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Research Article
Induction of Banana Autotetraploids "Klutuk Sukun" and their Reproductive Function for Producing Triploid Hybrids

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Abstract

Background and Objective: The most commonly used direct method in breeding triploid banana is crossing tetraploids with diploids. "Pisang Klutuk Sukun" (*Musa balbisiana* Colla, BB) has been associated with improved vigor and tolerance to biotic and abiotic stresses and therefore, it is a target for Musa breeding program. The objective of the study was to produce banana autotetraploid plants from diploid "Pisang Klutuk Sukun" and to evaluate the morphological characters and the reproductive potential of the autotetraploid plants. **Materials and Methods:** Induction of the autotetraploid was conducted using *in vitro*-cultured shoots of diploid "Pisang Klutuk Sukun" treated with oryzalin at a concentration of 60 μM for 7 days in a liquid MS basal medium with addition of 2 mg L^{-1} BA. The morphology characters of the autotetraploids were evaluated which based on 52 characters of UPOV for two reproductive cycles. The reproductive potential of the autotetraploid plants was conducted by crossing the autotetraploids with a diploid banana cultivar; "Pisang Rejang" to produce triploids. **Results:** The induction of autotetraploid experiment showed that 12 plants out of 34 plants of oryzalin-treated "Pisang Klutuk Sukun" were autotetraploids. The autotetraploid plants showed drooping leaves instead of erect leaves as in its diploid plants. They had fewer suckers compared to its diploid plants. The fruit diameter (width) of the autoteraploid was larger than those of its diploid. The autotetraploid "Pisang Klutuk Sukun" was successfully crossed with diploid banana cultivar, 'Pisang Rejang' and produced triploid hybrids. **Conclusion:** This study concluded that the banana autotetraploid plants of "Pisang Klutuk Sukun" were produced at a concentration of 60 μM for 7 days in a liquid MS basal medium with addition of 2 mg L^{-1} BA. The autotetraploids were fertile as well.

Key words: "Pisang Klutuk Sukun", "Pisang Rejang", *Musa balbisiana*, oryzalin, autoteraploid, triploid hybrids, Musa breeding program

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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

Bananas (*Musa* spp.) are important fruit crop in Indonesia and the world. Indonesia is the center of origin and diversity of cultivated banana[1]. Based on FAO Statistics, in 2016, Indonesia was the third largest banana producer in the world with a production of about 7.1 Mt. Banana production in Indonesia is threatened by many fungal, bacterial and virus diseases as well as abiotic stress, such as drought. To create new varieties with desired characteristics is quiet challenging. Bananas are difficult to breed. Cross breeding programs are difficult because of the asexual behavior of edible clones, which are sterile and polyploid. High sterility and parthenocarpic nature of most edible bananas as well as a lack of knowledge regarding the type of inheritance of resistance limits success of banana breeding program[2]. Breeding of most cultivated bananas rely upon conventional sexual hybridization, involving the crossing of triploid cultivars with wild or cultivated diploid parents. Generally, crossing triploid (3x) cultivars with diploid (2x) parents, generates tetraploid (4x) hybrids[3]. Induced polyploidy in banana plants has been conducted[2,4-6]. In a research performed, amplification of the leaves and fruits of autotetraploids in comparison to the original diploids were observed[4]. Likewise, increase in the plant height, number of living leaves at flowering and harvest, pseudo stem diameter and greater fruit and bunch were observed also in autotetraploid "Pisang Lilin"[2]. Tetraploids are usually induced via mitotic inhibition *in vitro*. Chromosome doubling of potential diploids allows the production of fertile autotetraploid plants that may be used in crosses with improved diploids to generate secondary triploids (AAA) with disease resistance and fruits with good agronomic characteristics[4,7]. The availability of 2n gametes is rare and unstable in banana plants and working with autotetraploid plants enables and systematizes the production of triploid hybrids directly from a large number of diploid germplasms[4].

"Pisang Klutuk Sukun" (*Musa balbisiana* Colla, BB) is a diploid local cultivar banana, non-parthenocarpic (seeded), tasty and sweet, medium size banana. It is used for its leaves for wrapping and young fruit for food. The *M. balbisiana* genome has been associated with improved vigor and tolerance to biotic and abiotic stresses and therefore, it is a target for Musa breeding program[8]. There are no available reports of tetraploid of "Pisang Klutuk Sukun". It is important to produce fertile tetraploid "Pisang Klutuk Sukun" to generate secondary triploids which has the best characters of "Pisang Klutuk Sukun". The present investigation was undertaken to obtain autotetraploid "Pisang Klutuk Sukun" using *in vitro* shoot treatment with oryzalin, to evaluate the morphological characters of the autotetraploid plants and to assess the reproductive potential of the autotetraploid to generate triploid hybrids.

MATERIALS AND METHODS

Materials: Diploid "Pisang Klutuk Sukun" was accessed from Banana Germplasm Repository, Yogyakarta.

Initiation and multiplication of shoot culture: Initiation and multiplication of shoot culture of diploid "Pisang Klutuk Sukun" was conducted on January-June, 2012. The culture was initiated from shoot tips of banana corm dissected and propagated aseptically on a solid Murashige and Skoog[9] (MS) medium as previously described[6]. Shoot cultures of "Pisang Klutuk Sukun" were established and multiplied in MS medium[9] supplemented with 30 g L^{-1} sucrose, 100 mg L^{-1} myo inositol, 4 mg L^{-1} thiamine HCl and 2 mg L^{-1} BA and solidified by 7 g L^{-1} agar. The media was adjusted to pH of 5.7-5.8.2. The cultures were maintained at 25°C.

