days after colchicine treatment, the plantlets that were treated with any colchicine concentrations 0.00, 0.05, 0.10, 0.15 and 0.20% for 1-4 days had a survival rate of 100%. Low

Table 1: Different physical parameters of *Mussa* AA group 'Kluai Khai' with different concentrations of BAP after 8 weeks in culture

BAP (mg L^{-1})	Shoot induction (%)	Number of shoots/explant±SE	Average shoot height (cm)±SE
0	0	0.00±0.00^{c}	0.00±0.00^{c}
2.5	100	2.20±0.13^{a}	4.27±0.23^{a}
5.0	100	1.00±0.00^{b}	1.31±0.09^{b}
7.5	100	1.10±0.10^{b}	1.35±0.18^{b}
10	100	1.20±0.13^{b}	1.51±0.18^{b}

Means within the column followed by different letters are significantly different at $p<0.05$, SE: Standard error

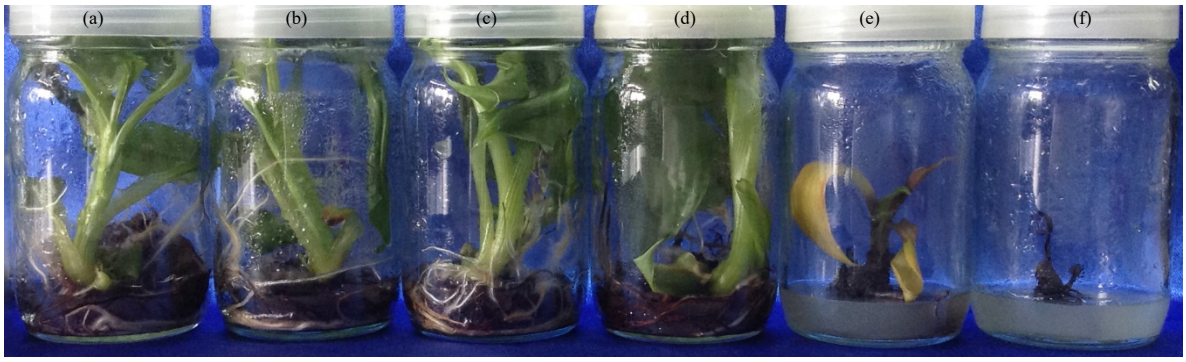

Fig. 1a-f: Survival rate of *Musa* AA group 'Kluai Khai' after 120 days of culture on MS medium

(a) Control, (b) Treated with 0.10% colchicine for 3 days, (c) 0.15%, colchicine for 3 days, (d) 0.20% colchicine for 3 days, (e) 0.15% colchicine for 5 days, (f) 0.20% colchicine for 5 days

Table 2: Root formation percentage, number of roots and average root length of *Mussa* AA group 'Kluai Khai' with different concentrations of NAA after 8 weeks in culture

NAA (mg L^{-1})	Root induction (%)	Number of roots/shoot±SE	Average root length (cm)±SE
0.0	80	4.20±1.22^{c}	5.83±1.25^{a}
0.5	100	9.30±0.80^{a}	5.48±0.52^{a}
1.0	80	4.70±1.07^{bc}	5.04±1.10^{a}
2.0	70	2.40±0.76^{c}	2.04±0.63^{b}
3.0	90	7.60±1.33^{ab}	2.62±0.34^{b}

Means within the column followed by different letters are significantly different at $p<0.05$, SE: Standard error

Table 3: Survival rate of *Musa* AA group 'Kluai Khai' subjected to different colchicine concentrations and soaking durations

	Survival rate (%)±SE				
	Colchicine concentration (%)				
Treatment duration (days)	0	0.05	0.10	0.15	0.20
1	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}
2	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}
3	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}
4	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}	100±0.00^{aA}
5	100±0.00 ^{aB}	100±0.00 ^{aB}	100±0.00 ^{aB}	76.66±5.77^{aB}	0±0.00^{bB}

