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Full Paper

RAPD marker analysis of genetic variation in Kluai Nam Wa (*Musa* ABB group) banana plants regenerated after 8 and 9 subcultures

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Abstract: Genetic variation is often found in plantlets propagated from *in vitro* cultures. In this study striped laminas were observed on Kluai Nam Wa (*Musa* ABB group) banana plantlets propagated in a temporary immersion bioreactor (TIB) after the eighth and ninth subcultures. For phenotypic confirmation, banana plants with altered morphology were further observed by transplanting in the greenhouse and observing new leaves generated thereafter. Twenty-one random amplified polymorphic DNA (RAPD) primers were used to screen the regenerated bananas for genetic variation. Six random primers were selected for confirming genetic stability between subcultures. Out of the 6 primers used, two revealed polymorphism, showing distinctly different patterns of striped laminas. OPA-14 primer presented a polymorphic pattern in one band (25%) in the eighth subculture plants and in three bands (50%) in the ninth subculture plants. OPD-07 primer showed polymorphism (16.7%) with striped laminas in both the eighth and ninth subcultures. The presence of specific bands in the regenerated bananas of different subcultures indicated that somaclonal variation in the regenerated plants increased with the number of subcultures. Morphological studies showed that the mean height of bananas with striped laminas was lower than that of normal plants when grown under field conditions for 6 months. The appearance of the striped phenotype of regenerated Kluai Nam Wa banana remained stable. Under these conditions, a maximum of 7 subcultures is recommended on account of possible somaclonal variation after 8 subcultures.

Keywords: banana, Kluai Nam Wa, striped laminas, temporary immersion bioreactor, subcultures, RAPD, somaclonal variation

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INTRODUCTION

Bananas (*Musa* sp.) are economically important tropical fruits consumed worldwide. They provide high energy and essential nutrients including vitamins, minerals (especially potassium), oligosaccharides, and phenolics with antioxidant activities [1-3]. In Thailand there are many cultivars of banana and Kluai Nam Wa (*Musa* ABB group) is one of the most popular and is locally consumed and widely cultivated [4]. Besides serving as a tasty and nutritious fruit, Kluai Nam Wa pseudo stems and leaves can be used for other purposes such as traditional food packaging and animal feed [5]. Demand for this banana has increased with the recognition of its benefits, hence the need for plantation expansion [3].

The propagation of banana using shoot apex suckers is a technique widely used throughout the world. This approach, however, has limitations in the number of suckers available and associated disease problems [5-7]. However, by using highly efficient propagation methods such as that employing temporary immersion bioreactors (TIB) [8, 9], commercial plant tissue culture laboratories have begun to supply large numbers of disease-free banana plantlets for plantation establishments [5, 10, 11]. To satisfy growers, the plants produced using *in vitro* propagation should maintain genetic fidelity to the parental plants and grow true to type. The *in vitro* multiplication of plants which leads to variation in the resulting plantlets, a phenomenon known as somaclonal variation, however, has been reported on numerous occasions. This may be associated with factors such as the use of plant growth regulators and/or the time that explants are maintained in culture [12-17].

Somaclonal variation has been reported in many crop species regenerated from plant tissue culture, including: rice [18-20], potato [21], sugarcane [22], rye [23] and banana [24-27]. While this may lead to undesirable variations in offspring and result in serious problems in commercial plant multiplication [28], some phenotypes caused by somaclonal variation have desirable characteristics and have been used for developing new commercial varieties [28-30]. The analysis of tissue-culture-derived plants for somaclonal variation is, therefore, a prerequisite for quality control [15, 28]. Detection of somaclonal variants derived from plant tissue culture is primarily based on the differences in morphology when compared with parental plants [29]. This approach requires specific expertise and frequently considerable time as some mutant characteristics can only be observed in different growth stages, e.g. at maturity [28].