Induction and multiplication of autotetraploid Pisang Klutuk Sukun: Induced tetraploidy was conducted on diploid "Pisang Klutuk Sukun" using oryzalin as described by Van Duren *et al.*[10] with minor modification[6]. The shoots were extracted from the medium and were treated with the antimitotic agent oryzalin at concentration of 60 µM for 7 days in liquid medium with agitation (60 rpm). After treatment, the shoots were washed three times with distilled water and transferred to a proliferation medium for multiplication. The shoots were then transferred to a rooting medium (MS supplemented with sucrose 30 g L^{-1} and solidified with 7 g L^{-1} agar). The explants were kept in a room with a photoperiod of 16 h and a temperature of 25±2°C during their growth phase. The cultures were sub-cultured for 5-6 times to reduce the frequency of mixoploids (plant material containing cells with chromosome number variations) and separate the mixoploids. All of the treated shoots were examined for their ploidy level by using Flow cytometer. Only tetraploid shoots were multiplied for characterization.

The autotetraploid induction and multiplication were conducted in Laboratory of Plant Cell and Tissue Culture of Research Center for Biology, Indonesian Institute of Sciences from June-December, 2012.

Acclimatization and cultivation of the autotetraploids: The plantlets (rooted plants) were transferred to a greenhouse and placed in cultivation pots with a substrate composed of sand, coco peat (coconut fiber) and soil compost (1:1:1) and

irrigated under 50% shading. After 60 days, the plants were transplanted to 20 L plastic polybags with the same substrate. After 2 months, banana plants were ready for planted in the field. All treated plants and control were planted in 5 plant rows in a random design and replicated three times.

Identification of ploidy level using flow cytometer: Ploidy detection was performed by using a Partec PAS II flow cytometer (FCM) (Partec GmbH, Munster, Germany). Samples were prepared according to Doležel *et al.*[11] with modification[6]. Approximately 20-30 mg of fresh leaf samples from cigar leaves (control and treated samples) were chopped with a sharp scalpel blade in a glass Petri dish containing 1 mL of LB01 buffer[12] of the following composition: 15 mM TRIS, 2 mM Na2 EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM mercaptoethanol, 0.1% Triton X-100, pH 7.5. The buffer was supplemented with DAPI (4', 6-diamidino-2-phenylindole) at final concentration of 2 μg mL^{-1} to stain nuclear DNA. The suspension of released nuclei was filtered through a 50 μm nylon mesh and kept on ice before analysis. The relative DNA content of the sample was then determined using FCM analysis.

Relative DNA content is given in C units. The 1C value is DNA content of haploid set of chromosomes (n). The distribution of fluorescence intensities (relative DNA content) obtained after flow cytometric analysis is usually in arbitrary units (channel numbers). For ploidy screening, this scale must be calibrated with a reference. In this study, it have used a sample prepared from *Musa acuminata* ssp. *malaccensis* (2n = 22) as a diploid reference[6] and the flow cytometer was adjusted so that the peak representing its G1 nuclei appeared at channel 200. This setting was kept constant and other samples were characterized by the relative position of their G1 peak.

Evaluation of morphology characters: Morphology characterization was conducted based on 52 characters of International Union for the Protection of New Varieties of Plants[13] for two reproduction cycles. The characterization was conducted in Cibinong Science Center, Indonesian Institute of Sciences, from August, 2013-December, 2016.

Crossing for evaluation of the reproductive potential of the autotetraploid bananas: The autotetraploid "Pisang Klutuk Sukun" (BBBB) and diploid "Pisang Rejang" (*Musa*, AA) were used for reciprocal crosses. The flowers were pollinated and covered with net bags. Seeds were collected from each fruit of the crosses at maturity. The crossings and harvesting were conducted on July-September, 2015. The numbers of hybrid seeds were recorded for each crossing.

Embryo rescue of the hybrid seeds: The hybrid seeds were separated from the pulp by continuous washing in tap water. Washed seeds were transferred to a beaker containing water for 15 min for embryo rescue procedure. Sunken seeds were used, since most of the floating seeds are having no either endosperm and/or embryo. Seed disinfection was performed under sterile conditions in a laminar flow hood. Seeds were treated with 5% sodium hypochlorite for 15 min. Before and after each treatment, the seeds were rinsed with sterile distilled water 2–3 times. Finally, the seeds were transferred to a sterile Petri plate and used for embryo extraction. Embryos were extracted in a chamber under laminar flow. A longitudinal fissure was made in each seed and the whitish, mushroom-shaped embryo was removed. The excised embryos were cultured in medium consisting of Murashige and Skoog[9] salts with addition of 2 mg L^{-1} of 6-benzyl adenine (BA) and the pH was adjusted to 5.8 before auto-claving at 121°C for 20 min. The embryo cultures were kept on dark until shoots were growing. The shoots were then transferred to a media containing a proliferation medium for multiplication. The shoots were then transferred to a rooting medium (MS supplemented with sucrose 30 g L^{-1} and solidified with 7 g L^{-1} with a photoperiod of 16 h and a temperature of 25±2°C during their growth phase).

Acclimatization and cultivation of the hybrids: The acclimation and cultivation of the hybrids were conducted similar to those of the autotetraploid plantlets and plants, as mentioned above.

