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Research article

Efficient *in vitro* regeneration and genetic fidelity analysis of shea tree (*Vitellaria paradoxa* Gaertn) using ISSR markers ☆

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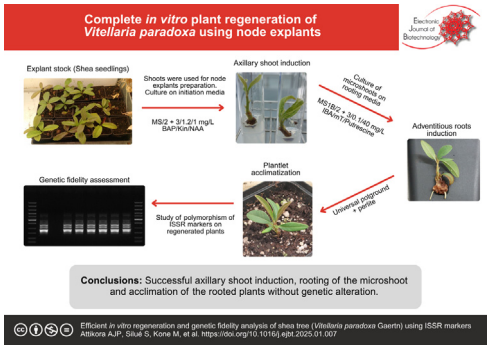
GRAPHICAL ABSTRACT

Efficient *in vitro* regeneration and genetic fidelity analysis of shea tree (*Vitellaria paradoxa* Gaertn) using ISSR markers.



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ABSTRACT

Background: Shea tree is an economically valuable tree crop in the food, cosmetic, and pharmaceutical industries due to its seed oil, known as shea butter. Rapid propagation of superior shea trees through *in vitro* culture is essential to support domestication and conservation efforts. This study aimed to establish an efficient *in vitro* propagation protocol for the regeneration of shea true-to-type plantlets. Nodal explants were cultured on half-strength Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP) and/or kinetin (Kin) combined with 1-naphthaleneacetic acid (NAA) for shoot induction. Rooting was tested on half- and quarter-strength MS and full- and half-strength modified MS (MS1B) media, enriched with indole-3-butyric acid (IBA) alone or combined with NAA, IAA, *meta*-topolin

☆ Audio abstract available in Supplementary material.

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In vitro regeneration
ISSR markers
Micropropagation
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Vitellaria paradoxa

(mT), and putrescine. Genetic fidelity of regenerated plants was assessed using inter-simple sequence repeat (ISSR) markers.
Results: The results showed that MS/2 medium containing 3:1.2:1 mg/L BAP:Kin:NAA gave the best regeneration of axillary shoots. Four-week-old axillary shoots were 100% rooted on MS1B/2 medium containing 3:0.1:40 mg/mL IBA:*m*T:putrescine. Rooted plantlets were successfully acclimated in vivo. The polymorphism of the ISSR markers ranged from 50 to 87.5%, with an average of 65%, and the polymorphism information content was 0.22. For genetic fidelity assessment, 34 scorable and reproducible markers were obtained. All markers were monomorphic and identical to the mother plant.
Conclusions: The micropropagation protocol proposed in this study is suitable for large-scale *in vitro* regeneration of shea without genetic alteration. However, further studies are needed for the induction of multiple micros-hoots.
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1. Introduction

Shea tree (*Vitellaria paradoxa*) grows across 21 countries that form a large belt in the semi-arid zone of sub-Saharan Africa [1]. In Côte d'Ivoire, shea trees are located in the northern part. It is an important economic plant species due to its multipurpose use [2]. The shea butter produced from its kernel is relevant in the food, cosmetic and pharmaceutical industries [1]. Income from shea has been shown to make a significant contribution to rural livelihoods throughout the Sahel-Savannah [3]. Shea tree is recognized as the second largest oil-producing plant after oil palm in Africa [4]. The global shea market was estimated at US$2.4 billion in 2024 and is expected to reach US$3.7 billion by 2030 [5]. This high demand is due to its use in the confectionery and cosmetics industries. In the European market, the demand for these natural and organic cosmetics reached a value of US$744.2 million in 2024 and is expected to reach US$1,133.8 million by 2030 [5].

Shea trees are the major constituents of the existing plant biomass in their growing area. In that way, they not only form a substantial carbon reserve with a huge potential for carbon sequestration (necessary for climate change mitigation) but also contribute to protect against the environmental degradation prevalent in their growing area [6]. However, the overexploitation of its timber for firewood and charcoal production [7] has led to the collapse of the tree's population at the extent that currently, it is indexed as a vulnerable plant species by the International Union for Conservation of Nature [8].

Indeed, the current population of shea trees are the result of natural regeneration, protection, and wild management during cultivation cycles [9]. Due to cross-pollination, these wild-managed stands result in heterogeneous populations. These heterogeneity often includes a prevalence of genotypes with poor genetic background and therefore produces an unreliable crop in terms of shea nut quantity and shea butter quality [10,11].

The necessity to produce superior true-to-type varieties that conserve the desired genetic traits [12] has prompted research into the clonal propagation strategy [13]. However, some vegetative propagation methods, such as the use of stem cuttings and the air-layering, were reported to have limited success [14,15,16,17,18]. However, studies have reported a successful grafting of shea tree plantlets on fields [19] and on one-year-old plantlets in nursery [20]. Grafting was recommended in a recent study to clone the individuals within the Design Core Collection of Ivorian shea trees for *ex situ* conservation [21]. Unfortunately, the grafting method can only be used on already established plantlets. Therefore, there is a need to develop a reliable asexual propagation technique that can be used to both produce superior true-to-type varieties and conserve shea tree desired traits to aid in the domestication of the species [11].

On the other hand, the *in vitro* culture is an alternative approach to improve the propagation and enhance the large-scale cultivation of the shea tree [22]. Explant culture methods have shown success in propagating various recalcitrant tree crops and woody plants [23] including various members of the Sapotaceae, such as *Argania spinosa* [24], *Madhuca latifolia* [25], *Pouteria lucuma* [26] and *Achras sapota* [27]. A variant of the *in vitro* approach, the micropropagation of woody species, has become a widely used technique for rapidly regenerating elite germplasm and reducing the juvenile post-propagation period for conservation purposes [28].

