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In Vitro Regeneration and ISSR-Based Genetic Fidelity Analysis of *Orthosiphon stamineus* Benth

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Abstract: *Orthosiphon stamineus* has been widely used as traditional remedy for various illnesses and diseases, such as cardiovascular diseases and epileptic seizures. In this study, direct regeneration through nodal segment of this species was attempted using Kinetin (6-Furfurylaminopurine) and IAA (indole-3-acetic acid). Optimum regeneration media was identified as MS media supplemented with 2.0 mg L^{-1} Kin plus 0.5 mg L^{-1} IAA. This yielded the highest number of shoots (5.57 ± 0.42) and leaves (20.53 ± 1.91) per explant. Acclimatization of the resulting in vitro regenerants was successful in all potting mixtures tested. However, potting mixture PF (1:1:1 ratio of black soil/red soil/compost) was identified as the best medium for acclimatization of this species, as it yielded 100% survival percentage after 90 days of acclimatization. Ten in vitro regenerants of *O. stamineus* were randomly collected after the third subculture and subjected to genetic variation analysis using inter-simple sequence repeat (ISSR) markers. Out of 20 ISSR markers tested, 10 working primers were observed to produce satisfactory amplification of bands, with an average of 7.11 bands per primer. A total of 610 bands were produced by the 10 primers. The percentage of polymorphism was observed to be very low, yielding only 7.32% polymorphism among all samples. Jaccard dissimilarity analysis was also conducted and very low genetic distance (about 0.1) was found among the in vitro regenerants and between the regenerants with the mother plant, thus ascertaining the clonal nature of the plantlets produced in this study.

Keywords: *Orthosiphon stamineus*; micropropagation; genetic fidelity; clonal; genetic distance; ISSR

1. Introduction

Orthosiphon stamineus, commonly known as Misai Kucing, has been widely used as a traditional remedy for various illnesses. This species can be found throughout Southeast Asia and tropical Australia. The leaves have also been introduced to Europe and Japan as a health tea, known as Java tea. The flowers resemble cat whiskers, with long wispy-shaped stamen [1]. This species has been reported to improve general health effectively, as well as to be a known remedy for kidney diseases, bladder inflammation, gout, and diabetes [2]. Other than that, *O. stamineus* is also used to treat rheumatism, tonsillitis, and menstrual disorder [3–5]. The leaves were found to possess diuretic [6,7] and anti-hypertensive activities [3,8]. Several bioactive compounds that are present in this plant, such as sterols, terpenoids, and polyphenols, have contributed to its therapeutic effects. Bioactive polyphenols in *O. stamineus* have the ability to protect the human body from oxidative stress associated with many diseases such as cancer [8,9], cardiovascular diseases, and aging [10]. A recent study has

shown that the methanolic extract of *O. stamineus* leaves also possess hepatoprotective properties [11]. Java tea extracts are also used as food or feed additives to protect the intestine from oxidative stress [12], as a novel symptomatic treatment for epileptic seizures [13], and has anti-obesity effects [14]. This species has been studied extensively for its medicinal benefits. The vast medicinal properties of this species have rendered it to be extremely important and always in high demand, thus requiring for its mass propagation.

Traditionally, breeding of this species is accomplished via vegetative propagation through mature stem, but the supply was inadequate for market demand [15,16]. To fit the demand, plant growth regulators (PGRs) are used to produce a large number of crops and seed germination [17]. For example, NAA and BAP have been widely used to induce production of shoots from various explant types of *O. stamineus* [18–21]. Moreover, both PGRs have also been reported to yield production of callus for establishment of cell suspension culture of this species [22,23]. In comparison, the use of other PGRs in tissue culture of *O. stamineus* has not been widely highlighted. Therefore, the present study aims to evaluate the efficiency of indole-3-acetic acid (IAA) and kinetin for efficient micropropagation of this species.

In vitro micropropagation is an efficient technique to generate a large supply of crops in shorter time. During in vitro culture, variability can also occur spontaneously and can be a result of temporary changes or permanent genetic changes in cells or tissues. Temporary changes result from epigenetic or physiological effects and are non-heritable and reversible. In contrast, permanent changes are heritable and often represent expression of pre-existing variation in the source plant [24]. There are a lot of factors that contribute to the occurrence of somaclonal variation phenomenon. These include the system by which the regeneration is induced, type of tissue, explant source, media components, and the duration of the culture cycle [25]. Somaclonal variation has been reported to cause regenerants to exhibit variations in terms of morphological, cytological, cytochemical, biochemical, and also at the molecular level [26]. Genetic variability is usually influenced by several factors, such as natural selection, mutation, migration, and population size, in different ways. Variation in chromosome numbers and structures, and chromosome irregularities such as breaks, acentric and centric fragments, ring chromosomes, deletions, and inversions, may result in the loss of genes or their function, the activation of genes which were previously silent, and the expression of recessive genes, once they become haploid [27,28]. There is a high risk that the regenerated plant will lose its fidelity to the parent plant due to the multicellular origin of adventitious buds used as the explant source.

