BIODIVERSITAS
Volume 24, Number 9, September 2023
Pages: 4628-4634

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240902

The effect of plant growth regulators on micropropagation of *Melientha suavis* Pierre. and assessment of genetic fidelity of regenerants based on iPBS and SRAP markers

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Manuscript received: 22 July 2023. Revision accepted: 1 September 2023.

Abstract. *Siringam T, Vanijajiva O. 2023. The effect of plant growth regulators on micropropagation of* Melientha suavis *Pierre. and assessment of genetic fidelity of regenerants based on iPBS and SRAP markers. Biodiversitas 24: 4628-4634. Melientha suavis* Pierre, a significant deciduous edible plant species with high nutritional value and belonging to the Opiliaceae family, holds paramount importance for both agricultural and conservation purposes in its native Southeast Asia. However, the species faces challenges due to habitat fragmentation and negative anthropogenic impacts, leading to a decline in its population. This study aimed to explore the effects of various concentrations of plant growth regulators (cytokinin and auxin) on the shoot and root development of *M. suavis*, and to assess the genetic stability of micropropagated plants obtained from nodal explants. In vitro shoot regeneration and proliferation were conducted on Murashige and Skoog (MS) semi-solid medium, supplemented with 6-benzylaminopurine (BAP) or kinetin (Kn) at different doses. The optimal shoot length, shoot number, and leaf volume were observed in the modified MS medium with 1.0 mg/L BAP after an 8-week incubation period. Efforts to enhance the rooting of micropropagated shoots included the addition of auxins, such as α-naphthalene acetic acid (NAA) or indole-3-butyric acid (IBA). However, the species exhibited recalcitrant behavior during the reproduction and rooting stages, as the rooting percentage did not correlate with the increase in auxin concentration. Interestingly, the highest root number and length were achieved in the MS medium without plant growth regulators after 8 weeks of incubation. To ensure genetic fidelity, regenerants were subjected to inter-primer binding site (iPBS) and sequence-related amplified polymorphism (SRAP) marker analysis. The results revealed no genetic variation between the micropropagated plants and the mother trees, confirming the production of genetically stable progeny. Overall, this protocol presents a promising alternative for profitable propagation and establishment of genetically constant progeny of *M. suavis*, with important implications for sustainable applications and germplasm conservation in Southeast Asia. Addressing the challenges faced by this valuable species through micropropagation and genetic fidelity assessment can contribute significantly to its preservation and utilization for agricultural and conservation initiatives.

Keywords: Genetic fidelity**,** iPBS, *Melientha suavis*, micropropagation, SRAP

INTRODUCTION

Melientha suavis Pierre, an edible deciduous plant in the Opiliaceae family, is endemic to Southeast Asia, primarily found on limestone mountains with scattered growth (Le et al. 2018). However, habitat fragmentation and human-induced destruction have led to a decline in its population. In Thailand, *M. suavis* is locally known as "Pak Wan Pa" and its young shoots and leaves are popular culinary vegetables, available in markets from June to July at a high price, providing substantial earnings for agriculturalists (Premjet et al. 2020). The plant's appeal lies in its delectable young leaves and stems, frequently used in various local dishes (Yotapakdee et al. 2015). Nutritionally, *M. suavis* is rich in bioactive compounds, including alkaloids and flavonoids, predominantly found in its leaves and stems (Ruttanaphan et al. 2022). Unfortunately, current harvesting practices are unsustainable, as communities gather the species from the wild and engage in harmful practices like burning woodlands. Attempts at cultivation through young seedlings have had limited success (Yotapakdee et al. 2015; Premjet et al. 2020). Therefore, developing an effective and reproducible propagation method is crucial to meet the market demand for *M. suavis* and ensure its long-term conservation.