Means followed by the different lowercase letters within the column and uppercase letters in the row are significantly different at $p<0.05$, SE: Standard error

colchicine concentration treatment (0.00, 0.05 and 0.10%) for 5 days provided a 100% survival rate. In contrast, treatment combinations, such as 0.15 and 0.20% colchicine for 5 days had a lower survival rate of 76.66 and 0.00%, respectively, when compared with the low colchicine concentrations (Table 3). Under both colchicine-untreated and colchicine-treated for 3 days, plantlets appeared normal and healthy and performed impressive plantlet growth and size (Fig. 1a,d). In addition, colchicine-treated plantlets showed stress symptoms, as symptoms were observed in 0.15 and 0.20% colchicine treatments with a 5-day soaking time. In the higher colchicine concentrations treated for 5 days, yellow leaves (Fig. 1e) and bruised stems were observed on plantlets and the damaged plantlet died (Fig. 1f).

All treated *Musa* AA group 'Kluai Khai' plantlets from the colchicine treatments were evaluated after 120 days for chromosome number determination. Chromosome numbers were different between colchicine treated and non-treated plantlets. Initially, the colchicine-free plantlets were diploids with 2n = 2x = 22 (Fig. 2a), whereas plantlets in the colchicine application group were either diploid or tetraploid (2n = 2x = 44, Fig. 2b). Moreover, colchicine successfully induced tetraploid plantlets (doubling of diploid plantlets) in those treated with 0.01, 0.15 and 0.20% colchicine for 3 days and 0.15% for 5 days.

After colchicine induction, flow cytometry measurements were used to detect the ploidy level. Plantlets with different colchicine concentrations and treatment durations provided different ploidy levels, namely diploids, mixoploids and tetraploids. The nuclear DNA content results showed that 0.15% colchicine for 3 days successfully induced all (100%) tetraploid plantlets of *Musa* AA group 'Kluai Khai', while increasing the colchicine application period significantly decreased the tetraploid-induction percentage by 50%. Higher colchicine concentrations, such as 0.10 and 0.20%, produced the most tetraploid plantlets (60%). Moreover, mixoploid

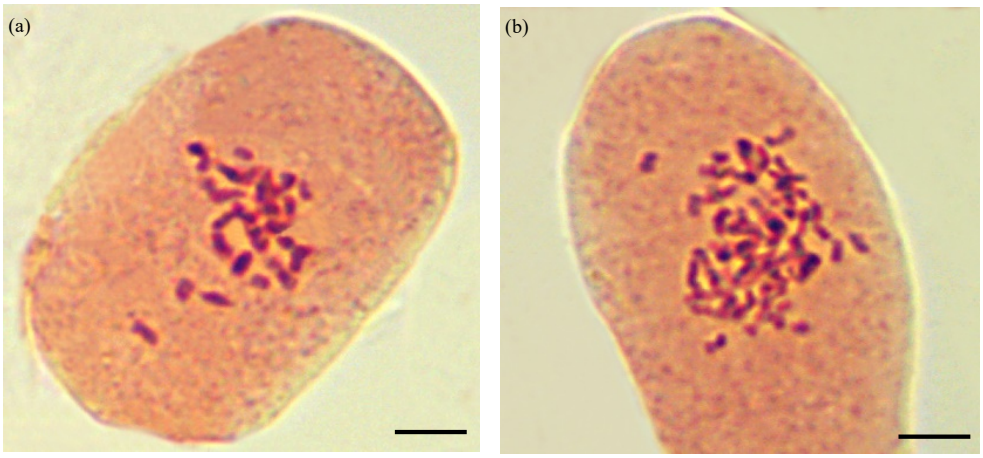

Fig. 2a-b: Chromosome number of *Musa* AA group 'Kluai Khai'
(a) Diploid plant with 2n = 2x = 22 and (b) Tetraploid plant with 2n = 4x = 44, Bars = 20 µm