Previous shea micropropagation attempts have used nodal and shoot tip explants [10,22,29], young leaf explants [30,31,32], and immature cotyledon and pulp techniques [33,34]. Most of the studies using nodal and shoot tip explants reported low rates of axillary shoot induction and did not attempt root induction [22,29]. However, in the study conducted by Lovett and Haq [10], the authors reported very low rates (8%) of rooted plantlets, but did not mention acclimatization, suggesting that they failed at this important stage. Also, none of the shea micropropagation studies mentioned multiple shoot regeneration. With other explants mentioned above, the authors reported callus formation but failed with shoot regeneration. All authors of these previous studies recommended further research to standardize micropropagation techniques for *V. paradoxa*. The main causes of these failures or low rates of micro-shoot induction were associated with the combination and/or concentration of growth regulators used.

It is essential to determine the genetic uniformity of *in vitro* plants, as the goal of micropropagated plants is to produce a uniform genetic material for commercial use [35]. *In vitro* plantlets often have genetic variants due to exposure to artificial conditions [36,37,38]. An effective method for studying somaclonal changes in *in vitro* plantlets is the use of molecular markers [36,38,39]. The inter-simple sequence repeat (ISSR) markers are the most commonly used PCR-based molecular markers in genetic stability studies [38,40,41]. ISSR markers are dominant, reproducible, and capable of amplifying genome-wide regions without prior sequence information [42]. These characteristics make ISSR markers valuable tools for various applications in plant genetics, such as genetic diversity analysis and marker-assisted genetic fidelity [38,40,43].

The aim of this study was to establish an efficient *in vitro* culture protocol for the regeneration of true-to-type shea plantlets.

2. Materials and methods

2.1. Preparation of explants and surface sterilization

Shoots (stems and buds) from a well-managed stock of *V. paradoxa* seedlings maintained in the tropical green-house of Gembloux Agro Bio-Tech, Plant Genetics Laboratory, were used for the preparation of explants (Fig. 1a). Excised shoots (Fig. 1b) were either pink or green in color. The collected shoots were rinsed under running tap water for 5 min to remove any surface dirt. The explants were prepared by carefully removing older leaves, leaving 1 to 2 new leaves per shoot (Fig. 1c). They were agitated in a disinfectant solution of 3% liquid soap for 10 min and left under running tap water for 10 min. The shoots were then dipped in a 2% (w/v) fungicide solution (Benlate WP) for 5 min and left under running tap water for 5 min. The shoots were then transferred to a sterile environment under a horizontal laminar air flow cabinet (Clean Air Technology; Filtest, Benelux, Belgium) for aseptic sterilization.

The shoots were surface sterilized with 70% (v/v) ethanol for 2 min and further sterilized with 10% (v/v) sodium hypochlorite solution (14% Cl_2; VWR, Belgium) for 10 min. After sterilization, the shoots were rinsed five times with sterile distilled water. The shoots were then placed on sterilized filter paper in a Petri dish to dry, and the remaining leaves and petioles were removed (Fig. 1d). The node was trimmed to a size between 1 and 2 cm and used as explants for culture initiation (Fig. 1e). Explants were randomly aseptically placed vertically on shoot induction media (Fig. 1f). Cultures were maintained in the growth chamber at 26°C ± 1 under a 16/8 h light/dark photoperiod.

2.1.1. Reference standard protocols for media preparation

Standard procedures were used to prepare all media. Murashige and Skoog medium micro and macronutrients including vitamins and modified Murashige and Skoog medium micro and ½ macronutrients including vitamins were used in this study, as defined by Murashige and Skoog [44]. The different base media, their composition and the strengths used in this study are shown in Table 1. An identification of the different strengths of each medium has also been defined in Table 1 and will be used throughout this paper. Media were supplemented with 30 g sucrose, pH was adjusted to 5.8, gelled by 8 g/L Agar. All media and all instruments (scalpels, forceps, scissors, and paperwork) were sterilized by autoclaving at 121°C and 15 psi for 15 min.

For the axillary shoot induction, only MS/2 was considered because it showed more promising results in shea axillary shoot induction than MS in previous studies [10,22,29]. The MS/2 medium was supplemented with three plant growth regulators (PGRs) BAP, Kin and NAA. Three regimes of these plant growth regulators were applied: (1) use of cytokinin alone (BAP or Kin), (2) combination of cytokinin (BAP) and auxin (NAA) and (3) combination of two cytokinins (BAP and Kin) and one Auxin (NAA) (Table 2). For the first regime, the concentrations of BAP or Kin were: 0, 0.5, 1, 2.5 and 3.5 mg/L. The second regime concentrations of BAP:NAA are: 3.5:0.1 and 3:1 mg/L. Finally, the combination of BAP:NAA: Kin concentrations were: 3:1:1 and 3:1.2:1 mg/L. The selection of concentrations and combinations of growth regulators was done by consulting previous studies on Sapotaceae family or other recalcitrant plants *in vitro* culture [10,22,24,25,26,27,29].

Regenerated shoots from lateral bud breaks were transferred to rooting media consisting of MS, MS/2, MS1B and MS1B/2 with different PGRs. The different PGR combinations and concentrations are listed in Table 2. The plant growth regulators, indole-3-butyric acid (IBA), *meta*-topolin (*m*T), 1-Napthaleneacetic acid (NAA), Indole-3-acetic acid (IAA), and Putrescine were added to stimulate adventitious root induction. The cultures were maintained in a growth room under the same conditions as described above. The different concentrations of the combination (IBA:*m*T: Putrescine) in MS1B medium were 3:0:0; 3:0.1:40 and 3:0.1:75 mg/L. Concentrations of plant growth regulators in MS medium were 3 mg/L (IBA); 1.5:0.5:160 mg/L (IBA:NAA:Putrescine) or 1.5:0.5:1 mg/L (IBA:NAA:IAA). The choice of concentrations and combinations was the same as mentioned above.

For the rooting experiment, axillary shoots of different ages were used: young axillary shoots (4–6 weeks) and old axillary shoots (8 weeks and older).