Micropropagation has become a highly effective technique for large-scale propagation and conservation of important plant species (Majumder and Rahman 2019; Tejavathi and Padma 2020). This method has been linked to the sustainable utilization of plant resources (Poeaim et al. 2022; Yoosumran et al. 2022). Plant micropropagation allows regenerating entire plants from different organs like buds, leaves, shoots, anthers, and roots due to the totipotent nature of plant cells. Node culture is one such method where axillary shoot buds from live or dormant shoots are cultured on artificial nutrient media to develop new plants. This technique has been successfully applied to produce numerous plant species (Aroonpong and Chang 2015; Cheruvathur et al. 2015; Li et al. 2015;) and offers advantages like disease-free and pest-resistant plants, uniformity in characteristics, propagation of difficult-to-propagate species, and obtaining new cultivars through somaclonal variation. However, ensuring genetic stability of in vitro regenerated plantlets is crucial, and molecular

techniques are valuable for assessing the genetic fidelity (Rohela et al. 2020; Chirumamilla et al. 2021).

Among the various molecular markers, nuclear DNA-based markers such as iPBS (inter primer binding site) and SRAP (Sequence-Related Amplified Polymorphism) have demonstrated successful application in evaluating the genomic stability of regenerated in vitro plants. These molecular markers offer rapid, cost-effective, highly discriminating, and reliable methods for assessing genetic homogeneity (Çetin 2018; Bychappa et al. 2019; Borsai et al. 2020; Akgur and Aasim 2022). Consequently, genetic homogeneity analyses are essential, particularly when considering commercial applications of micropropagated plants. Ensuring the genetic stability of propagated plants becomes paramount to guarantee their suitability and value in profitable applications.

The utilization of plant growth regulators is crucial for successful plant regeneration during micropropagation, with auxins and cytokinins playing pivotal roles in shoot organogenesis. Research studies have extensively investigated their application in tissue culture. Cytokinins such as 6-benzylaminopurine (BAP) and kinetin (Kn) stimulate both axillary and adventitious shoot formation from meristematic explants in various plant species but higher BAP concentrations impede adventitious meristem elongation and conversion into complete plants. Auxins, including α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA), have been reported to promote in vitro plant rooting (Ngomuo et al. 2013; Rahman et al. 2018; Poeaim et al. 2022; Saleem et al. 2022). However, investigations on the effective implementation of plant growth regulators in *M. suavis* have been limited, necessitating more extensive inquiries and trials to develop robust propagation and expansion protocols for this species (Premjet et al. 2020). Determining the ideal concentration of cytokinins and auxins in the tissue culture medium, tailored to the specific cultivar, becomes imperative for the efficient in vitro propagation of *M. suavis*.

This study aimed to investigate the micropropagation responses of *M. suavis* under the influence of different plant growth regulators. Notably, we present, for the first time, findings on the successful implementation of nodal segment culture and the utilization of iPBS and SRAP markers to assess genetic fidelity in *M. suavis*. The establishment of this regeneration system holds promise for the enhancement of multiplication potential and subsequent experiments involving genetic transformation in *M. suavis.*

MATERIALS AND METHODS

Plant material and preparation of explants

The plant material of *M. suavis* was collected from the Khao Phluang area in the Chaibadan district of Lopburi province, Thailand, comprising approximately 10 mother plants. A comprehensive assembly of living specimens was gathered, and voucher specimens of all *M. suavis* samples were deposited at Chaibadanpiphat College, Phranakhon Rajabhat University, establishing a valuable repository for future scholarly reference.

To initiate the tissue culture process, the collected plant material underwent initial washing with running tap water. Subsequently, nodal segments of *M. suavis*, measuring 3.00±0.50 cm in length, were excised and subjected to a 15-minute water rinse. The next step involved immersing the nodal explants in 70% (v/v) ethanol for 1 minute, followed by a sterilization process. This sterilization process began with a treatment involving 10% (v/v) NaOCl and two drops of Tween 20, followed by gentle agitation at 200 rpm for 10 minutes. Subsequently, the nodes underwent sterilization with 5% (v/v) NaOCl, gently agitated at 200 rpm for 5 minutes. Thorough washing with sterilized water was performed, and this washing process was repeated five times.

The excised nodal explants, now measuring 1.50±0.50 cm in length, were cultured on MS (Murashige and Skoog) medium supplemented with 30 g/L sucrose and 4.0 g/L Gellan gum (Kelcogel® CP Kelco A HUBER COMPANY, USA). Before autoclaving, the medium's pH was adjusted to 5.6±0.2. The nodal segments were then incubated under a light regime of 16 hours per day, followed by a dark period of 8 hours, at a constant temperature of 25°C ± 1°C, for a duration of 8 weeks.