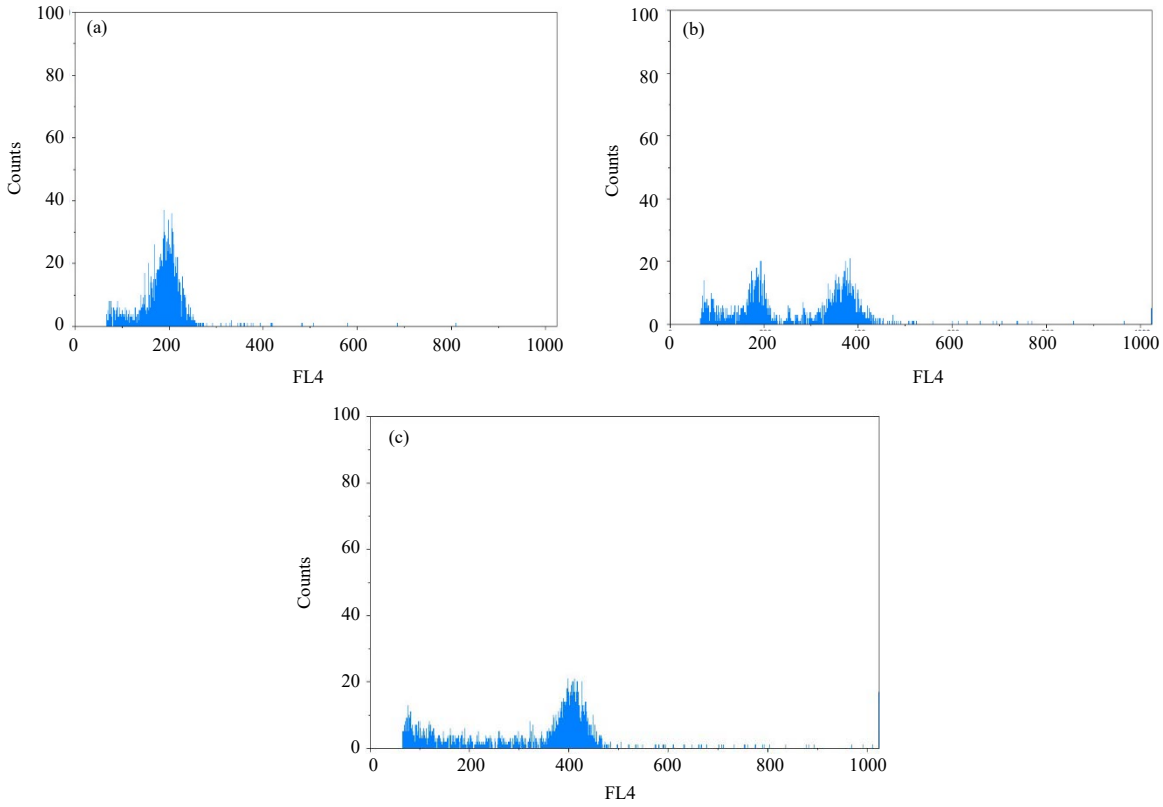

Fig. 3a-c: Flow cytometric histograms of relative nuclear DNA content of *Musa* AA group 'Kluai Khai'
(a) Diploid, (b) Mixoploid and (c) Tetraploid

plantlets were observed at a 0.10% colchicine concentration for 3, 4 and 5-day treatment durations, 0.15% colchicine for 2, 4 and 5 days and 0.20% colchicine for 2, 3 and 4 days. Only diploids were produced from the control treatment (0%) and the lower colchicine concentration of 0.05%. The 0.10% concentration for 1- and 2-day soaking periods also only produced diploids (Table 4 and Fig. 3a-c).