Identification and verification of the hybrids: The hybrids were analyzed for their ploidy by flow cytometry as described above. The confirmation of hybrids was conducted based on DNA profile of ISSR analysis. DNA extraction protocol of Delaporta *et al.*[14] with modification[15] and DNA amplification using ISSR marker protocol[16,17] were employed for the ISSR analysis. Hybrid identification and cultivation were conducted from January, 2016-June, 2018.

Statistical analysis: Quantitative data of the experiment on evaluation of morphological characters was analyzed for their means and standard deviation on five individuals for three replicates of a completely random design. The data were combined for two cycles of plant reproduction.

RESULTS

Ploidy identification: In this study, flow cytometry was performed on regenerated plants to give an estimation of nuclear DNA content. Data in Fig. 1 showed the result of

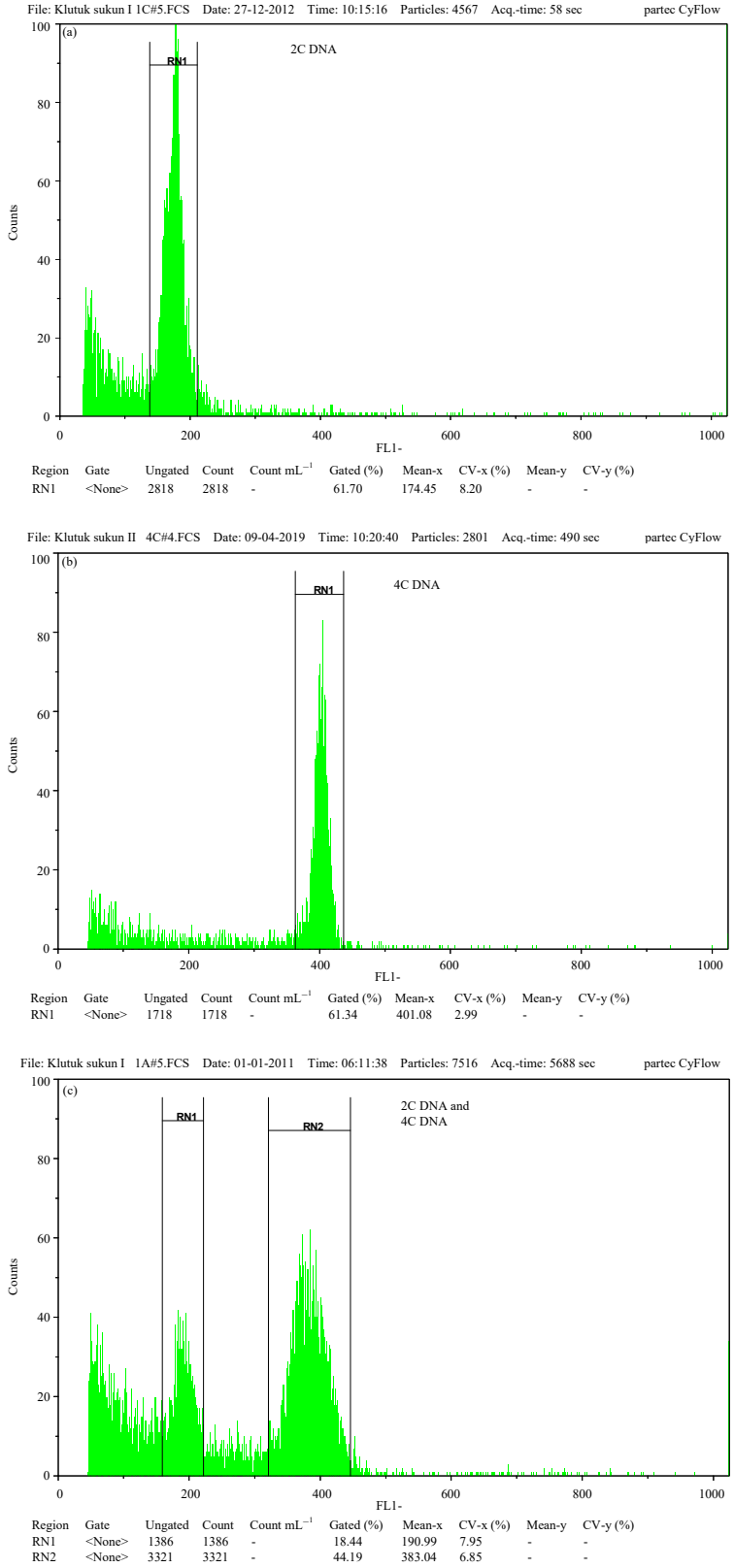

Fig. 1(a-c): (a) Histogram of diploid control of "Pisang Klutuk Sukun", (b) Histogram of autotetraploid "Pisang Klutuk Sukun" and (c) Histogram of mixoploid "Pisang Klutuk Sukun"

flow cytometry measurement with three types of histograms. Control diploid "Pisang Klutuk Sukun" containing 2C DNA showed peak at channel 200 (Fig. 1a), autotetraploid "Pisang Klutuk Sukun" containing 4C DNA showed peak at channel 400 (Fig. 1b) and mixoploid "Pisang Klutuk Sukun" containing 2C and 4C DNA showed peak at channel 200 and 400 (Fig. 1c).