Molecular markers, on the other hand, can also be used to detect somaclonal variation at the DNA level [31]. This is rapid and requires less knowledge of the original growth patterns and methods of production of the parental material. Random amplified polymorphic DNA (RAPD) marker analysis is a powerful technique for detecting somaclonal variation [23]. This approach to screening genetic differences offers the advantages of being simple, less expensive and rapid [15]. Furthermore, RAPD markers are useful for genetic identification of different banana varieties. The examination of somaclonal variation in bananas using RAPD has produced variable results. Thirty RAPD primers were used to study somaclonal variation in the banana (*M. acuminate* L.) cultivar 'Valery' regenerated at different subcultures [31]. Eighteen out of the thirty primers produced 51.40% polymorphic bands. The presence and loss of specific bands in the regenerated plants of different subcultures compared with the parental plants indicated somaclonal variation and suggested that more genetic variation might occur with continued culture. RAPD and morphological investigation were also used to screen for somaclonal variants in tissue-culture-derived banana plants cultivar 'Grand Naine' [25]. RAPD was carried out to study the differences between true-to-

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type plants and 23 variants using 17 arbitrary primers and to analyse the relationships between the variant bananas compared with the normal plant [25]. Recently, morphological investigation and RAPD were also used to monitor somaclonal variations of the banana cultivar 'Ney Poovan (AB)' during micropropagation [27]. For molecular analysis, 6 primers were used and two gave polymorphic banded patterns. The results obtained using RAPD showed recordable monomorphic and polymorphic banded patterns: 94.10% monomorphism and 5.90 % polymorphism. These results indicate that RAPD markers can be effectively used to detect somaclonal variation in several commercial banana varieties. However, no somaclonal variation of Kluai Nam Wa banana (*Musa* ABB group) propagated in TIB seems to have been reported. The present study aims to use RAPD markers, together with morphological and plant growth characteristics, to detect somaclonal variation in Kluai Nam Wa plants produced in a TIB system.

MATERIALS AND METHODS

Plant Material and Growth

Kluai Nam Wa suckers were collected from Maejo University, Chiang Mai, and the suckers were proliferated at the Plant Tissue Culture Laboratory. The outer leaves were removed and shoot tips inside were used as initial explants. They were cultured in solid Murashige and Skoog (MS) medium [32] supplemented with 0.25 mg/L thidiazuron (TDZ) (Sigma, USA). After one month, multiple shoots were separated into individual shoots and multiplied in liquid MS medium containing 0.125 mg/L TDZ in twin-flask temporary immersion bioreactors (TIB) [33]. The explants were immersed for 5 min. every 3 hr and subculturing was carried out at 4-week intervals. When the 8th and 9th subcultured plantlets showed striped laminas, the plantlets, along with normal (non-striped) cultures, were separated into individual shoots and transferred to solid MS medium without plant growth regulators for one month. The plantlets were then transferred to a greenhouse under natural daylight with regular watering in order to be examined for morphological characteristics and growth. After acclimatisation in the greenhouse for 4 weeks, morphological identification was recorded 2 months after transplanting. Four traits: plant height (cm), leaf width (cm) leaf length (cm) and leaf number were measured.

DNA Extraction

Genomic DNA was extracted with cetyltrimethylammonium bromide extraction buffer according to Doyle and Doyle [34] with modification. One gram of the leaf of Kluai Nam Wa plants was sampled after they had been grown in the greenhouse for one month. Each leaf sample was ground with liquid nitrogen and mixed with 800 µL of the pre-warmed extraction buffer with the addition of 1% polyvinylpyrrolidone and vigorously homogenised and kept in a water bath at 60^{o}C for 60 min. The extract was gently mixed every 10 min. and then centrifuged at 14,000 rpm for 10 min. at 4°C. The supernatant was mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) solution and extracted with chloroform twice. The aqueous layer was separated and an equal volume of ice-cold absolute ethanol was added and the mixture was kept at -20^{o}C overnight. The DNA in the mixture was pelleted by centrifugation and allowed to air-dry before being re-suspended in 40 µL of diethyl pyrocarbonate-treated water (Life Science Products, USA). RNase A (Bio Basic, Canada) treatment was given to the DNA mixture by incubating the mixture with a final concentration of 1 mg/mL of RNase A at 37°C for 3 hr. The isolated DNA sample was quantified by an absorbance plate reader with a cuvette port (BMG Labtech, Germany). The DNA was then

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separated on 0.8% agarose gel (Invitrogen, USA) using ethidium bromide and photographed with a Gel Documentation System (GelMax@ Imager, Germany).