In this paper, the efficiency of indole-3-acetic acid (IAA) and kinetin in aiding micropropagation of *O. stamineus* was highlighted, to complement and further add to the knowledge in tissue culture protocols of this species. The genetic fidelity of the regenerants were also assessed and compared to the mother plant using inter-simple sequence repeat (ISSR) markers, to elucidate any occurrence of somaclonal variation. Acclimatization of the plantlets to ex vitro environments was also carried out to ensure the success of the tissue culture experiments conducted.

2. Materials and Methods

2.1. Preparation of Growth Media and Plant Material

In this study, MS [29] media was prepared using 4.4 g L^{-1} Murashige and Skoog (MS) powder added with 30 g L^{-1} sucrose as carbon source and 2 g L^{-1} Gelrite Gellan Gum (Duchefa Biochemie B.V, 2003 Netherlands) as the gelling agent. Then, 0.5–2.0 mg L^{-1} indole-3-acetic acid (IAA) and Kinetin (Kin) were supplemented to the media, either singly or at different combinations. The pH of the media was adjusted to 5.7–5.8 using 0.1M NaOH or 0.1M HCl before being autoclaved at 121 °C for 20 min.

Explants were excised from the nodal segment of a field-grown *O. stamineus* mother plant. Surface sterilization was conducted by washing all samples taken from the field-grown plant under tap water for 1 h, and subsequently treated with commercial bleach, i.e., Clorox (50% and 30%), (v/v) for 1 min, then rinsed with sterile distilled water after each treatment to remove excess Clorox. Two drops of

Tween 20 were added with 50% of Clorox. Then samples were treated with 70% ethanol (v/v) for 1 min, followed by rinsing three times with sterile distilled water. Samples were blot dried on sterile tissue paper before being cultured.

2.2. Induction of Direct Regeneration

The nodal region was excised (approximately 1.0 cm in height) from the mother plant and cultured in sterile tubes containing MS media supplemented with various concentrations of IAA and Kin. All cultures were maintained in a culture room at 25 ± 1 °C for 16 h light and 8 h dark under 1000 lux (14–15 μmol m^{-2} s^{-1}) of light intensity. The morphology of the resulting regenerants (number of shoots, leaves, and roots), as well as production of callus, were observed for 4 weeks. The multiplication index was also analyzed by calculating the number of newly formed shoots per initial shoot tip recorded [30]. Experiments were conducted in triplicates of $n = 10$ and followed a randomized complete block design (RCBD). Data analysis was conducted using analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at 5% significance level.

2.3. Acclimatization

Fifteen rooted plantlets with fully expanded leaves were acclimatized on 6 different types of sterilized commercial potting mixture, which are black soil, red soil, black soil/red soil (1:1), black soil/compost (1:1), red soil/compost (1:1) and black soil/red soil/compost (1:1:1). The potting mixtures were purchased from a nearby nursery. Acclimatization was conducted at 25 ± 1 °C under 16-h photoperiod with 1000 lux (14–15 μmol m^{-2} s^{-1}) of light intensity. The plantlets were transferred to a jam jar containing the different potting mixtures, sealed with plastic wrap, and left to acclimatize for 1 month. The plastic wrap was pricked in stages (at timely interval) until the jam jar was fully exposed. Plantlets were subsequently transferred into plastic pots after 3 months. Survival data were collected after 30, 60, and 90 days, and data were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at 5% significance level.

2.4. DNA Isolation and ISSR Analysis

Ten random in vitro plantlets were harvested and used for molecular analysis. Genomic DNA was isolated using 50 mg fresh leaves of *O. stamineus* (after the third subculture) according to i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology Inc., Gyeonggi-do, South Korea). The concentration of DNA extracted was determined using a nanophotometer (IMPLEN, Munich, Germany).