In vitro shoot multiplication

The disinfected nodal segments of *M. suavis* were excised to a length of 1.50±0.50 cm and subsequently placed on a modified MS medium supplemented with various concentrations of BAP and Kn (0.5, 1.0, or 2.0 mg/L), as well as a control group without any plant growth regulators. The culture medium also contained 30 g/L sucrose and 4.0 g/L Gellan gum (Kelcogel® CP Kelco A HUBER COMPANY, USA), and its pH was adjusted to 5.6±0.2 prior to autoclaving. The nodal explants were subjected to a light regime of 16 hours per day, followed by a dark period of 8 hours, at a constant temperature of 25°C ± 1°C, over an incubation period of 8 weeks. Following the incubation period, measurements were taken to assess shoot length, shoot number, and leaf number.

In vitro rooting

The shoot explants of *M. suavis* derived from the previous experiment were excised to a standardized length of 1.50±0.50 cm and placed on a modified MS medium supplemented with different concentrations of NAA and IBA (0.5, 1.0, or 2.0 mg/L), along with a control group without any plant growth regulators. The culture medium also contained 30 g/L sucrose and 4.0 g/L Gellan gum (Kelcogel® CP Kelco A HUBER COMPANY, USA), and its pH was adjusted to 5.6±0.2 before undergoing autoclaving. The shoot explants were subjected to a light regime of 16 hours per day, followed by a dark period of 8 hours, at a constant temperature of 25°C ± 1°C, during an 8-week incubation period. Subsequent to the incubation period, evaluations were conducted to assess the root number and root length resulting from the 8-week culture.

Establishment of plants in soil

Following in vitro rooting, the plantlets were meticulously washed with distilled unsterile water to eliminate any

remnants of the culture medium adhering to the roots. Subsequently, these plantlets were transplanted into plastic pots filled with a mixture of soil and covered with plastic bags to facilitate acclimatization over a duration of 4 weeks. Following this acclimatization period, the plantlets were transplanted into garden pots comprising a mixture of soil, sand, and compost in a ratio of 1: 1: 1. The *ex vitro* cultivation of these plantlets took place within a greenhouse environment, where illumination was reduced by 75% for a period of 90 days, and a twice-daily irrigation regime was employed to maintain appropriate moisture levels.

DNA isolation and assessment of genetic stability using iPBS and SRAP markers

To assess the genetic fidelity of the regenerated *M. suavis* plantlets, genomic DNA was extracted from 500 mg of fresh leaf tissue of the mother plant and 20 randomly selected acclimated plantlets using a modified cetyl trimethyl ammonium bromide (CTAB) method (Vanijajiva 2020). The quality of the isolated DNA was verified using a Nanodrop Spectrophotometer (Thermo Scientific Nanodrop 1000, USA) by measuring the absorbance ratio at 260 and 280 nm, aiming for an optimal range of 1.7-1.8, indicating clean DNA preparation. Additionally, the quality of DNA fragments was assessed through 0.8% agarose gel electrophoresis in 1X TAE buffer. The extracted DNA was stored at -20°C until utilized as templates for PCR amplification.

To evaluate genetic stability, two molecular marker techniques, inter primer binding site (iPBS) (Kalendar et al. 2010) and sequence-related amplified polymorphism (SRAP) (Li and Quiros 2001) were employed in this study. For the iPBS analysis, a set of 27 primers were utilized, all of which yielded scorable amplification patterns (Table 1). As for the SRAP analysis, 30 primers were initially screened, and 27 primer combinations were identified as reproducible, generating unambiguous amplification profiles for all samples (Table 1).