Primer Screening and RAPD Analysis

A total of 21 arbitrary primers (Macrogen, Korea) were used for primer screening of the striped lamina plants compared with normal plants. Primers that showed good amplification were deemed useful for polymorphic analysis. The polymerase chain reaction (PCR) mixture contained 20 ng of genomic DNA, 10 µM primer and 25 µL Quick Taq HS dye mix (Toyobo, Japan). The PCR was performed at 94ºC for 2 min. followed by 39 cycles of 94ºC for 30 sec., 36ºC for 30 sec., and 68ºC for 1 min. Sterile distilled water was used as a negative control in place of DNA template. The PCR products were separated on 2% agarose gel (Invitrogen, USA). After electrophoresis, the gel was stained with ethidium bromide and photographed with a Gel Documentation System (GelMax@ Imager, Germany).

Data Analysis

The data on plant height (cm), leaf number, leaf width (cm) and leaf length (cm) were analysed by ANOVA followed by Duncan's Multiple Range Test (0.05) (SPSS version 16.0).

RESULTS AND DISCUSSION

Detection of Somaclonal Variation by RAPD

Some *in vitro* Kluai Nam Wa plantlets, mass-propagated in a TIB using TDZ-supplemented MS media, showed white or yellow stripes on the lamina after the eighth and ninth subcultures (Figure1A). The plantlets with altered leaf morphology and the normal plants (non-striped) were further observed for phenotypic confirmation by transferring to the greenhouse. New leaves generated thereafter were found to retain the striped appearance (Figures 1B, 1C). To assess the genetic variation at the eighth and ninth subcultures of the regenerated bananas using RAPD markers, 21 arbitrary primers were screened for production of polymorphic DNA bands in the variant plants compared with normal plants. Six primers which showed clear amplification were selected for more detailed analysis. Out of the 6 primers used, 2 primers, OPA-14 and OPD-07 (Table 1), revealed polymorphism, showing distinctly different band patterns in the bananas with striped laminas at the eighth and ninth subcultures when compared with the normal plants (Figure 2). One specific band (25%) of the OPA-14 primer appeared in the striped-lamina plantlets in the eighth subculture while three bands (50%) with the same primer appeared in the ninth subcultured plants and was absent in normal plants. OPD-07 primer showed polymorphic band patterns (16.7%) when tested in plantlets with striped laminas from both the eighth and ninth subcultures. Among the primers used, OPD-02 produced the highest number of bands (13) while OPA-14 produced the lowest (4) (Figure 2, Table 1). RAPD analysis showed clear differences between the DNA patterns of the bananas with striped-laminas and non-striped ones. These results correlated with the leaf morphology of the plants in the greenhouse. This was especially apparent in striped-lamina plants from the ninth subculture, which differed in 3 bands or 50% (Figures 1C, 2). These findings confirm that the striped-lamina variants observed in the eighth and ninth subcultures have undergone somaclonal variation, which tends to be more prevalent with repeated subculturing.