Twenty ISSR primers [31] were tested in this study (Table S1 in Supplementary Material). PCR amplification was performed in a total of 20 μl reaction volume containing 1 μl of template DNA at concentration of 50 ng μl^{-1}, 0.5 μl of primer at concentration of 0.50 μM, 0.2 μl at concentration of 20 mg μl^{-1} of BSA (Fermentas, Vilnius, Lithuania), 2.0 μl of buffer (EURx, Gdańsk, Poland) at 10X concentration, 1.0 μl of dNTP's at concentration of 4.0 mM (Invitrogen, Carlsbad, California, USA), 1.0 μl of MgCl2 (EURx, Gdańsk, Poland) at a concentration of 50 mM, 2.5 unit of 0.5 μl Taq polymerase (EURx, Gdańsk, Poland). Then, 10x Pol Buffer C was used (enriched with two gel tracking dyes and a gel loading reagent). Amplification was performed using Thermocycler (Eppendorf, Hamburg, Germany) with the following conditions: Initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation step at 94 °C for 1 min, 1 min at specific annealing temperature, extension step at 72 °C for 2 min, and 1 cycle of final extension step at 72 °C for 10 min. Next, 1.5% agarose gel (1X TAE) was used to resolve the amplified product, then visualized under UV light. TrackIt™ 1Kb Plus DNA ladder (Invitrogen, Carlsbad, California, USA) was used for sizing of the DNA bands.

All data were compiled as a binary character matrix. The bands were scored as presence (1) and absence (0) for each sample through manual scoring, and triple-checked to minimize errors. Faint and ambiguous bands were excluded from the statistical analysis. Jaccard's coefficients were used to calculate the genetic dissimilarity between the samples using Darwin 5.0 software [32].

3. Results

3.1. Direct Regeneration In Vitro

Emergence of new shoots could be observed after 1 week of culture. Most samples showed direct regeneration of shoots and roots. Morphological data such as number of shoots, leaves, and roots, as well as number of explants producing callus, were collected for 4 weeks (Tables 1 and 2). No roots and callus formation were observed in control media; MS basal (Tables 1 and 2). Generally, media supplemented with combinations of Kin and IAA hormones showed better production of shoots and leaves than media supplemented with either Kin or IAA only.

Table 1. Effects of single hormone on number of shoots, multiplication index, leaves and roots per explant, and percentage (%) of explant producing callus.

MS + Hormone (mg L^{-1})	Number of Shoots per Explant	Multiplication Index	Number of Leaves per Explant	Number of Roots per Explant	Percentage (%) of Explant Producing Callus
Control	2.67 ± 0.09 a	1.67 ± 0.09 a	4.21 ± 0.32 a	NR	NR
0.5 Kin	2.90 ± 0.11 a	1.89 ± 0.11 b	8.93 ± 0.47 b	0.31 ± 0.10 a	93.33 ± 0.05 b
1.0 Kin	3.77 ± 0.16 b	2.77 ± 0.16 b	11.70 ± 0.70 c	1.03 ± 0.30 a	40.00 ± 0.09 a
1.5 Kin	4.00 ± 0.32 b	3.00 ± 0.32 b	15.10 ± 0.15 d	0.34 ± 0.12 a	90.00 ± 0.06 b
2.0 Kin	3.90 ± 0.23 b	2.90 ± 0.23 b	13.00 ± 1.09 c	0.23 ± 0.10 a	96.67 ± 0.03 b
0.5 IAA	3.63 ± 0.09 b	2.63 ± 0.09 b	9.23 ± 0.49 b	2.93 ± 0.64 b	50.00 ± 0.09 a
1.0 IAA	2.62 ± 0.13 a	1.62 ± 0.13 a	8.69 ± 0.55 b	4.00 ± 0.74 bc	80.00 ± 0.07 b
1.5 IAA	2.55 ± 0.11 a	1.55 ± 0.11 a	9.28 ± 0.65 b	3.31 ± 0.61 bc	96.67 ± 0.03 b
2.0 IAA	2.63 ± 0.12 a	1.63 ± 0.12 a	7.77 ± 0.67 b	4.60 ± 0.65 c	96.67 ± 0.03 b

Data represent mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters within the same column are significantly different at $p < 0.05$, according to Duncan's multiple range test (DMRT). Kin, Kinetin; IAA, indole-acetic acid; NR, no response.

Table 2. Effects of combination hormones on number of shoots, multiplication index, leaves and roots per explant, and percentage (%) of explant producing callus.