For the iPBS analysis, PCR amplifications were conducted using a Thermohybaid Px2 (Roche Molecular Systems, Inc., USA), following the method of Vanijajiva and Pornpongrungrueng (2020). Each 20 µL reaction mixture comprised PCR buffer (Promega; 20 mM Tris-HCl (pH 8.4), 50 mM KCl), 2 mM $MgCl_2$, 0.24 mM of each deoxyribonucleotide triphosphate, 0.5 units of Taq polymerase (Promega), and 0.8 µm of primer. Each reaction contained 50 ng of template DNA. The thermocycler was programmed as follows: an initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at a temperature ranging from 50-65°C (depending on the primer) for 1 minute, and a final extension step at 72°C for 5 minutes.

For the SRAP analysis, PCR reactions were performed using a total volume of 20 µL, consisting of PCR buffer (Promega; 20 mM Tris-HCl (pH 8.4), 50 mM KCl), 2 mM $MgCl_2$, 0.24 mM of each deoxyribonucleotide triphosphate, 0.5 units of Taq polymerase (Promega), and 0.6 µm of each primer. The SRAP amplification conditions involved an initial denaturation at 94°C for 5 minutes, followed by 5 cycles of denaturation at 94°C for 1 minute, primer annealing at 35°C for 1 minute, and extension at 72°C for 2 minutes. Subsequently, 30 additional cycles were performed with an annealing temperature of 50°C, and a final extension step at 72°C for 8 minutes.

Each PCR amplification was repeated twice for each iPBS and SRAP primer combination to ensure the reproducibility of the results. The amplified products for both methods were separated through agarose gel electrophoresis (1.8% w/v) at 150 volts for 30 minutes in 0.04 M TAE buffer (Tris-acetate 0.001 M-EDTA) at pH 8. The gels were stained with ethidium bromide (10 mg/mL) and visualized and photographed using a Bio-Imaging System (Syngene, Genegenuis). To determine iPBS and SRAP profiles, the size of each DNA band was estimated by comparing them with a 100 bp DNA ladder (Promega) used as a molecular weight marker (M). Polymorphisms at all loci were confirmed by conducting three repeating tests for each primer at different times.

Statistical analysis

The experimental design employed in this study followed a completely randomized design (CRD) with five replications for each treatment. The obtained data were subjected to statistical analysis using one-way analysis of variance (ANOVA), and significant differences between means were determined using Duncan's multiple range test ($P \leq 0.05$) with the aid of SPSS software (IBM SPSS Statistics).

RESULTS AND DISCUSSION

In vitro shoot multiplication

The findings of the current study elucidated the impacts of cytokinins on shoot formation and multiplication in *M. suavis*. The effects of cytokinins on shoot development regeneration were investigated by culturing nodal segments on MS medium supplemented with varying concentrations of BAP and Kn (0.0, 0.5, 1, or 2 mg/L), and the results are summarized in Table 2. After an 8-week culture period, the nodal segments cultured on MS medium supplemented with 1.0 mg/L BAP exhibited the greatest shoot length (2.42±0.20 cm), shoot number (5.60±0.40 shoots/explant), and leaf number (12.80±1.20 leaves/explant). This initial response of explants to shoot formation, facilitated by the addition of cytokinin, is mediated by an increase in cytosolic calcium concentration, which is promoted by its high uptake from the culture medium. This in turn influences the cytoskeleton and regulates exocytosis.

Consistent with previous studies by Kodad et al. (2021) and Majumder and Rahman (2019), who observed that 1 mg/L BAP was the most efficient concentration for in vitro shoot proliferation in various plant cultivars, our study also found that concentrations higher than 1 mg/L did not lead to further enhancement in shoot production. In fact, concentrations of 2 mg/L BAP resulted in fewer shoots compared to concentrations of 0.5 mg/L and 1 mg/L. Higher cytokinin concentrations tend to have adverse effects on the multiplication rate and morphology of the culture. Our study indicated that increasing the concentration of BAP for this particular variety of *M. suavis* improved

fresh weight and shoot formation, with the addition of 1 mg/L BAP in the growth medium yielding the best results (Figure 1). Thus, it appears that 1 mg/L is the optimal concentration for this variety.