Morphological characteristics of autotetraploid "Pisang Klutuk Sukun": The 52 morphological characteristics of the autotetraploid and diploid "Pisang Klutuk Sukun" were presented in Table 1. Five morphological characters (numbers of suckers above ground, plant growth habit, compactness of bunch, fruit width and shape of fruit apex) of the autotetraploids were different than those

Table 1: Morphology characters of autotetraploid "Pisang Klutuk Sukun"

Characters	Autotetraploid "Pisang Klutuk Sukun"	Diploid "Pisang Klutuk Sukun"
Ploidy	Tetraploid (BBBB)	Diploid (BB)
Rhizome: number suckers above ground	Medium: 8±3	Many: 14.0±4
Pseudostem: length (m)	Long: 4.76±0.31	Long: 5.95±0.6
Pseudostem: diameter (cm)	Large: 24.60±2.36	Large: 22.3±3.0
Pseudostem: overlapping of leaf sheaths	Medium	Medium
Pseudostem: tapering	Medium	Medium
Pseudostem: color	Dark green RHS 141D	Dark pink RHS 61D
Pseudostem: anthocyanin coloration	Very strong	Medium
Pseudostem: color of inner side of basal sheath	Light yellow green RHS 2D	Light yellow green RHS 2C
Plant: compactness of crown	Medium	Medium
Plant: growth habit	Drooping	Upright
Petiole: attitude wings at base	Curved outwards	Curved outwards
Petiole: length (cm)	Short 50±3.5 (<50 cm)	Short: 44±4.45 (<50 cm)
Leaf blade: color of midrib on lower side	Yellow green (RHS 149D)	Light yellow green RHS 2D
Leaf blade: shape of base	Both sides acute	Both sides acute
Leaf blade: waxiness on lower side	Very weak	Very weak
Leaf blade: length (cm)	234±4.62	220±3.80
Leaf blade: width (cm)	61.5±7.5	Narrow: 56±5.6
Leaf blade: ratio length/width	3.8	4.06
Leaf blade: glossiness at upper side	Present	Present
Peduncle: length (cm)	Short: 30±2.0	Short: 25±1.4
Peduncle: diameter (cm)	Small: 4.10±0.9 (<6 cm)	Small: 2.8±0.8 (<6 cm)
Peduncle: pubescence	Present	Present
Peduncle: curvature	Weak	Weak
Bunch: length (cm)	Short : 35.0±4.6	Short : 23.0±2.0
Bunch: diameter (cm)	Narrow: 35±3.7	Narrow: 30±4.0
Bunch: shape	Irregular	Irregular
Bunch: attitude of fruits	Horizontal to slightly turned up	Horizontal to slightly turned up
Bunch: compactness	Compact	Medium
Bunch: number of hands	Medium: 5.0±0.7	Medium: 5.0±0.8
Rachis: attitude of male parts	Vertical	Vertical
Rachis: prominence of scars	Strong	Strong
Rachis: persistence of bracts	Absent	Absent
Rachis: persistence of hermaphrodite flowers	Absent	Absent
Fruit: curvature	Straight	Straight
Fruit: longitudinal ridges	Absent	Absent
Fruit: length (cm)	Short: 12.5±0.5 (<15 cm)	Short : 10.17±0.35 (<15cm)
Fruit: width (excluding ridges) (cm)	2.84±0.11	2.57±0.06
Fruit: length of pedicel (mm)	Short : 10±0.9 (<15 mm)	Short: 12±0.6
Fruit: shape of apex	Rounded	Truncate
Fruit: thickness of peel (mm)	Thin: 1.7±0.2	Thin: 1.0±0.1
Fruit: color of peel before maturity	Light green RHS 140D	Light green RHS 140D
Fruit: color of peel	Light yellow (RHS 15D)	Light yellow (RHS 20A)
Fruit: adherence of peel	Strong	Strong
Fruit: persistence of floral organs	Present	Present
Fruit: color of flesh	Dark orange yellow RHS 21B	Dark orange yellow RHS 21B
Fruit: firmness of flesh	Firm	Firm
Male inflorescence: persistence	Present	Present
Male inflorescence: shape	Narrow ovate	Narrow ovate
Male inflorescence: opening of bracts	Closed	Closed
Bract: color of inner side	Dark pink red RHS 52A	Orange red RHS 41B
Bract: shape of apex	Emarginate	Emarginate