MS + Hormone (mg L^{-1})	Number of Shoots Per Explant	Multiplication Index	Number of Leaves per Explant	Number of Roots per Explant	Percentage (%) of Explant Producing Callus
Control	2.67 ± 0.09 a	1.67 ± 0.09 a	4.21 ± 0.32 a	NR	NR
0.5 Kin + 0.5 IAA	2.93 ± 0.14 ab	1.93 ± 0.14 ab	10.20 ± 0.65 b	0.73 ± 0.21 abcd	100.00 ± 0.00 b
0.5 Kin + 1.0 IAA	3.23 ± 0.11 abcd	2.23 ± 0.11 abcd	12.70 ± 0.51 bcde	1.60 ± 0.28 de	93.33 ± 0.05 b
0.5 Kin + 1.5 IAA	3.13 ± 0.20 abc	2.13 ± 0.19 abc	11.20 ± 0.58 b	1.07 ± 0.42 bcde	80.00 ± 0.07 a
0.5 Kin + 2.0 IAA	2.87 ± 0.14 ab	1.87 ± 0.14 ab	12.11 ± 0.84 bcd	1.93 ± 0.59 e	96.67 ± 0.03 b
1.0 Kin + 0.5 IAA	3.77 ± 0.16 ab	2.77 ± 0.16 cdefg	11.30 ± 1.01 bc	1.80 ± 0.60 cde	93.33 ± 0.05 b
1.0 Kin + 1.0 IAA	4.53 ± 0.25 cdefg	3.53 ± 0.25 g	12.03 ± 0.86 bcd	1.47 ± 0.48 de	96.67± 0.03 b
1.0 Kin + 1.5 IAA	4.10 ± 0.16 efg	3.10 ± 0.16 efg	10.52 ± 0.60 b	0.10 ± 0.07 a	100.00 ± 0.00 b
1.0 Kin + 2.0 IAA	4.43 ± 0.17 g	3.43 ± 0.18 g	11.60 ± 0.91 b	0.43 ± 0.18 abc	96.67 ± 0.03 b
1.5 Kin + 0.5 IAA	4.23 ± 0.28 fg	3.23 ± 0.28 fg	15.80 ± 0.89 ef	0.07 ± 0.05 a	70.00 ± 0.09 a
1.5 Kin + 1.0 IAA	3.93 ± 0.29 defg	2.93 ± 0.29 defg	15.53 ± 1.29 ef	0.27 ± 0.14 abc	100.00 ± 0.00 b
1.5 Kin + 1.5 IAA	3.40 ± 0.17 abcde	2.40 ± 0.17 abcde	12.11 ± 0.87 bcd	0.15 ± 0.08 ab	100.00 ± 0.00 b
1.5 Kin + 2.0 IAA	4.20 ± 0.26 fg	3.20 ± 0.26 fg	15.23 ± 1.12 def	0.70 ± 0.23 abcd	93.33 ± 0.05 b
2.0 Kin + 0.5 IAA	5.57 ± 0.42 h	4.57 ± 0.42 h	20.53 ± 1.91 g	0.73 ± 0.27 abcd	93.33 ± 0.05 b
2.0 Kin + 1.0 IAA	3.60 ± 0.24 bcdef	2.60 ± 0.24 bcdef	16.83 ± 1.21 f	1.69 ± 0.29 de	100.00 ± 0.00 b
2.0 Kin + 1.5 IAA	4.20 ± 0.36 fg	3.20 ± 0.36 fg	14.50 ± 1.42 cdef	0.27 ± 0.15 abc	96.67 ± 0.03 b
2.0 Kin + 2.0 IAA	4.40 ± 0.27 g	3.40 ± 0.27 g	15.07 ± 1.08 def	0.83 ± 0.30 abcd	96.67 ± 0.03 b

Data represent mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters within the same column are significantly different at $p < 0.05$, according to Duncan's multiple range test (DMRT). Kin, Kinetin; IAA, indole-acetic acid; NR, no response.

The presence of Kin (singly applied) in the media was found to improve production of new shoots and leaves, but did not affect root formation (Table 1). Contrasting results were observed when only IAA was added to media, where it only aided root formation but yielded no effect on shoot or leaf production (Table 1). Supplementation of high Kin concentration (1.0–2.0 mg L^{-1}) yielded the most number of shoots and leaves among all single PGR treatments (Table 1).

When both PGRs were used in combination, production of new shoots and leaves were observed to be significantly improved. As shown in Table 2, addition of 2.0 mg L^{-1} Kin plus 0.5 mg L^{-1} IAA in the media (identified as the optimum regeneration media) resulted in the highest number of shoots (5.57 ± 0.42) and leaves (20.53 ± 1.91) per explant. The optimum regeneration media also yielded the highest multiplication index (4.57 ± 0.42) among other treatments. However, combined PGRs were observed to reduce production of roots, where more number of roots were produced when only IAA was added in the media (Tables 1 and 2).