It should be noted that in vitro buds proliferation of the plant is reported to be cultivar-dependent, and the rate of shoot multiplication is influenced by both the cytokinin concentration and the genotype of the plant species, as mentioned by Arab et al. (2014) and Gogoi et al. (2017). However, in the present study, the shoot length and leaf number did not exhibit significant differences when comparing the nodal segments cultured on MS medium supplemented with 1.0 mg/L Kn, which resulted in shoot lengths of 2.38±0.30 cm and 11.20±0.73 leaves/explant, respectively.

Overall, the shoot length, shoot number, and leaf number were enhanced when the nodal segments were subjected to concentrations of 0.5-1.0 mg/L BAP or Kn in the MS medium, whereas MS medium supplemented with 2.0 mg/L BAP or Kn tended to hinder shoot development. These results suggest that BAP was more effective than Kn in promoting shoot development.

In vitro rooting

The successful establishment of a micropropagation protocol relies significantly on the in vitro rhizogenesis of microshoots, wherein the production of high-quality adventitious roots is of utmost importance. Auxins, well-recognized for their role in rooting, play a vital role in promoting adventitious root formation in most plant species (Aslam et al. 2013; Wei et al. 2019; Kulus 2020). In the case of many woody species, auxins have been identified as effective inducers of adventitious root development. Moreover, they play a crucial role in modulating lateral root formation in various woody perennials, necessitating higher concentrations of auxin for the induction of adventitious rooting. To ascertain the optimal conditions for adventitious root induction in *M. suavis*, different types and concentrations of auxin supplements (IBA and NAA) were tested on MS media.

Table 1. The features of inter primer binding site (iPBS) (Kalendar et al. 2010) and sequence-related amplified polymorphism (SRAP) primers (Li and Quiros 2001) used in the present study

iPBS primers			
Primer	**Sequence (5'-3')**	**Primer**	**Sequence (5'-3')**
2076	GCTCCGATGCCA	2271	GGCTCGGATGCCA
2077	CTCACGATGCCA	2273	GCTCATCATGCCA
2079	AGGTGGGCGCCA	2295	AGAACGGCTCTGATACCA
2083	CTTCTAGCGCCA	2374	CCCAGCAAACCG
2095	GCTCGGATACC	2378	GGTCCTCATCCA
2220	ACTTGGCTCATGATGCCA	2383	GCATGGCCTCCA
2222	ACTTGGATGCCGATACCA	2385	CCATTGGGTCCA
2224	GGAAGGCTCTGATTACCA	2389	ACATCCTTCCCA
2232	AGAGAGGCTCGGATACCA	2394	GAGCCTAGGCCA
2237	CCCCTACCTGGCGTGCCA	2395	TCCCCAGCGGAGTCGCCA
2242	GCCCCATGGTGGGCGCCA	2399	AAACTGGCAACGGCGCCA
2253	TCGAGGCTCTAGATACCA	2400	CCCCTCCTTCTAGCGCCA
2256	GACCTAGCTCTAATACCA	2415	CATCGTAGGTGGGCGCCA
2270	ACCTGGCGTGCCA		
SRAP primers			
Forward primer	**Sequence (5'-3')**	**Reverse primer**	**Sequence (5'-3')**
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGAATTTGC
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTTGA
Me5	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTAAC
		Em6	GACTGCGTACGAATTGCA

Table 2. Effect of BAP and Kn concentrations on shoot length (cm), shoot number (shoots) and leaf number (leaves) from nodes of *Melientha suavis* Pierre. cultured on modified MS medium for 8 weeks

Plant growth regulators (mg/L)	**Shoot length (cm)**	**Shoot number (shoots/explant)**	**Leaf number (leaves/explant)**
0 (control)	1.26±0.02 bc[1/]	1.20±0.20 f	6.40±0.24 c
BAP 0.5	1.60±0.06 b	4.00±0.10 c	9.00±0.32 b
BAP 1.0	2.42±0.20 a	5.60±0.40 a	12.80±1.20 a
BAP 2.0	1.42±0.02 b	2.60±0.24 d	4.40±0.24 d
Kn 0.5	1.56±0.09 b	3.00±0.15 d	7.80±0.66 bc
Kn 1.0	2.38±0.30 a	4.80±0.20 b	11.20±0.73 a
Kn 2.0	0.92±0.18 c	2.00±0.10 e	3.20±0.37 d
ANOVA	*	*	*