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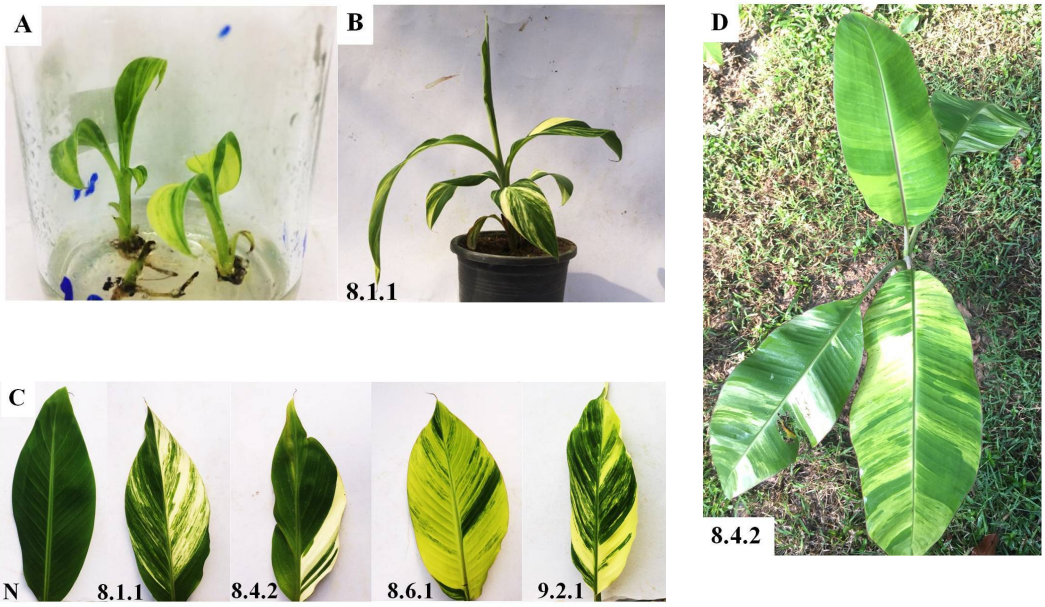

Figure 1. Striped laminas of Kluai Nam Wa banana: (A) plantlets in MS medium (hormone-free); (B) a plant after two months in the greenhouse; (C) laminas observed in the 8th and 9th subcultures of regenerated plants; (D) a plant grown under natural conditions (6-months old).

Abbreviations: N - normal banana leaf; Coding x.y.z – x = passage no., y = initial sprout no., z = final sprout no. For example, 8.1.1 refers to banana sprout no. 1 isolated from initial sprout no. 1 in the 8th subculture.

Table 1. Total and polymorphic band numbers generated by six RAPD primers from plants regenerated after 8th (S8) and 9th (S9) subcultures of Kluai Nam Wa banana

Primer	Primer sequence	Total band no.		Monomorphic band no.		Polymorphic band no.		% Polymorphism	
		S8	S9	S8	S9	S8	S9	S8	S9
OPA-14	CTCGTGCTGG	4	6	3	3	1	3	25.0	50.0
OPD-02	GGACCCAACC	13	13	13	13	0	0	0	0
OPD-07	TTGGCACGGG	6	6	5	5	1	1	16.7	16.7
OPD-20	ACCCGGTCAC	11	11	11	11	0	0	0	0
OPK-08	GTGACATGCC	10	10	10	10	0	0	0	0
OPM-16	GTAACCAGCC	7	7	7	7	0	0	0	0

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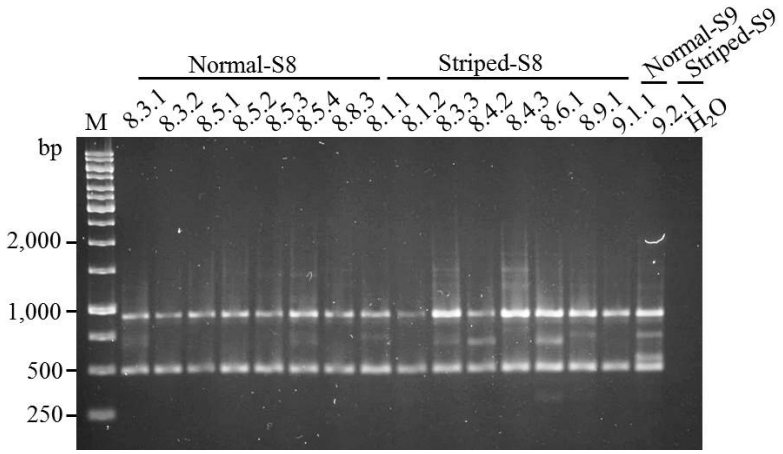

Figure 2. RAPD profiles of regenerated Kluai Nam Wa banana from 8th and 9th subcultures obtained using OPA-14 primer: M = 1 kb DNA ladder; Normal-S8 and Normal-S9 = normal plants regenerated after 8th and 9th subcultures respectively; Striped-S8 and Striped-S9 = striped lamina plants regenerated after 8th and 9th subcultures respectively; H_2O = negative control. (For number codes, see Figure 1.)