3.2. Production of Callus

In contrast to the control (cultures on MS basal), all cultures produced on MS media supplemented with PGRs (either singly applied or combined) showed production of callus, indicating that callus induction in this species relied on supplementation of PGRs (Tables 1 and 2). However, the degree of callus production was observed to be non-uniform and not concentration-dependent, except when the media was added with IAA (singly applied). The lowest percentage of explants producing callus was observed in MS media supplemented with 1.0 mg L^{-1} Kin (40%) and 0.5 mg L^{-1} IAA (50%). On the other hand, more than 70% of the explants produced callus when cultured on MS media added to with both IAA and Kin (Table 2).

3.3. Acclimatization

In this study, most of the regenerated plantlets were successfully acclimatized (Table 3). Only plantlets with fully expanded roots and leaves were used as samples in this assessment (Figure 1). After 30 days of acclimatization, only plantlets transplanted into potting mixture PD and PF showed 100% survival rate. However, the survival rate of the acclimatized plantlets on potting mixture PD declined after 60 days. Data analysis also indicated that potting mixture containing only black soil (PA) was the most unfavorable medium for acclimatization of this species, as it resulted in the highest decrease of survival rate with time (Table 3). On the other hand, the best medium for acclimatization of *O. stamineus* plantlets was observed to be potting mixture PF (1:1:1 ratio of black soil/red soil/compost), as 100% of the plantlets survived after 90 days of acclimatization (Table 3).

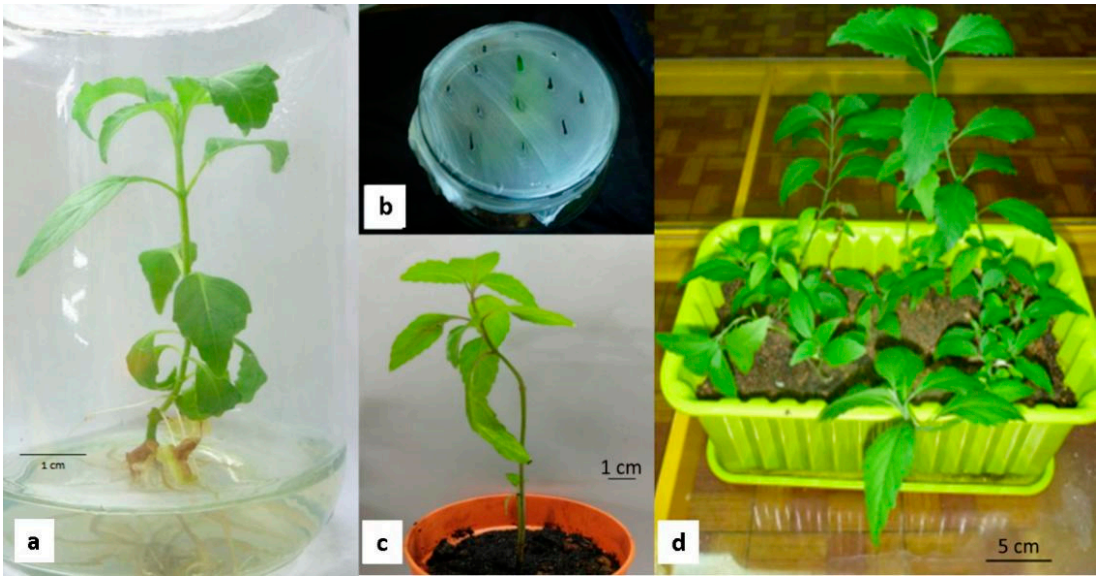

Figure 1. Acclimatization of regenerated plantlets of *Orthosiphon stamineus*; (**a**) in vitro grown plantlet, (**b**) acclimatization after 30 days, (**c**) acclimatization after 90 days, and (**d**) several successfully acclimatized plantlets after 6 months.

Table 3. Acclimatization of regenerated plantlets of *Orthosiphon stamineus*.

Sample ID	Potting Mixture	Survival % after 30 Days	Survival % after 60 Days	Survival % after 90 Days
PA	Black soil	66.67 ± 12.60 ab	40.00 ± 13.09 a	26.67 ± 11.82 a
PB	Red soil	57.14 ± 12.78 a	57.14 ± 12.78 ab	57.14 ± 12.78 b
PC	Black soil + red soil	78.57 ± 10.60 abc	64.33 ± 12.37 abc	64.33 ± 12.37 bc
PD	Black soil + compost	100.00 ± 0.00 c	92.31 ± 6.63 cd	92.31 ± 6.63 cd
PE	Red soil + compost	86.67 ± 9.09 bc	86.67 ± 9.09 bcd	80.00 ± 10.69 bcd
PF	Black soil + red soil + compost	100.00 ± 0.00 c	100.00 ± 0.00 d	100.00 ± 0.00 d

Data represent mean value ± standard error (SE) with 15 explants in each treatment. Means with different letters within the same column are significantly different at $p < 0.05$, according to Duncan's multiple range test (DMRT).