Note: *Significant difference at $P \leq 0.05$. [1/]Mean±S.E. (n=5) within column followed by the same letter was not significant difference as determined by Duncan's multiple range test

In this study, healthy and uniform-sized individual shoots from proliferated cultures were selected and subjected to rooting media supplemented with different types and concentrations of auxins (IBA and NAA) ranging from 0.0 to 2.0 mg/L. However, it was observed that an increase in auxin concentration led to a decrease in all rooting parameters (Table 4). Additionally, no significant difference was noted between MS media supplemented with IBA and NAA concerning the number of roots produced per explant. The results demonstrated that the highest root number and root length, reaching 3.80±0.58 roots/explant and 1.96±0.19 cm, respectively, were achieved when cultured on MS medium without NAA or IBA. These findings suggest that higher auxin concentrations inhibit root elongation while promoting adventitious root formation.

These observations align with previous studies in plant micropropagation that have reported IBA and NAA as suitable for root induction and optimal root length at low auxin concentration levels (Aroonpong and Chang 2015; Nazir et al. 2022; Hejazi et al. 2023).

Establishment of plants in soil

The percentage survival of the plantlets was 75% after transfer to garden pot survived. The regenerated plants did not show any detectable variation in morphology when compared with the mother plant (Figure 1).

DNA isolation and assessment genetic stability using iPBS and SRAP markers

Micropropagation plays a pivotal role in plant biotechnology due to its potential for regenerating selected plants. However, as the commercialization of micropropagation systems increases, there is a higher risk of encountering somaclonal variations among the sub-clones derived from the selected parental line (Rohela et al. 2020). Therefore, it becomes imperative to regularly screen for genetic variations among in vitro-raised plantlets to ensure the generation of progeny that closely resemble the parent plant. Plants derived from organized meristems are less susceptible to genetic modifications, as these organized meristems exhibit resistance to genetic changes (Borsai et al. 2020). In the present study, iPBS and SRAP profiles were conducted on twenty randomly selected in vitro regenerated *M. suavis* plants and compared with the mother plant to assess genetic stability.

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Table 4. Effect of NAA and IBA concentrations on root number (roots) and root length (cm) from shoots of *Melientha suavis* Pierre. cultured on modified MS medium for 8 weeks

Plant growth regulators (mg/L)	Root number (roots/explant)	Root length (cm)
0 (control)	3.80±0.58 a	1.96±0.19 a
NAA 0.5	1.80±0.37 bc[1/]	1.06±0.09 b
NAA 1.0	0.80±0.37 cd	0.92±0.17 b
NAA 2.0	0.00±0.00 d	0.00±0.00 c
IBA 0.5	2.60±0.40 b	1.02±0.10 b
IBA 1.0	2.00±0.32 b	0.88±0.08 b
IBA 2.0	0.00±0.00 d	0.00±0.00 c
ANOVA	*	*

Note: *Significant difference at $P \leq 0.05$. [1/]Mean±S.E. (n=5) within column followed by the same letter was not significant difference as determined by Duncan's multiple range test