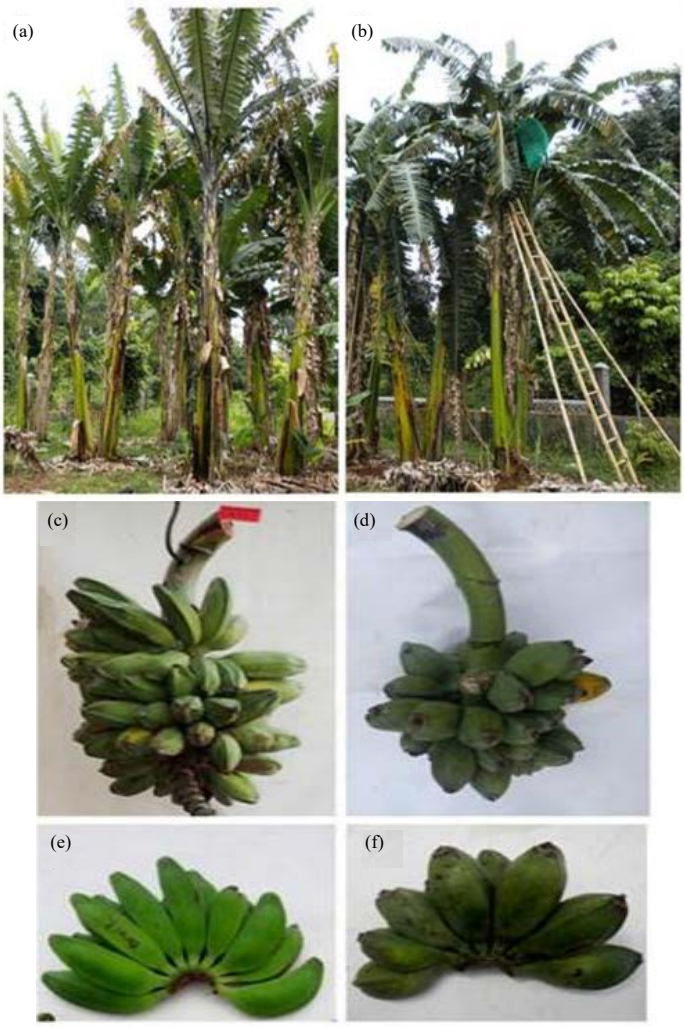

Fig. 2(a-f): (a) Diploid "Pisang Klutuk Sukun" with upright leaves, (b) Autotetraploid "Pisang Klutuk Sukun" with drooping leaves, (c) Bunch of Diploid "Pisang Klutuk Sukun", (d) Bunch of autotetraploid "Pisang Klutuk Sukun", (e) Fruit of Diploid Diploid "Pisang Klutuk Sukun" and (f) Fruit of autotetraploid "Pisang Klutuk Sukun"

of the diploids. The number of suckers above ground of the autotetraploid was fewer than that of the diploid.

The plant growth habit of the diploid showed upright leaves (Table 1, Fig. 2a), while the autotetraploid exhibited drooping leaves (Table 1, Fig. 2b). The fruit bunch of the diploid were medium (Table 1, Fig. 2c) while the autotetraploids had a compacted fruit bunch (Table 1, Fig. 2d). The fruit width of the autotetraploid was bigger than that of the diploid (Table 1, Fig. 2e, f). The diploid had a truncated fruit apex (Table 1, Fig. 2e), while the autotetraploid has a rounded fruit apex (Fig. 2f).

Crossing for evaluation of the reproductive potential of the autotetraploid bananas: In order to evaluate the reproductive potential of the autotetraploid, autotetraploid "Pisang Klutuk Sukun" were crossed with diploid 'Pisang Rejang' to produce triploid hybrids. In this research, from 106 seeds, only 42 seeds had embryos. Twelve out of 20 seedlings were triploids (Table 2).

Flow cytometry was performed on the two parents (i.e., the autotetraploid "Pisang Klutuk Sukun" as a female parent and diploid "Pisang Rejang" as male parent) and the hybrids to give an estimation of nuclear DNA content. Results in Fig. 3 showed the flow cytometry measurement with three types of histograms. The autotetraploid "Pisang Klutuk Sukun" containing 4C DNA showed peak at channel 400 (Fig. 3a), diploid "Pisang Rejang" containing 2C DNA showed peak at channel 200 (Fig. 3b) and triploid hybrid containing 3C DNA showed peak at channel 300 (Fig. 3c).