3.4. ISSR Analysis

Ten out of 20 tested ISSR primers produced satisfactory amplification of bands in this study (data not shown). Bands observed were fairly clear (Figure 2). A total of 610 scorable bands were generated from 10 random *O. stamineus* in vitro plantlets (Table 4). The total number of bands amplified per primer varied from 36 to 100 per primer. The range of amplification was between 300 to 2500 bp. Only 4 primers (UBC845, UBC836, UBC841, and UBC856) showed polymorphism with 7.32% polymorphism in total number of bands.

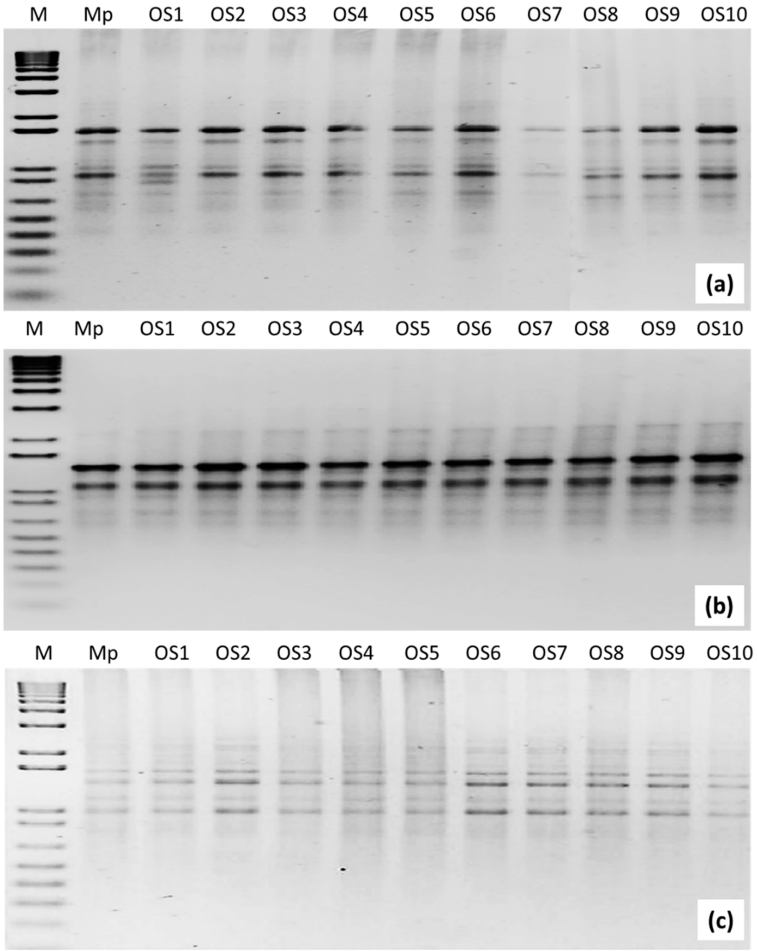

Figure 2. Polymerase chain reaction (PCR) amplification products obtained with inter-simple sequence repeat (ISSR) markers; (**a**) Primer UBC841, (**b**) Primer UBC835, and (**c**) Primer UBC855. Lane M: Molecular marker (100 bp–15 kbp); Lane Mp: Mother plant; Lanes OS1–OS10: In vitro-raised *Ortosiphon stamineus* plantlets.

Table 4. Primer used in ISSR polymorphism analysis, number and size of amplified fragments of *Orthosiphon stamineus*.

Primer Code(UBC)	Sequence 5′–3′	Annealing Temperature (°C)	Total Number of Bands Amplified	Number of Scorable Bands per Primer	No. and Frequency of Polymorphic Bands per Primer	Range of Amplification (bp)
UBC807	$(AG)_8T$	46.5	68	8	0	500–2000
UBC829	$(TG)_8C$	52.5	80	8	0	600–1300
UBC835	$(AG)_8YC$	50.0	59	7	2 (28.57%)	550–2500
UBC836	$(AG)_8YA$	48.0	68	11	1 (9.90%)	400–2300
UBC840	$(GA)_8YT$	46.5	49	6	0	300–1300
UBC841	$(GA)_8YC$	52.0	36	5	2 (40.00%)	400–2500
UBC845	$(CT)_8RG$	47.5	71	13	1 (7.69%)	400–1800
UBC854	$(TC)_8RG$	50.0	40	10	0	500–1800
UBC855	$(AC)_8YT$	53.0	100	10	0	400–2000
UBC856	$(AC)_8YA$	54.0	39	4	0	700–1500
Total			610	82	6 (7.32%)	