2.2. Acclimatization

Rooted plantlets were removed from the rooting medium, and the roots were carefully washed with distilled water to remove adherent agar. Plantlets were transferred to plastic pots (one per

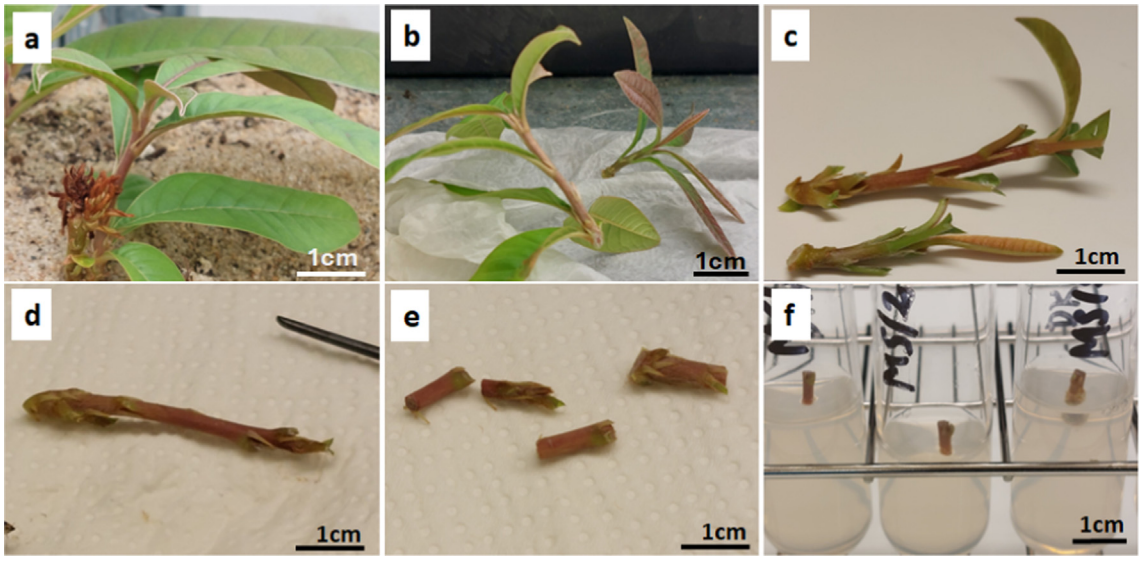

Fig. 1. Preparation of explants for culture initiation. (a) location of a shoot on the donor tree, (b) Excised shoots; (c) shoot after removal of old leaves, (d) shoot after remaining petiole and leaf, (e) nodal explants, (f) explants on shooting medium.

Table 1
Basal media composition and strength length used during this study.

Media	Composition (mg/L)			Strength	Code
	Macronutrients	Micronutrients	Vitamins		
Murashige and Skoog medium	$CaCl_2$:332.02 KH_2PO_4:170 KNO_3:1900 $MgSO_4$:180.54 NH_4NO_3: 1650	$CoCl_2$.6H2O: 0.025 $CuSO_4{\cdot}5H_2O$: 0.025 FeNaEDTA: 36.7 H_3BO_3: 6.2 KI: 0.83 $MnSO_4{\cdot}H_2O$: 16.9 $Na_2MoO_4{\cdot}2H_2O$: 0.25 $ZnSO_4{\cdot}7H_2O$: 8.6	Glycine: 2 myo-Inositol: 100 Nicotinic acid:0.5 Pyridoxine HCl:0.5 Thiamine HCl: 0.1	Full (100%, w/v) Half (50%, w/v) Quarter (25%,w/v)	MS MS/2 MS/4
Murashige and Skoog medium mod. No. 1B	$CaCl_2$: 166 KH_2PO_4: 85 KNO_3: 950 $MgSO_4$: 87.86 NH_4NO_3: 825	Idem	Idem	Full (100%, w/v) Half (50%, w/v)	MS1B MS1B/2

Table 2
Plant growth regulator combinations and concentration used for shoot and root induction.

PGR Comb.	C1 (mg/L)	C2 (mg/L)	C3 (mg/L)	C4 (mg/L)	C5 (mg/L)	Medium
Shoot induction						
BAP	0	0.5	1	2.5	3.5	MS/2
Kin		0.5	1	2.5	3.5	MS/2
BAP:NAA	3.5:0.1	3:1				MS/2
BAP:Kin:NAA	3:1:1	3:1.2:1				MS/2
Root induction						
IBA	0	3			MS/2, MS/4, MS1B, MS1B/2	
IBA:NAA:Putrescine	1.5:0.5:160				MS/2	
IBA:NAA:IAA	1.5:0.5:1				MS/2	
IBA:*m*T:Putrescine	3:0.1:40	3:0.1:75*			MS1B, MS1B/2	

PGR Comb.: plant growth regulator combinations; C1-C5: different concentrations; concentration with an asterisk was only used with MS1B.

pot) containing a mixed substrate of autoclaved universal potting soil consisting of peat moss, fertilizer, lime, and perlite. They were then transferred to the greenhouse under the following conditions: 28 ± 2°C temperature, 85 ± 5% relative humidity and 16/8h light/-dark photoperiod. The pots were covered with clear polyethylene plastic to maintain high humidity.

2.3. Genetic fidelity assessment using ISSR markers

2.3.1. DNA extraction and quantification

To assess the genetic fidelity of regenerated plantlets of *V. paradoxa* plants to the donor (plant from which explants are taken), fresh leaf samples of 100 mg were randomly collected from 5 acclimated plantlets and the donor plant. Leaf tissue was finely ground in liquid nitrogen using a pre-chilled mortar and pestle. Genomic DNA was isolated from the resultant tissue powder using the "Innovative Shea DNA extraction protocol" [45]. The DNA pellet was resuspended in sterile double-distilled water and stored at −20°C until further analysis. The concentration of the isolated DNA was determined using a Quantus Fluorometer (Promega, Madison, USA) in a total volume of 100 µl, containing 98.5 µl (1XTE Buffer), 0.5 µl (QuantiFluor dsDNA System; Madison, Promega) and 1 µl (DNA template).