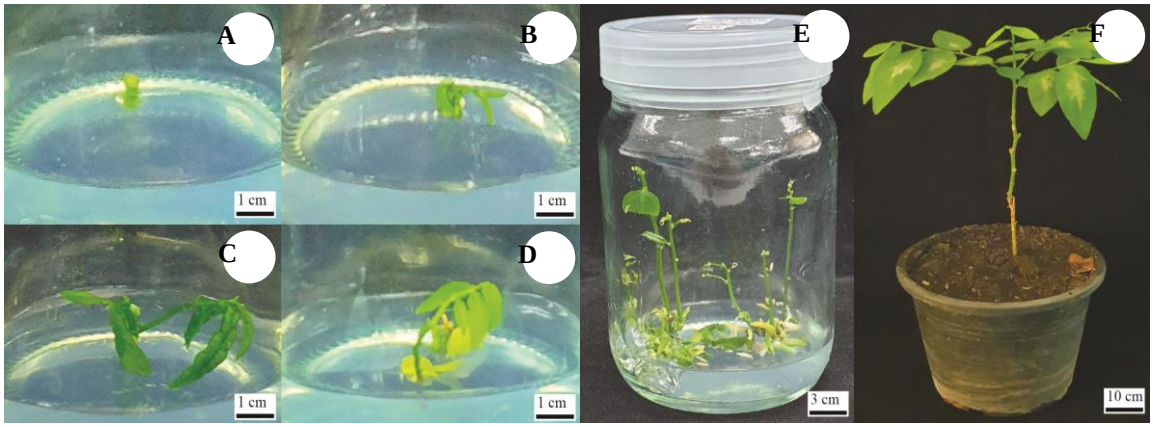

Figure 1. In vitro plant regeneration from nodal explant in *Melientha suavis*. A. Disinfected explants. B. Shoots regenerated from nodal segment cultured on MS medium for 4 weeks. C. Shoots regenerated from nodal segment cultured on MS medium for 8 weeks. D. Shoots regenerated from nodal segment cultured on MS medium without cytokinins for 8 weeks. E. Shoots and root regenerated from nodal segment cultured on MS medium for 8 weeks. F. Plantlets from to the greenhouse for 90 days

The extraction of high-quality DNA from *M. suavis* is of particular interest due to the presence of abundant polyphenolics in its tissues. An efficient and high-throughput DNA extraction procedure is essential in this context. The occurrence of polyphenols, known as potent oxidizing agents present in many tropical plant species, can substantially reduce the yield and purity of DNA by covalently binding to it, rendering it unsuitable for various research applications (Chirumamilla et al. 2021). To address this challenge, we improved the extraction of high-quality DNA by incorporating a re-extraction step using the CTAB DNA isolation procedure and phenol: chloroform: isoamyl alcohol extraction, instead of using chloroform: isoamyl alcohol extraction alone (Vanijajiva 2020). This modification effectively removed the polyphenolics associated with the DNA, leading to satisfactory iPBS and SRAP electrophoretograms for all the samples.

The DNA extracted from *M. suavis* foliage using the modified method exhibited good and satisfactory quality, suitable for PCR reactions. The minor modification in the extraction method resulted in strong and reliable amplification products, with DNA quantities ranging from 100 to 125 µg per milligram of fresh weight leaf material. The ratios of A260/A280 ranged from 1.81 to 1.98, indicating the integrity and purity of the extracted DNA. Furthermore, the quality of the DNA was confirmed through PCR, affirming its suitability for further molecular analyses.

During the optimization of the iPBS and SRAP protocols for *M. suavis* samples, various parameters were experimented with, including the concentration of dNTPs, magnesium chloride, enzyme, primer, and template DNA. Among these factors, the concentration of template DNA and magnesium chloride exhibited the most significant influence on banding patterns and reproducibility. Based on the results obtained, an optimal template DNA concentration of 50 ng and a magnesium chloride concentration of 2.5 mM were found to be suitable for subsequent PCR analyses.

The iPBS and SRAP techniques have proven to be successful tools employed by numerous researchers for confirming genetic uniformity and identifying somaclonal variants in tissue culture-raised plants (Borsai et al. 2020; Akgur and Aasim 2022). In the present study, iPBS and SRAP analyses were conducted, and no genetic variability was detected, indicating the suitability of the current protocol in producing true-to-type plants. This finding aligns with previous research that reported no variation between mother and micropropagated plants using iPBS and SRAP markers (Çetin 2018; Bychappa et al. 2019; Borsai et al. 2020; Akgur and Aasim 2022).

Initially, 27 iPBS primers were tested, and all 27 primers produced unambiguous and clear amplification patterns (Vanijajiva and Pornpongrungrueng 2020). These 27 iPBS primers yielded a total of 262 scorable bands, with an average of 9.70±2.26 bands per primer (Figure 2). For SRAP analysis, 30 SRAP primer combinations were tested, of which 27 primer combinations produced explicit and certain amplification patterns (Kaewpongumpai et al. 2016). These 27 SRAP primer pairs generated a total of 129 scorable bands, with an average of 4.3±2.00 bands per primer pair (Figure 2). Thus, the iPBS and SRAP markers demonstrate technical efficiency, simplicity, cost-effectiveness, reliability, high discriminative power, and the ability to work with small and relatively crude amounts of DNA without the need for prior information about the genome.