Very low genetic distance values (about 0.1) were recorded between all samples (Table 5). This confirmed the clonal identity of the samples and that no somaclonal variation had resulted from the PGR treatments. Other than that, the morphology of the leaves of the in vitro grown plantlet and the mother plant was also compared. Figure 3 shows the morphology of a leaf from an in vitro grown *Orthosiphon stamineus* plantlet having a similar pattern with that of the mother plant. Both leaves showed serrated margin and visible pinnate veins. The uniform morphological characteristics between the mother plant and in vitro grown plantlet further strengthen the ISSR analysis results, thus confirming the clonal nature of the plantlets.

Table 5. Genetic distance based on Jaccard distance coefficient for of 10 random in vitro grown *O. stamineus* (OS) plantlets.

	OS1	OS2	OS3	OS4	OS5	OS6	OS7	OS8	OS9	OS10
OS2	0.110625									
OS3	0.077377	0.110625								
OS4	0.021594	0.110625	0.077377							
OS5	0.021594	0.110625	0.077377	0.021594						
OS6	0.077377	0.110625	0.077377	0.077377	0.077377					
OS7	0.077377	0.110625	0.077377	0.077377	0.077377	0.077377				
OS8	0.077377	0.110625	0.077377	0.077377	0.077377	0.077377	0.000000			
OS9	0.077377	0.110625	0.077377	0.077377	0.077377	0.077377	0.077377	0.077377		
OS10	0.077377	0.110625	0.077377	0.077377	0.077377	0.077377	0.000000	0.000000	0.077377	
Mp	0.124336	0.124336	0.124336	0.124336	0.124336	0.124336	0.124336	0.124336	0.124336	0.124336

Mp, mother plant; OS1 – OS10, in vitro-raised *Ortosiphon stamineus* plantlets.

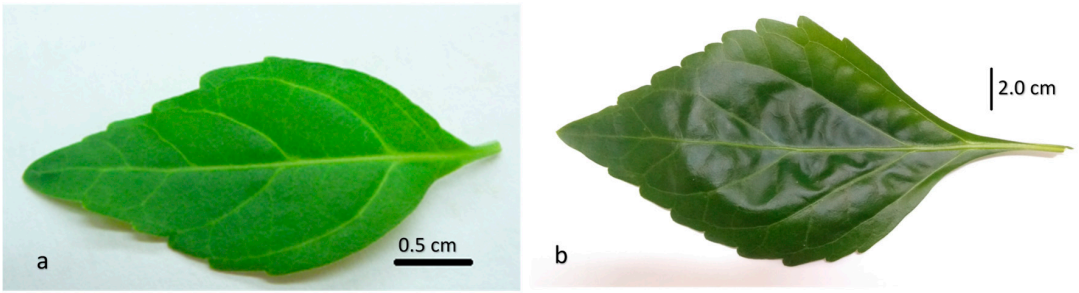

Figure 3. Morphology of leaves of *Orthosiphon stamineus*; (**a**) in vitro grown plantlet, and (**b**) mother plant.

4. Discussion

Various PGRs have been used in tissue culture studies. Most tissue culture studies conducted on *Orthosiphon* employed the use of BAP (6-benzylaminopurinehormone) [18,33–35]. On the other hand, the current study evaluated the effectiveness of Kinetin as the exogenous cytokinin in improving shoot induction of this species. Both Kinetin and BAP were reported to generate high response (up to 90%) in tissue culture of this species [36]. BAP was reported to be better for induction of multiple shoots, while Kinetin was found to induce longer shoot length compared to BAP [20,35,37]. In this study, 1.5–2.0 mg L^{-1} Kinetin supplemented singly was recorded to yield the highest number of shoots, similar to a previous report [20]. On the other hand, IAA was identified as the best auxin in adventitious root formation from leaf explants of *O. stamineus*, better than IBA and NAA [38]. Similar observation was recorded in this study, where media supplemented with 2.0 mg L^{-1} IAA was observed to produce the highest number of roots per explant (4.60 ± 0.65). Nevertheless, other studies have reported that IBA is a more effective rooting inducer than IAA [33,39–41]. However, it was observed that application of Kinetin together with IAA was not able to induce regeneration of roots.