2.3.2. ISSR-PCR analysis

Five (5) ISSR primers were used to assess the genetic fidelity of regenerated plantlets. ISSR markers have not been yet mentioned in *V. paradoxa* study. Therefore, the polymorphic level of the 5 ISSR primers was first assessed using 10 shea trees randomly selected from the shea seedling established in the tropical greenhouse as mentioned in the "Preparation of Explants and Surface Sterilization" section. The seeds used to establish the seedlings were collected in Côte d'Ivoire in the districts of Poro and Bagoué. The five ISSR markers used were polymorphic and produced highly readable and reproducible bands. It should be noted that the effect of genotype on propagation efficiency was not considered in this study. However, one shea tree was identified as a donor prior to shoot induction to study the genetic fidelity of the regenerated shea trees from this donor using our method. The five ISSR primers were then used to assess the genetic fidelity of five regenerated shea trees. The sequence of the primers used and their characteristics are shown in Table 3. Polymerase chain amplification (PCR) was performed in a total volume of 20 µL containing: 10 µL of Go Taq Green Master Mix (2x), 1 µL of the primer (10 pmol/µL), 1 µL of DNA template (15 ng/µL) and 8 µL of sterile ddH_2O. The PCR run program was performed on a Bio-Rad T100 Thermal Cycler the PCR machine, with a denaturation step at 94°C for 4 min, followed by 30 cycles of amplification at 94°C for 30 s, 40 s at the primer's annealing temperature (Table 3), and a 72°C for 2 min and a final extension at 72°C for 5 min.

ISSR amplification was performed three times, and only reproducible PCR products were scored. Amplified fragments were separated on a 1% (w/v) agarose gel using 1 × TAE buffer and stained with 0.1 µL/mL Gelred. Electrophoretic separation was performed at 180 V for 25 min. Bands were visualized using a gel documenta-

Table 3
Sequence and characteristics of ISSR primers.

Locus code	Primer sequence	Tm (°C)
IS-1	5′ AGA GAG AGA GAG AGAGG 3′	52.8
IS-2	5′ GAG AGA GAG AGA GAGAT 3′	50.4
IS-3	5′ GAG AGA GAG AGA GAGAC 3′	52.8
IS-4	5′ TCT CTC TCT CTC TCTCC 3′	52.8
IS-5	5′ AGA GAG AGA GAG AGA GYT 3′	52.6

Tm (°C): annealing temperature.

tion system (Gel Doc XR+ system; Bio-Rad model; Universal Hood II). The size of each amplicon was estimated by comparison with the Fast DNA Ladder (BioLabs, England). The banding patterns were compared to evaluate the genetic variability between samples. The fragments were scored as either present (1) or absent (0). The polymorphism information content (PIC) was determined following the general equation [**Equation 1**] established by Serrote et al. [42] for dominant markers:

$$PIC = 1 - (p^2 + q^2) \quad (1)$$

where p is the frequency of present bands and q, the frequency of absent bands.

2.4. Collection of experimental data and statistical analysis

Cultures were observed weekly for signs of growth. For shoot induction, the data recorded were time for shoot induction (week), callus formation, percentage of explants that induced shoot, number of shoots per explant, percentage of elongated shoot, average shoot length (cm) and number of leaves. The experiment was repeated three times with 130 explants in each replicate (10 explants per treatment).

For root induction, the following data were recorded: callus formation, time for root induction (week), number of roots per shoot, root length and percentage of healthy shoots in the rooting media. Five new axillary shoots were used for each treatment with two replicates.

The collected data were analyzed using the R version 4.3.3. Analysis of variance (ANOVA) was used to determine the differences between treatment means. Multiple comparisons between means were performed using Tukey's pairwise comparison.

3. Results

Like many plant species of the Sapotaceae family, *V. paradoxa* is recalcitrant in tissue culture. In this section, we present the main findings of an efficient protocol for *in vitro* propagation of shea tree for axillary shoot proliferation, root induction and *ex vitro* acclimatization. We further assess the genetic fidelity of the regenerated plants using ISSR markers.

3.1. Axillary shoot regeneration

Axillary shoots were induced when explants were cultured on MS/2 media supplemented with various combinations of the plant growth regulators BAP, Kin and NAA.

Axillary shoots were induced after 1 week on explants grown on media supplemented with Kin alone (1 mg/L) and the combination BAP:Kin:NAA (3:1.2:1 mg/L). Media containing BAP alone and the combination of BAP:NAA (3:1 mg/L) induced axillary buds after 2 weeks (Table 4). However, axillary shoots induced on media containing BAP alone showed no signs of elongation. Induced shoots were elongated on media supplemented with Kin alone, the combinations BAP:NAA, and BAP:Kin:NAA. The highest percentage of elongated axillary buds was obtained on MS/2 medium supplemented with the combination of 3:1.2:1 mg/L BAP:Kin:NAA (Table 4).

Media containing the hormone concentration of 3:1.2:1 mg/L BAP:Kin:NAA had the highest average length of axillary shoot per explant (5.2 cm). In contrast, media supplemented with Kin alone (1 mg/L) had the lowest average shoot length in the elongated axillary buds. A highly significant difference ($p < 0.001$) in mean length was observed between treatments (Table 4). After 4 weeks of culture on medium containing 3:1.2:1 mg/L BAP:Kin:NAA, Fig. 2a and Fig. 2c show the elongation of the microshoots, while Fig. 2e shows the development of the microshoot on medium containing 1 mg/L Kin.

Globally, the first induced leaves were observed on the shoot tip explants after 2 weeks, while leaves were observed on the nodal explants after 4 weeks in culture (Fig. 2a and Fig. 2c). However, the number of leaves per explant was higher on nodal explants than on shoot tip explants after 6 weeks of culture (Fig. 2b and Fig. 2d). The number of leaves per explant was higher in the BAP:Kin:NAA treatment with an average of 6.7 leaves per explant after 8 weeks (Table 4), while the lowest average number of leaves (0.84 leaves/explant) was obtained from media supplemented with Kin alone. The number of leaves from the BAP:Kin:NAA combination was significantly different ($p < 0.001$) from the number of leaves from the BAP:NAA combination and from the treatment with Kin alone. Explants cultured on media containing BAP:Kin:NAA formed moderate callus at the basal side (Fig. 2b), while media containing only Kin did not form callus (Fig. 2f).