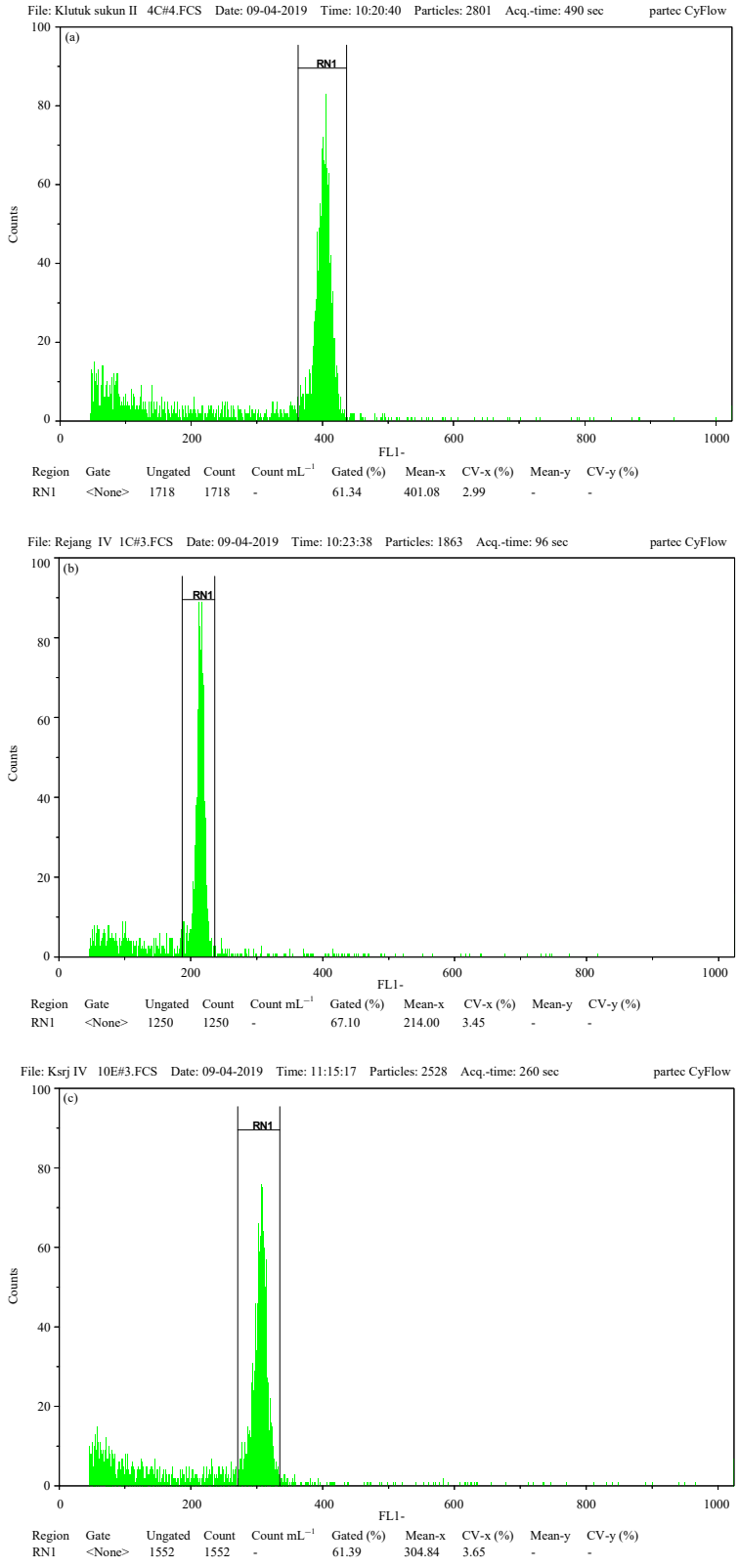

Fig. 3(a-c): (a) Histogram of autotetraploid "Pisang Klutuk Sukun" (female parent), (b) Histogram of diploid "Pisang Rejang" (male parent) and (c) Histogram of triploid hybrid (Autotetraploid "Pisang Klutuk Sukun" x diploid "Pisang Rejang")

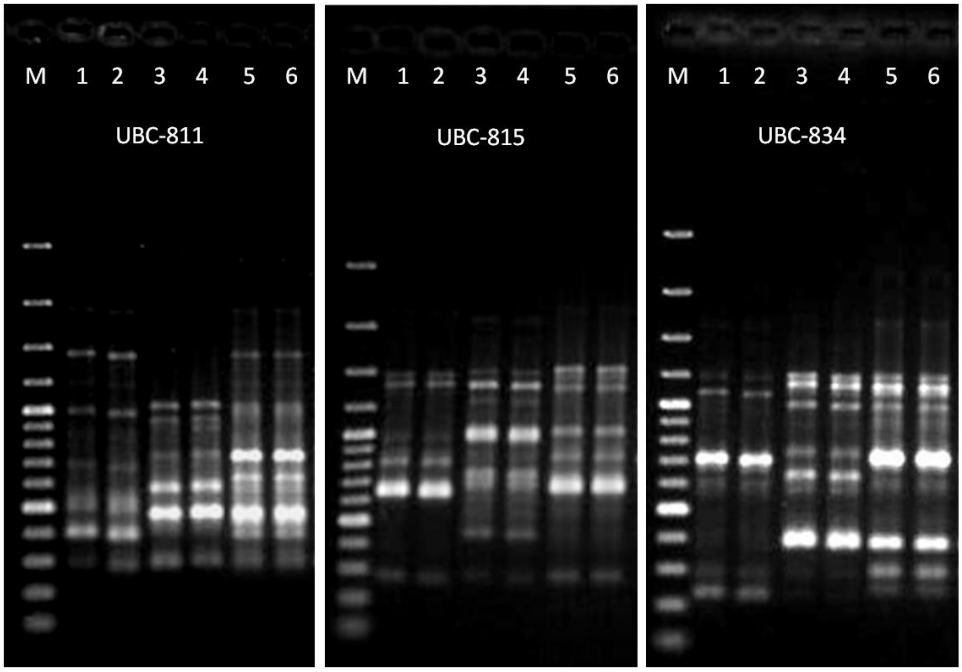

Fig. 4: DNA profile of banana hybrids and their parents (autotetraploid "Pisang Klutuk Sukun" and diploid "Pisang Rejang")
M: 100 pb plus DNA ladder (Fermentas), 1, 2: Female parent (Autotetraploid "Pisang Klutuk Sukun"), 3, 4: Male parent (Diploid "Pisang Rejang"), 5, 6: Hybrids

Table 2: Production of secondary triploid hybrid from autotetaploid "Pisang Klutuk Sukun" x diploid "Pisang Rejang" crosses

Cross combination						Ploidy levels	
Female parent	Male parent	No. of flowers pollinated	No. of seeds	No. of embryos	No. of seedlings	Diploids	Triploids
"Pisang Klutuk Sukun" 4x	"Pisang Rejang" 2x	17	106	42	20	4	12
"Pisang Rejang" 2x	"Pisang Klutuk Sukun" 4x	84	24	15	2	2	0

The hybrids were confirmed by DNA profiling using ISSR marker i.e., UBC-811, UBC-815 and UBC-834. Hybrids were identified by the presence of bands of the both parents using three different primers (Fig. 4).