Acclimatization of in vitro grown plantlets is a very crucial step, as it determines the success of any plant tissue culture experiment. During field transfer, in vitro grown plantlets are often unable to cope with the environmental conditions, such as exposure to high light intensity [42] and to compete with soil microbes [43]. One of the major limiting factors affecting the survival of acclimatized plantlets is humidity [42,43]. Gradually reducing humidity during hardening of the plantlets is key in ensuring survival of in vitro grown plantlets when taken ex vitro. In this study, the in vitro *O. stamineus* regenerants were gradually exposed to reduced levels of humidity, thus generating high survival percentages in all potting mixtures tested. A 100% survival rate was maintained, even after 90 days of acclimatization, when the regenerants were grown on potting mixture PF, suggesting that 1:1:1 ratio of black soil/red soil/compost is the most suitable acclimatization medium for this species. The composition of potting mixture PF ensures good aeration with excellent water holding capacity, thus supporting the growth and survival of the plantlets after acclimatization.

Other than that, one of the main aims of mass propagation of plants through tissue culture is to generate clonal plantlets with superior quality. Thus, ensuring the genetic uniformity of tissue culture-raised plants is important for mass production of elite crops or cultivars such as rice [44], cotton [45], and sugarcane [46], as well as for conservation of endangered plant species [47–49]. Furthermore, plant tissue culture also allows for uniform production of clonal plants to be used as plant factories for production of novel and important bioactive compounds with medicinal properties [50,51]. Many marker systems have been used in assessments of genetic variation among clonal plants, such as random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), simple sequence repeat (SSR), and amplified fragment length polymorphism (AFLP). ISSR has been reported as a more effective molecular marker than RAPD and SSR [52–55]. Various reports have indicated that ISSR can reveal higher polymorphism than RAPD, such as in studies on *Tilletia indica* [56]. Usage of ISSR markers reveals a larger number of polymorphic fragments per primer than RAPD because of the occurrence of abundant SSR regions [57]. Due to this, ISSR has been suggested as an alternative to replace RAPD in genetic diversity assessment of coconut germplasm [52]. Moreover, ISSR is also able to detect higher similarity index than RAPD [55,58], possibly due to the abundant and highly polymorphic nature of the ISSR microsatellites caused by slippage in DNA replication [58]. ISSR has been widely used to help ascertain the clonal fidelity and to reveal any occurrence of somaclonal variation among tissue culture plants, such as *Magnolia sirindhorniae* Noot. & Chalermglin [48], *Abutilon indicum* [59], *Fritillaria dagana* [60], *Eleusine coracana* (L.) Gaertn. [61], *Smallanthus sonchifolius* (Poepp. and Endl.) H. Robinson [31], *Morus* sp. [62], and bamboos [31,63].

Considered as one of the most important medicinal plants in Malaysia, *Ortosiphon stamineus* is widely planted in the country to support the nation's growing herbal industry [64]. However, environmental factors such as availability of soil nutrients, as well as other biotic and abiotic stresses, have been reported to influence and in some cases, hamper the production of important secondary

metabolites in field-grown plants [65–67]. In this study, usage of ISSR markers revealed 7.32% polymorphism and low genetic variation among the randomly collected samples. The low genetic distance among the tested samples confirms the clonal nature of the in vitro *O. stamineus* regenerants produced in this study. Thus, the in vitro regeneration protocol reported in this study is beneficial for both researchers and industry players alike, and is suitable for commercial mass propagation of this species.

5. Conclusions

Optimum regeneration media for micropropagation of *O. stamineus* has been successfully identified (MS media supplemented with 2.0 mg L^{-1} Kin plus 0.5 mg L^{-1} IAA) and yielded the highest number of shoots (5.57 ± 0.42) and leaves (20.53 ± 1.91) per explant. A 1:1:1 ratio of black soil/red soil/compost (potting mixture PF) was found to be the best acclimatization medium for this species, resulting in 100% survival percentage after 90 days of ex vitro transfer. Inter-simple sequence repeat (ISSR) was found to be a very useful tool to determine genetic differences among 10 randomly collected in vitro samples of *O. stamineus*. Very low polymorphism (7.32%) was detected between all samples, thus ascertaining the clonal nature of the plantlets produced in this study.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/12/778/s1, Table S1: List of primers used in the analysis [31].

Author Contributions: J.S.Y. and S.A.K. conceived and designed the experiments; H.A., I.F.M. and N.A.A.B. performed the experiments; H.A. and J.S.Y. analyzed the data; J.S.Y. and S.A.K. contributed reagents/materials/analysis tools; H.A. and J.S.Y. wrote the paper.

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