3.2. Root induction

Adventitious roots were induced on regenerated axillary buds of *V. paradoxa* after 12 weeks on rooting media consisting of MS1B/2 supplemented with a combination of IBA, *m*T, and putrescine (3:0.1:40 mg/L). All 4- or 5-week-old axillary buds were rooted and formed 1 or 2 roots on the MS1B/2 medium supplemented with 3 mg/L IBA, 0.1 mg/L *m*T, and 40 mg/L putrescine (Fig. 3a). It should be noted that the color of the medium changes

Table 4
Effect of BAP, Kin and NAA concentrations on growth, callus formation and axillary shoot induction of *V. paradoxa* explants after 8 weeks culture on half-strength MS media.

Media	PGRs (mg/L)			TSI (week)	CFo	EIS (%)	PES (%)	NSE	ASL	NoL
	BAP	Kin	NAA							
MS/2	0	0	0	*NA*	N	0	0	0	0	0
	0.5	0	0	*NA*	N	0	0	0	0	0
	1	0	0	*NA*	N	0	0	0	0	0
	2.5	0	0	*NA*	N	0	0	0	0	0
	3.5	0	0	*NA*	N	0	0	0	0	0
	0	0.5	0	*NA*	N	0	0	0	0	0
	0	1	0	1	N	100	53.33	1	1.3 ± 0.15a	0.84 ± 0.04a
	0	2.5	0	*NA*	N	0	0	0	0	0
	0	3.5	0	*NA*	N	0	0	0	0	0
	3.5	0	0.1	2	L	46.67	13.33	1	1.5 ± 0.3a	1.4 ± 0.2a
	3	0	1	2	L	53.33	26.67	1	2.2 ± 0.44a	1.7 ± 0.26a
	3	1	1	1	M	100	86.67	1	3.5 ± 0.5b	4.6 ± 0.55b
	3.3	1.2	1	1	M	100	93.33	1	5.2 ± 0.36c	6.7 ± 0.58c

PGRs: plant growth regulators; TSI: Time for shoot induction; NA: None; CFo: callus formation; N: None; L: Low; M: Moderate; EIS: percentage of explants that induced a shoot; PES: percentage of elongated shoots; NSE: number of induced shoots per explant; ASL: average shoot length, NoL: number of leaves. Means with the same letter are not significantly different from each other ($p > 0.05$ ANOVA followed by Tukey test) in ALS and NoL columns.

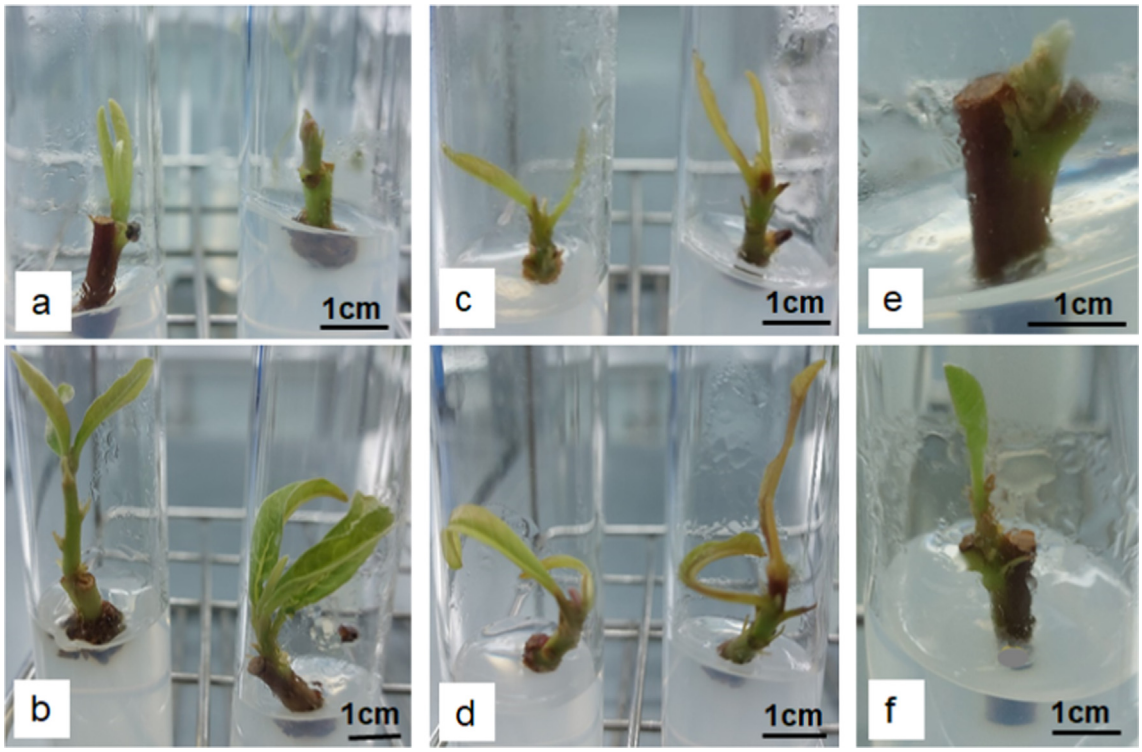

Fig. 2. Growth and development of *V. paradoxa* microshoots on MS/2 medium supplemented with: (1) 3:1.2:1 mg/L BAP:Kin:NAA: **a** and **c** (4 weeks), and **b** and **d** (6 weeks) on culture initiation media respectively; and (2) 1 mg/L Kin **e** and **f:** 4 and 6 weeks respectively on initiation media.

in rooted tubes due to callus degradation (Fig. 3b and Fig. 3c). The average root length measured was 2.2 cm (Table 5). However, no roots were observed on axillary buds of 8 weeks and older from the same treatment (Fig. 3d). No evidence of rooting was observed when shoots were cultured on media containing MS1B (Fig. 3e) and MS/2 media or MS/4.