DISCUSSION

In this study, ploidy estimation was conducted by using flow cytometry method. The method was suitable for observing large numbers of samples and for identifying the mixoploids. The ploidy estimation was usually conducted by chromosome counting[18]. However, this technique was not recommended because the chromosome size of *Musa* was very small, time consuming and mixoploid was difficult to identified[10]. Furthermore, this method cannot be used to establish ploidy of non-dividing cells in differentiated tissues, such as leaves. Flow cytometry is used as the method of choice for large-scale ploidy screening in *Musa* spp. because flow cytometry provides rapidity, convenience and accuracy[11].

In this study, the mixoploids were eliminated by sub-culturing until six times, however, the mixoploids were still existed. Mixoploids with different ploidy levels in different plant cells or organs were observed in chromosome[5,6] doubling experiment[2,12,19-21]. Mixoploids may arise because antimitotic agents may not reach all of the meristems on a plant (or those that are actively dividing)[12]. The ultimate goal was to develop protocols for regeneration of solid autotetraploid plants from mixoploid plants. Roux *et al.*[21] showed that the type of propagation system has an effect on cytochimera dissociation specially when used at an early stage of clonal propagation of mixoploids. Marcotrigiano[22] suggested that by destroying the main shoot after one or two weeks post-subculture, a higher proliferation rate could be accomplished and dissociation of chimeras would be more effective.

In this study, all tetraploids were maintained in the field for almost 3 years for evaluation of polyploidy stability; so far, no major change has been seen on the morphological level. The autotetraploid had fewer suckers, drooping leaves

and a rounded fruit apex. Similar results were also observed by other researchers[2,4-6]. Tetraploidy was found to affect fruit size and shape of "Pisang Lilin"[2] as well as Mas Jambe"[6]. Autotetraploid plants had bigger bunch size compared to diploid plants of "Pisang Lilin"[2] and "Kluai Leb Mu Nang" and "Kluai Sa"[5].

In this study, the autotetraploids produced flowers and could be crossed with diploid and generating triploid hybrids. Bakry *et al*.[4] showed similar results. Induction of banana tetraploid plants is a step to obtain sterile triploid genotypes resulting from a cross between a diploid and a chromosome doubled plant[4,14]. In the crosses between the autotetraploid and diploid cultivar, the autotetraploid as a male parent (pollen donor) showed fewer developed seeds. On the other hand, when the autotetraploid was used a female parent, many seeds were obtained. Therefore, triploid hybrids were successfully obtained from crossing autotetraploid x diploid, when the autotetraploid were used as female parent. Similar result was also found in the work of Oselebe *et al*.[3].

Although somatic chromosome doubling does not introduce new genetic material and produces only additional copies of existing genes and chromosomes, many genome alterations occur after mitotic polyploidization[23]. Studies showed that genetic changes often result in polyploid crops being superior to diploids with respect to morphological changes, genetic adaptability and tolerance to environmental stresses[24-29]. However, the next step will be to evaluate the autotetraploid of "Pisang Klutuk Sukun" to exploit the superiority to vigor, disease resiststance and tolerance to environmental stresses.

CONCLUSION

This study concluded that the banana autotetraploid plants of " Pisang Klutuk Sukun" *(Musa balbisiana,* BBBB) were successfully obtained by oryzalin treatment of shoot culture of diploid "Pisang Klutuk Sukun" at a concentration of 60 µM for 7 days in a liquid MS basal medium with addition of 2 mg L^{-1} BA. The autotetraploids had different morphology characters from their diploid plants, specifically in their drooping leaves, fewer suckers and larger fruit diameter. The autotetraploids were fertile and could be used to produce triploid hybrids.

SIGNIFICANCE STATEMENT

This study discovered the induction of autotetraploid of "Pisang Klutuk Sukun" using oryzalin treatment that can be beneficial for producing 2n gamete for triploid hybrid production. This study revealed that ploidy and morphology of the autotetraploid plants of "Pisang Klutuk Sukun" were stable during two cycles of reproduction. The autotetraploid plants were fertile and can be used a female parent for producing sterile triploid hybrids. This study will help the researchers to uncover the critical areas of providing fertile autotetraploids for producing sterile triploid hybrids that many researchers were not able to explore.

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REFERENCES

1. Perrier, X., E. De Langhe, M. Donohue, C. Lentfer and L. Vrydaghs *et al*., 2011. Multidisciplinary perspectives on banana (*Musa* spp.) domestication. Proc. Nat. Acad. Sci., 108: 11311-11318.
2. Do Amaral, C.M., J.D.A. dos Santos-Serejo, S.D.O. e Silva, C.A. da Silva Ledo and E.P. Amorim, 2015. Agronomic characterization of autotetraploid banana plants derived from 'Pisang Lilin' (AA) obtained through chromosome doubling. Euphytica, 202: 435-443.
3. Oselebe, H.O., A. Tenkouano and M. Pillay, 2006. Ploidy variation of *Musa* hybrids from crosses. Afr. J. Biotechnol., 5: 1048-1053.
4. Bakry, F., N.P. de la Reberdiere, S. Pichot and C. Jenny, 2007. In liquid medium colchicine treatment induces non chimerical doubled-diploids in a wide range of mono- and interspecific diploid banana clones. Fruits, 62: 3-12.
5. Kanchanapoom, K. and K. Koarapatchaikul, 2012. *In vitro* induction of tetraploid plants from callus cultures of diploid bananas (*Musa acuminata*, AA group) 'Kluai Leb Mu Nang' and 'Kluai Sa'. Euphytica, 183: 111-117.
6. Poerba, Y.S., D. Martanti, T. Handayani and Witjaksono, 2018. Induction and characterization of autotetraploid Mas Jambe Banana using oryzalin treatment. AIP Conf. Proc., Vol. 2021, No. 1. 10.1063/1.5062817.
7. Silva, S.D.O.E., M.T.S. Junior, É.J. Alves, J.R.S. Silveira and M.B. Lima, 2001. Banana breeding program at Embrapa. Crop Breed. Applied Biotechnol., 1: 399-436.