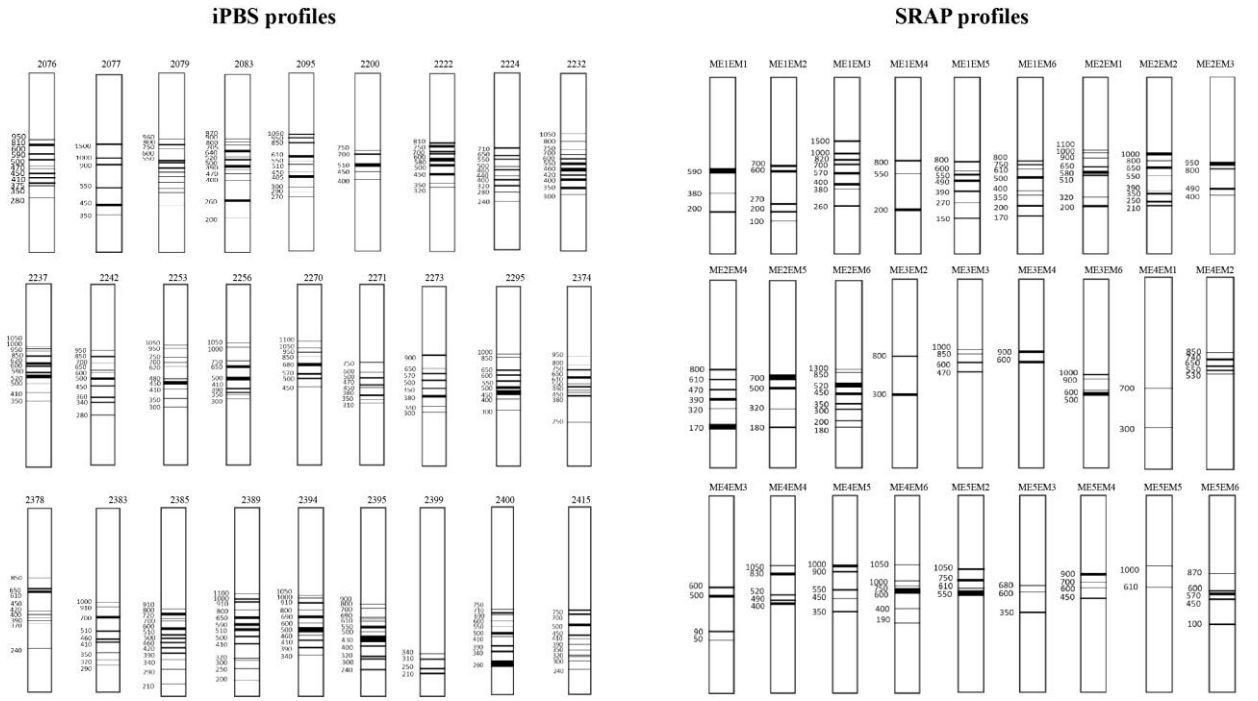

Figure 2. The iPBS and SRAP profiles in this study, depicted in the electrophoretograms, are clear and stable, with no genetic variation between micropropagated plants and mother trees, confirming genetically stable progeny production

In conclusion, the protocol developed in this study, along with the employed techniques, enhances the potential for consistent and sustainable in vitro preservation of nodal segment-derived clones, characterized by the absence of DNA modifications or variations. The established in vitro system sheds light on the crucial role of nodes in maintaining their regeneration frequency and morphogenetic competence. Furthermore, the developed protocol offers a promising alternative for large-scale multiplication and successful establishment of genetically stable plants, making it a valuable candidate for agricultural exploitation upon future release.

ACKNOWLEDGEMENTS

The research was supported by the Fundamental Fund (65A140000005) of Phranakhon Rajabhat University, Thailand. The author expresses sincere gratitude to all the participants who provided the plant materials and their support throughout the study.

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