These results show that *V. paradoxa* can be successfully rooted, although the axillary shoots must be very young. All microshoots

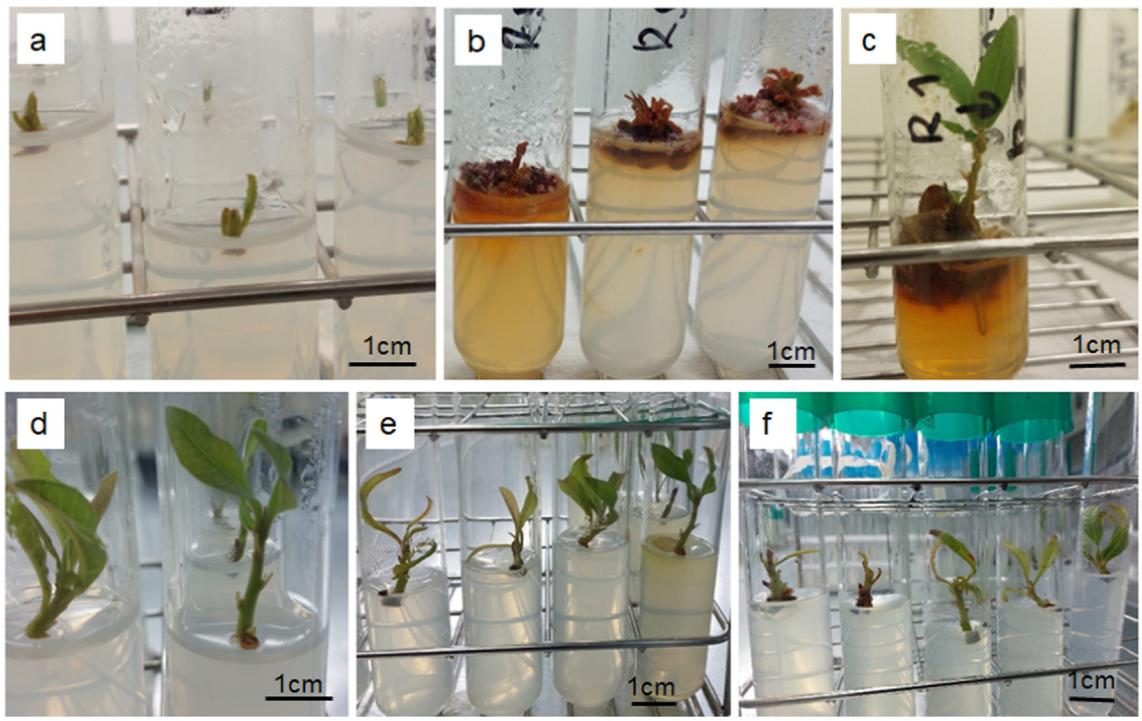

Fig. 3. Microshoots onto rooting media MS1B/2 containing 3:0.1:40 mg/mL IBA:mT:putrescine. (a) Four weeks (young) axillary buds on rooting media; (b) young axillary buds after 6 weeks; (c) *in vitro* rooted plantlet after 12 weeks, (d) 8 weeks axillary buds (old) on rooting media; (e) old axillary buds after a week; (f) old axillary buds after 12 weeks.

Table 5
Effect of culture media, putrescine, indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and 1-naphthalene acetic acid (NAA) on rooting percentage, root length, callus formation for shea tree (*V. paradoxa*) after 12 weeks of culture.

Medium	Plant growth regulators (mg/L)					CFo	Root Induction		RAL (cm)	HAB
	IBA	mT	IAA	NAA	Putrescine		4–6 ws	≥8 ws		
MS1B	0	0	0	0	0	N	–	–	–	100
	3	0	0	0	0	L	–	–	–	100
	3	0.1	0	0	40	M	–	–	–	100
	3	0.1	0	0	75	N	–	–	–	100
MS1B/2	0	0	0	0	0	N	–	–	–	100
	3	0	0	0	0	L	–	–	–	100
	3	0.1	0	0	40	M	R+	–	2.2 ± 0.3	100
MS/2	0	0	0	0	0	N	–	–	–	100
	3	0	0	0	0	L	–	–	–	100
	1.5	0	1	0.5	0	N	–	–	–	100
	1.5	0	0	0.5	160	N	–	–	–	100
MS/4	0	0	0	0	0	N	–	–	–	100
	3	0	0	0	0	L	–	–	–	100
	1.5	0	1	0.5	0	N	–	–	–	100
	1.5	0	0	0.5	160	N	–	–	–	100

CFo: Callus formation; N: None; L: Low; M: Moderate; 4–6 ws: 4 to 6 weeks axillary shoot; ≥ 8 ws: 8 weeks or older axillary buds; RAL: Root average length; HAB: healthy axillary bud; R+: root induction; (–): None.

that did not exhibit roots were still healthy at the end of the experiment (Fig. 3f).

3.3. Ex vitro acclimatization

The rooted plantlets were transferred to a substrate containing universal compost and perlite acclimated successfully. A survival rate of 100% was observed on both plantlets with one or two roots. Some plantlets showed signs of growth after 1 week and exhibited more green leaves compared to the initial color (Fig. 4b and Fig. 4d). The plantlets continued to grow, producing new leaves and elongating shoots.