8. Davey, M.W., R. Gudimella, J.A. Harikrishna, L.W. Sin, N. Khalid and J. Keulemans, 2013. A draft *Musa balbisiana* genom sequence for molecular genetics in polyploidy, inter- and intra-specific *Musa* hybrids. BMC Genomics, Vol. 14. 10.1186/1471-2164-14-683.
9. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Planta., 15: 473-497.
10. Van Duren, M., R. Morpurgon, J. Dolezel and R. Afza, 1996. Induction and verification of autotetraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. Euphytica, 88: 25-34.
11. Doležel, J., M.A. Lysák, I.V. den Houwe, M. Dolezelová and N. Roux, 1997. Use of flow cytometry for rapid ploidy determination in *Musa* species. InfoMusa, 6: 6-9.
12. De Carvalho, J.F.R.P., C.R.D.P. de Carvalho and W.C. Otoni, 2005. *In vitro* induction of polyploidy in annatto (*Bixa orellana*). Plant Cell Tissue Organ Culture, 80: 69-75.
13. International Union for the Protection of New Varieties of Plants, 2010. Banana: Guidelines for the conduct of tests for distinctness, uniformity and satbility. UPOV., TG/123/4(proj.8). https://www.upov.int/edocs/mdocs/upov/en/tc/46/tg_123_4_proj_8.pdf
14. Dellaporta, S.L., J. Wood and J.B. Hicks, 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep., 1: 19-21.
15. Poerba, Y.S., F. Ahmad and Witjaksono, 2012. [Hybridization of wild diploid *Musa acuminate* Colla var *malaccensis* (Ridl.) nasution as pollen source with teraploid pisang madu]. J. Biol. Indones., 8: 181-196.
16. Witono, J.R., T. Konishi and K. Kondo, 2008. DNA polymorphisms analysis of *Alocasia odora* and *A. cucullata* in Ishigaki Island, Japan generated by RAPD and ISSR markers and ITS nrDNA sequence data. Chromosome Bot., 3: 11-18.
17. Poerba, Y.S. and F. Ahmad, 2013. Analisis keragaman genetik *Musa balbisiana* colla berdasarkan marka rapd dan ISSR [Genetic variation analyses of *Musa balbisiana* Colla based on RAPD and ISSR markers]. Berita Biol., 12: 259-267.
18. Osuji, J., B.E. Okoli and R. Ortiz, 1996. An improved procedure for mitotic studies of the *Eumusa* section of the genus *Musa* L. (Musaceae). Infomusa, 5: 12-14.
19. Roux, N.S., J. Doležel and F.J. Zapata-Arias, 1999. Cytochimera Dissociation Through Shoot-Tip Culture of Mixoploid Bananas. In: Plant Biotechnology and *In vitro* Biology in the 21st Century, Altman, A., M. Ziv and S. Izhar (Eds.)., Springer, Dordrecht, ISBN 978-94-010-5966-4, pp: 255-258.
20. Pio, L.A.S., M. Pasqual, S.O. Silva, H.S. Rocha, H.M. Magalhaes, J.A. Santos-Serejo and H.S. Rocha, 2014. Inducing and identifying artificially-induced polyploidy in bananas. Afr. J. Biotechnol., 13: 3748-3758.
21. Roux, N., J. Dolezel, R. Swennen and F.J. Zapata-Arias, 2001. Effectiveness of three micropropagation techniques to dissociate cytochimeras in *Musa* spp. Plant Cell Tissue Organ Cult., 66: 189-197.
22. Marcotrigiano, M., 1997. Chimeras and variegation: Patterns of deceit. HortScience, 32: 773-784.
23. Ranney, T.G., 2006. Polyploidy: From evolution to new plant development. Combined Proc. Int. Plant Propagators Soc., 56: 137-142.
24. Osborn, T.C., J.C. Pires, J.A. Birchler, D.L. Auger and Z.J. Chen *et al*., 2003. Understanding mechanisms of novel gene expression in polyploids. Trends Genet., 19: 141-147.
25. Otto, S.P., 2007. The evolutionary consequences of polyploidy. Cell, 131: 452-462.
26. Parisod, C., R. Holderegger and C. Brochmann, 2010. Evolutionary consequences of autopolyploidy. New Phytol., 186: 5-17.
27. Marfil, C.F., P.F. Duarte and R.W. Masuelli, 2018. Phenotypic and epigenetic variation induced in newly synthesized allopolyploids and autopolyploids of potato. Scient. Hortic., 234: 101-109.
28. Soltis, P.S. and D.E. Soltis, 2000. The role of genetic and genomic attributes in the success of polyploids. Proc. Natl. Acad. Sci. USA., 97: 7051-7057.
29. Li, W.D., X. Hu, J.K. Liu, G.M. Jiang, O. Li and D. Xing, 2011. Chromosome doubling can increase heat tolerance in *Lonicera japonica* as indicated by chlorophyll fluorescence imaging. Biol Plant, Vol. 55. 10.1007/s10535-011-0039-1.