Somaclonal variation is generally not desirable in the plant tissue culture industry because it affects the quality of the planting material. The variation can be difficult to detect and eliminate in the early stages of *in vitro* propagation [28]. Obvious morphological changes such as variegated leaves or colour changes may be detectable *in vitro*. However, most somaclonal variations, such as the traits that affect plant growth, are not observable until the explants are removed from culture and grown in the field; even then, they may not be detected [17].Normally, it is the goal in plant tissue culture to multiply new plantlets repeatedly through many successive subcultures to obtain large numbers of plants. However, as the number of subcultures increases, so does the likelihood that variant plants will appear [28]. It was reported that the percentage of somaclonal variation increased with the number of subcultures in micropropagated shoots of the Brazilian banana cultivar 'Nanicão' (*Musa* spp., AAA group) [13]. No variants were produced after three subcultures, but somaclonal variants appeared from the fifth subculture. So it is important to screen plants propagated through tissue culture to ensure the genetic stability of subclones derived from the parental plants**.**

In Kluai Nam Wa banana the RAPD marker analysis showed that variants with striped-laminas that appeared in the eighth and ninth subcultures were indeed examples of somaclonal variation. Similarly, somaclonal variation in regenerated banana plants of the cultivar 'Valery' from the first, third, fifth, seventh and ninth subcultures as detected by RAPD markers has been reported [31]. RAPD markers were also used for detection of somaclonal variation in 12 successive subculture levels of micropropagated 'Cavendish Basrai' banana [15]. Minor morphological variations were observed in the leaves of some regenerated plants. These were found to be typical of the parent plants up until the eighth subculture, after which the number of somaclonal variants increased. This observation was also associated with the decreased multiplication rate of the propagules.

Nowadays Kluai Nam Wa banana is micropropagated in large numbers to meet the grower's demands. The present study examined the genetic uniformity of regenerated Kluai Nam Wa plantlets that exhibited the striped lamina phenotype in different subcultures. In the RAPD marker

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analysis, the presence of specific bands in samples prepared from striped laminas from the eighth and ninth subcultures indicated that somaclonal variation could occur in this variety and that such variation increased with the increasing number of subcultures (data not shown)**.** Therefore, it was suggested that the number of subcultures of Kluai Nam Wa banana should be limited to 7 subcultures. In order to ensure genetic fidelity, the number of cycles should not exceed 8 subcultures and the propagators should start with new suckers of known genetic conformity when they require mass production using tissue culture.

TDZ is used to induce rapid shoot multiplication in the commercial production of Kluai Nam Wa banana and the use of high concentrations of TDZ could be a contributing factor leading to somaclonal variation. It has been reported that when somaclones of banana cultivars 'Berangan Intan', 'Berangan' and 'Rastali' were regenerated using higher concentrations of both the cytokinin 6-benzylaminopurin and TDZ, they accumulated more genetic variation [35]. Somaclonal variation was also found in banana cultivar 'Ney Poovan' that was micropropagated using high concentrations of TDZ [27]. By contrast, it was established that tissue culture conditions did not induce somaclonal variation in the banana cultivar 'Nanjanagudu Rasabale' (AAB) despite the use of high levels of cytokinins (N6-benzyladenine and kinetin) [36]. The prevalence of somaclonal variation in banana is probably dependent partly on the cultivar [26] and the type of cytokinin used to induce multiplication.