3.4. Genetic fidelity studies of acclimated plantlets of V. paradoxa

3.4.1. Polymorphism of the 5 ISSR primers in V. paradoxa

To access the genetic fidelity of the regenerated shea trees, 5 ISSR primers were used. Due to the lack of knowledge of the level of polymorphism of these five markers in *V. paradoxa*, 10 shea trees were used to evaluate their variation. The total number of bands produced was 34, consisting of 23 polymorphic and 11 monomorphic bands (Table 6). The average polymorphic percentage was 65%. The highest polymorphic percentage was found in IS-4 (87.5%), while the lowest polymorphic percentage was found in IS-2 and IS-5 (50%). The polymorphism information content (PIC)

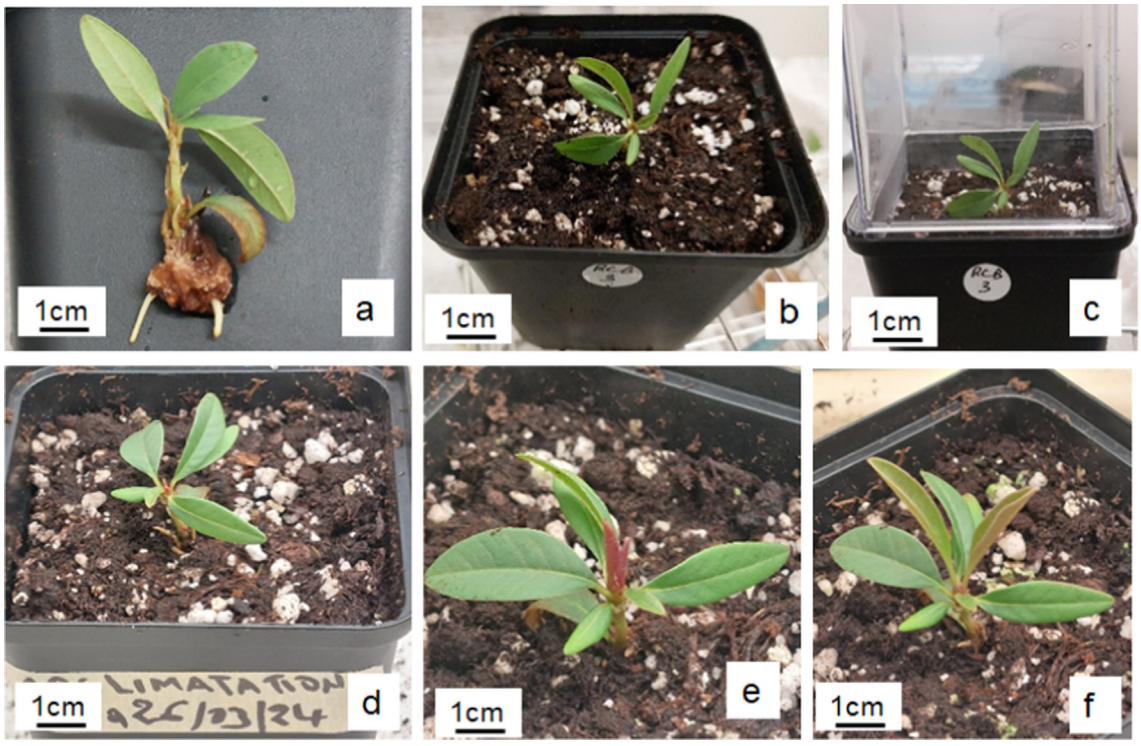

Fig. 4. *Ex vitro* acclimatization in universal compost-perlite mixture substrate. (a) Washed plantlet to remove adherent agar; (b) Plantlet transferred to substrate; (c) Plantlet in pot covered to keep moisture; (d) Plantlet after one week in substrate; (e) Plantlet after 2 weeks in substrate; (f) Plantlet after 3 weeks in substrate.

ranged from 0.17 (IS-5) to 0.32 (IS-3) with an average of 0.22 (Table 6). The ISSR markers IS-1, IS-3 and IS-4 produced the same number of bands, which was 8, while IS-5 produced the lowest number of bands, which consisted of 4.

Fig. 5 shows the polymorphism of the primer IS-4 within the 10 shea trees.

3.4.2. Genetic fidelity analysis

The five ISSR primers were then used to assess the genetic fidelity of five regenerated shea trees, since their polymorphic nature had been established. The screening process produced 34 scorable and reproducible bands, ranging from 200 to 2000 bp (Table 7). All ISSR primers successfully produced DNA bands with an average of 7 bands per primer.

The primer IS-1 produced the highest number of bands, with nine bands ranging from 200 to 1000 bp. In contrast, IS-2 produced the lowest number of bands, with 5 bands ranging from 300 to 900 bp. The banding patterns observed were monomorphic (Fig. 6a). Fig. 6b and Fig. 6c show the banding patterns between the regenerated shea trees and the donor plant.

4. Discussion

In vitro regeneration of *V. paradoxa* is challenging. Providing an efficient *in vitro* protocol for shea tree regeneration is a prerequisite for its domestication and conservation regarding climate change.

Plant growth can be influenced by the appropriate use of heterocyclic compounds as a factor of plant growth regulators. The efficiency of plant regeneration depends on the composition of the medium: macro- and micronutrients, type and concentration of growth regulators and type of carbon sources [46].

In this study, half-strength MS medium (MS/2) supplemented with Kin alone or combinations of BAP:Kin:NAA and BAP/NAA well-induced new axillary shoots and leaves. Previous studies reporting the use of half-strength MS on *V. paradoxa* have demonstrated its efficacy in axillary shoot induction [10,22,29]. Only half the strength of MS medium was used in this study since full strength has been shown to have an inhibitory effect on *V. paradoxa* axillary shoot induction [10,22,29]. Media containing full-strength MS may have exhibited an inhibitory effect due to excessive ion concentration [47].

In this study, cytokines and auxin were used as plant growth hormones to induce axillary shoots. Cytokines are known to stimulate axillary shoot development [48,49]. This is consistent with our investigation, in which media supplemented with Kin alone showed new axillary shoots on the explants. Several studies have used cytokines for *in vitro* shoot propagation of various woody plants and medicinal plants [22,49,50]. However, while BAP alone induced axillary buds, the induced shoots were not elongated in shea tree. Different results were obtained in the micropropagation of *Achras sapota* (Sapotaceae) [27].

Table 6
Polymorphism assessment of the 5 ISSR primers within 10 shea tree genotypes.