Table 4: Effects of colchicine treatment on polyploidization induction of *Musa* AA group 'Kluai Khai'

Colchicine concentration (%)	Treatment duration (days)	Number of plants examined	Ploidy level (%)		
			Diploid	Mixoploid	Tetraploid
0	0	5	5 (100)	0	0
0.05	1	5	5 (100)	0	0
	2	5	5 (100)	0	0
	3	5	5 (100)	0	0
	4	5	5 (100)	0	0
	5	5	5 (100)	0	0
0.10	1	5	5 (100)	0	0
	2	5	5 (100)	0	0
	3	5	0	2 (40)	3 (60)
	4	5	3 (60)	2 (40)	0
	5	5	1 (20)	4 (80)	0
0.15	1	5	5 (100)	0	0
	2	5	3 (60)	2 (40)	0
	3	5	0	0	5 (100)
	4	5	2 (40)	3 (60)	0
	5	5	2 (30)	1 (20)	3 (50)
0.20	1	5	5 (100)	0	0
	2	5	4 (80)	1 (20)	0
	3	5	1 (20)	1 (20)	3 (60)
	4	5	3 (60)	2 (40)	0
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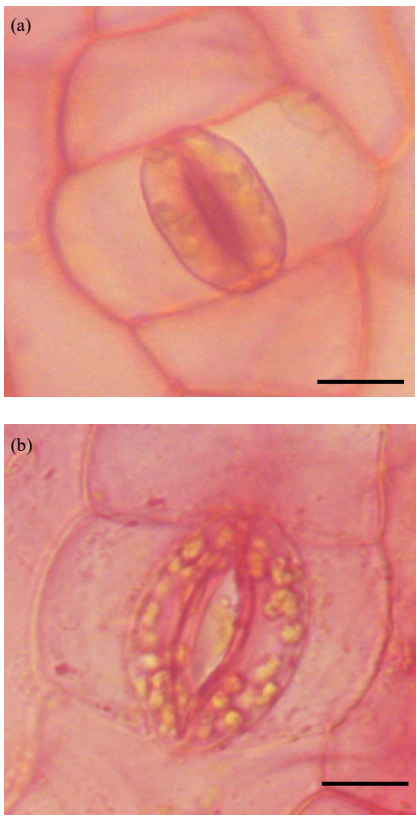

Fig. 4a-b: Stomatal size of (a) Diploid and (b) Tetraploid, *Musa* AA group 'Kluai Khai'
Bars = 20 µm

Table 5: Stomatal density and stoma size in diploid and tetraploid *Musa* AA group 'Kluai Khai'

Ploidy level	Stomatal width (µM)±SE	Stomatal length (µM)±SE
Diploid	23.13±0.18[a]	47.24±0.47[a]
Tetraploid	37.58±0.62[b]	68.23±0.85[b]

Means within the column followed by different letters are significantly different at $p<0.05$

Determination of stomatal traits between diploid and tetraploid of Kluai Khai banana: The length and width of stomata differed between tetraploid and diploid *Musa* AA group 'Kluai Khai' plantlets (Table 5 and Fig. 4a-b). Stomata length was 68.23 and 47.24 µm in tetraploid and diploid plantlets, respectively. Tetraploid plantlets had significantly higher stomatal width than diploid plantlets (Table 5).

DISCUSSION

Application of BAP with MS media in *in vitro* culture provided the effective shoot regeneration, shoot numbers and shoot height for Kluai Khai when compared to the BAP-free condition. The function of BAP is to effectively induce shoot regeneration for plantlet growth[6,13]. Cytokinins play an important role in cell division and enlargement and stimulate protein synthesis and enzyme activity[14]. Sofian *et al.*[15] found that BAP reduced the cell division period in the S phase due to their support in preparing DNA and proteins in this process. In *Musa* spp., the appropriate concentration of cytokinins added to MS media depends on the genetics and growth

development stage. Buah *et al.*[16] reported that the addition of BAP, kinetin and 2ip to MS caused more shoot induction than kinetin and 2ip in bananas. In addition, Rehana[17] investigated the effect of BAP concentration on four different banana varieties (Seeded, Amritsagar, Sabri and Anajee) and found that different banana varieties provided various suitable rates of BAP for plantlet regeneration. A high induction rate generally occurred at 4-8 mg L^{-1} BAP. However, a low fruit yield, such as that of *Musa* AA group Kluai Khai in this study showed that 2.5 mg L^{-1} BAP in the MS media was the best treatment.