The striped lamina trait may not be desirable for commercial banana production, partly because it may be associated with lower photosynthetic capacity. However, leaf variegation is an important trait in ornamental plants [37] and more than one third of the commercially produced ornamental foliage plants are valued for their variegated leaves [38]. When Kluai Nam Wa plants with striped laminas were grown under natural conditions (for more than 6 months), the striped lamina phenotype remained. This observation suggests that the striped-lamina phenotype likely stems from genetic change, not environmental factors such as stress generated from the culture conditions or a transient phenotype only observed in the juvenile growth stage. Thus, this Kluai Nam Wa banana with striped laminas has the potential for being developed into an ornamental plant.

Growth Characteristics

It was also important to know if the genetic variation brought about other morphological changes besides leaf variegation**.** When grown in the greenhouse, Kluai Nam Wa bananas with striped laminas from both the eighth and ninth subcultures had different heights compared to the normal plants: they grew significantly shorter than the normal bananas. There was also a difference in growth between the eighth and ninth subcultured plants. The latter was significantly shorter (Table 2). However, the number of leaves, their width and length showed no significant difference when compared with the normal plants. When the striped lamina bananas were grown under field conditions for six months, the phenotype still remained (Figure 1D). The smaller green area on the striped leaves represents a lower chlorophyll content, likely resulting in a lower photosynthetic capacity and slower growth.

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Table 2. Growth characteristics of Kluai Nam Wa banana plants regenerated from tissue culture at two months under greenhouse conditions

Plant Morphology	Plant height (cm)	Leaf number	Leaf width (cm)	Leaf length (cm)
Normal plant	31.2 ± 0.76^{a}	3.7 ± 1.15^{a}	7.2 ± 0.15^{a}	17.5 ± 0.81^{a}
Striped laminas-S8	28.3 ± 0.58^{b}	3.0 ± 1.00^{a}	7.0 ± 1.64^{a}	18.7 ± 1.57^{a}
Striped laminas-S9	17.5 ± 1.80^{c}	3.3 ± 1.15^{a}	7.2 ± 0.70^{a}	16.9 ± 2.35^{a}

Notes: Striped laminas-S8 and striped laminas-S9 = 8^{th} and 9^{th} subcultures of regenerated Kluai Nam Wa respectively. Standard errors were calculated from sample sizes of n = 3. Means in a column followed by the same letter are not significantly different from each other ($P < 0.05$) according to Duncan's Multiple Range Test.

It was found that the use of cytokinin in the culture media and the duration of tissue culture in the micropropagation by shoot tips of banana cultivars from the Cavendish subgroup resulted in genetic defects that gave rise to leaf variegation [39]. Variegated leaves were also found in the micropropagated banana plant of 'Grand Naine' cultivar, and tests showed its lower chlorophyll content compared to that of the normal leaves [39]. It is well known that the plant hormone cytokinin regulates chloroplast development including chlorophyll biosynthesis [40, 41]. This may explain why the amount of cytokinin in the tissue culture medium can affect the amount of chlorophyll in the regenerated plantlets. Variegated patterns on the leaf surface in the form of white and green streaks as well as dark and green patches on the leaves of 'Cavendish Basrai' banana *in vitro* were also reported; the variation was observed after 9 or more subcultures [15]. In the study of 'Grand Naine' cultivar, however, the variegated phenotype showed no change in size and leaf development [35]. Our results are different in that the regenerated Kluai Nam Wa bananas with striped laminas from the eighth and ninth subcultures (Figure 1C, Table 1) give rise to plants with shorter heights, which is favourable for most ornamental varieties.

CONCLUSIONS

Somaclonal variation in Kluai Nam Wa (*Musa* ABB group) banana plantlets, produced by TIB using TDZ supplemented medium, showed stripes on the laminas after the eighth and ninth subcultures. Therefore, the number of subcultures of this banana should be limited to a maximum of 7 subcultures for commercial plant propagation and the micropropagated plants should be screened in the nursery for early detection of variants. Since the appearance of the striped-lamina phenotype in the regenerated plantlets is stable, this variegated form has the potential for being developed into an ornamental plant.

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