The application of 0.5 mg L^{-1} NAA was appropriate for root induction of the Kluai Khai genotype in terms of root induction rate, root number per shoot and root length. NAA promotes cell division and enlargement[18] and controls root growth[19-21]. In tissue culture conditions, the requirements for root induction involve plant genetics, the physiological and developmental phases of the shoots and culture conditions[22]. The NAA concentration in our study agreed with Hossain *et al.*[6], who reported that 4 mg L^{-1} NAA promoted root initiation and provided root numbers ranging from 4.33-5.66 roots per shoot in plantlets of banana cultivars 'Grand Naine' 'Amritasagar' and 'Sabri'. Furthermore, using 2 ppm NAA induced root germination up to 26 roots per shoot and the average root length[3] was 92.22 cm.

Plantlet death was observed at a high concentration of colchicine and a long soaking duration (5 days). Colchicine influences the growth of tissue and is identified as a toxicant that can cause cell death in excessive doses[8,23]. The mortality of somatic embryos of 'Namwa' banana increased with increasing ABB concentrations and soaking duration from 0.1% colchicine concentration for 48 hrs 1.0% colchicine concentration for 72 hrs, the death rate increased to 90% under high concentrations and long durations[24]. Therefore, the death rate depends on the concentration and soaking duration of colchicine[25]. A higher rate was observed with a greater dose and soaking duration.

Moreover, a suitable colchicine concentration and treatment duration could completely induce chromosome doubling, producing tetraploids ($2n = 4\times = 44$) in banana cv. Kluai Khai. Colchicine inhibits the binding of the tubulin dimer and the polymerization of the mitotic spindle, causing an incomplete production of the spindle fibre and blocking the separation of chromosomes in metaphase, this causes an increase in the ploidy level[8]. Despite that colchicine is a polyploidy initiator, it has a toxic effect at high concentrations, causing high mortality and abnormal plants[26,27]. A high ploidy level in bananas generally increases vigour compared to the normal diploid but they spend a lot of energy and time duplication on their DNA[25]. In addition, this study found that tetraploid bananas had significantly higher stomatal width and length than diploid bananas. Stomatal size is usually used as a criterion to determine the different ploidy levels in many plants, such as in *Lilium regale*[28] and *Rhododendron fortunei* Lindl[29]. This information will be useful for providing an alternative protocol of Kluai Khai banana tissue culture technique to multiply plants in a short time that are disease-free and to improve genetic resources for developing breeding of Kluai Khai banana.

CONCLUSION

This study reported successful efforts to generate a high shoot and root induction rate and increase the growth of *Musa* AA group 'Kluai Khai' from shoot tips. Application of 2.5 mg L^{-1} BAP under light conditions provided the most effective shoot regeneration for Kluai Khai. For root induction, 0.5 mg L^{-1} NAA was promising, as it generated the highest number of roots per shoot and the highest root induction rate. Moreover, this report also explored a highly efficient method for inducing tetraploids in bananas (*Musa* AA group 'Kluai Khai'). Application of 0.15% colchicine for 3 days was the most suitable for inducing tetraploids in bananas (*Musa* AA group Kluai Khai). Future studies should focus on the traits and yields of tetraploid fruit, the molecular changes that occur internally and the triploid breeding of bananas.

SIGNIFICANCE STATEMENT

This study discovers the optimal conditions for plantlet regeneration and *in vitro* polyploidy induction in Kluai Khai that can be beneficial for Kluai Khai banana researchers who are interested in *in vitro* culture techniques and tetraploids generation. This study will help the researcher to uncover the multiply plants in a short time that are disease-free and to improve genetic resources for developing Kluai Khai banana that many researchers were not able to explore. Thus, a new theory of most appropriate for the shoot and root induction and the colchicine-induced may be arrived at.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Loei Rajabhat University and Northeast Thailand Cane and Sugar Research Center, Faculty of Agriculture, Khon Kaen University for providing financial support (grant number RP64-2-001) and the Faculty of Science and Technology, Loei Rajabhat University for the facilities used in this research.

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