Primers	Sequence	Tm (°C)	TB	PB	MB	%P	%M	PIC
IS-1	$(AG)_8G$	52.8	8	5	3	62.5	37.5	0.18
IS-2	$(GA)_8T$	50.4	6	3	3	50	50	0.14
IS-3	$(GA)_8C$	52.8	8	6	2	75	25	0.32
IS-4	$(TC)_8C$	52.8	8	7	1	87.5	12.5	0.3
IS-5	$(AG)_8YT$	52.6	4	2	2	50	50	0.17
Total/Mean	–	–	34	23	11	65	35	0.22

Tm (°C): annealing temperature; TB: total bands; PB: polymorphic bands; MB: monomorphic bands; %P: percentage of polymorphic bands; %M: percentage of monomorphic bands; PIC: polymorphism information content.

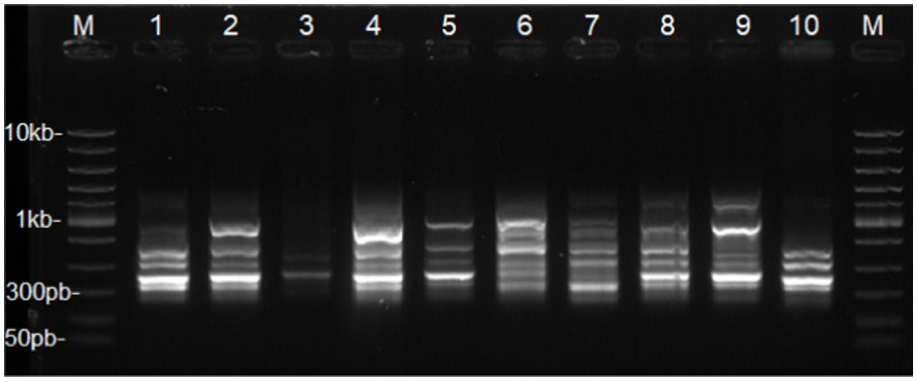

Fig. 5. Polymorphic band patterns of the ISSR primer IS-4 within the shea trees; M: Fast DNA Ladder; numbers represent the genotypes.

Table 7
Five ISSR primers used in genetic fidelity assessment of *V. paradoxa*.

Locus code	Primer sequence	Tm (°C)	Number of bands	Range of bands size (bp)
IS-1	5′ AGA GAG AGA GAG AGAGG 3′	52.8	9	200–1000
IS-2	5′ GAG AGA GAG AGA GAGAT 3′	50.4	5	300–900
IS-3	5′ GAG AGA GAG AGA GAGAC 3′	52.8	6	200–1200
IS-4	5′ TCT CTC TCT CTC TCTCC 3′	52.8	8	300–2000
IS-5	5′ AGA GAG AGA GAG AGA GYT 3′	52.6	6	300–1000

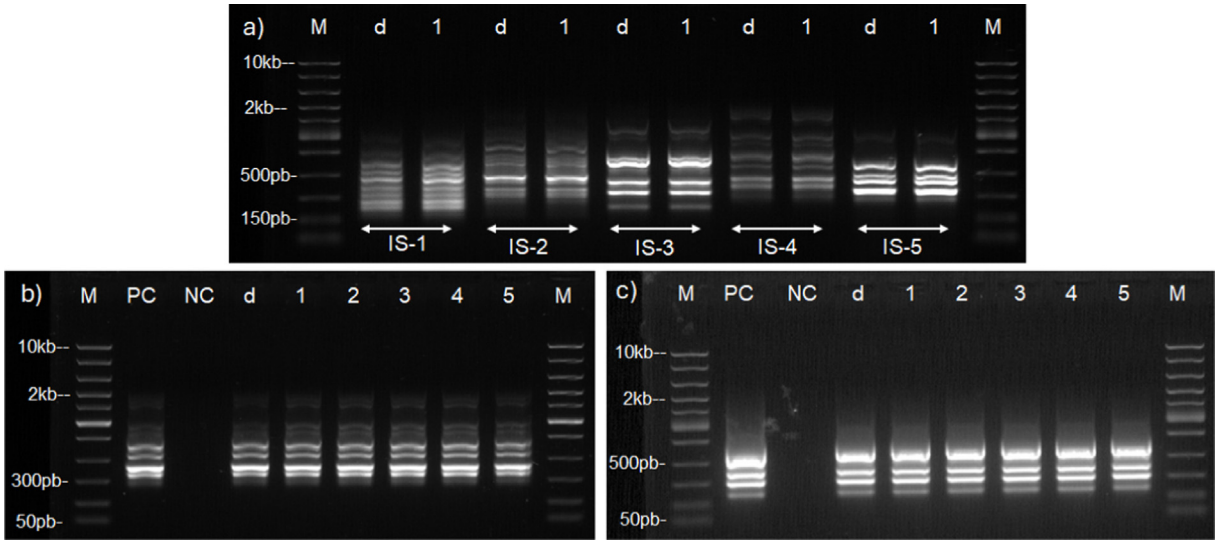

Fig. 6. Genetic fidelity assessment. (a) The five ISSR molecular markers profiling of *in vitro* regenerated plants with donor plants of V. paradoxa. M: Fast DNA Ladder; d: Donor plant DNA banding pattern; 1: in-vitro regenerated plants DNA banding patterns; (b) banding pattern between regenerated plants with IS-4; (c) banding pattern between regenerated plants with IS-3. PC and NC represent positive control and negative control respectively.

The combination of cytokines and auxin has often shown better growth and shoot proliferation in shea tree [10] and *Madhuca latifolia*, a Sapotaceae plant species [25]. Our results are consistent with this, as we obtained better shoot proliferation and growth when cytokines and auxin were combined.

Combinations of the cytokines used in different proportions affected the growth in different patterns. In this study, explants showed variable responses to different concentrations and combinations of the plant growth regulators BAP, NAA and Kin. Numerous morphogenetic responses have been reported for *V. paradoxa* in the presence of various plant growth regulators [10,22,29].

The concentration of cytokines used in different combinations promoted the growth of new axillary shoots and leaves as the experiment progressed. Similar results have been reported with increasing shoot length of *V. paradoxa* cultured on media supplemented with different ratios of BAP:NAA [10] and GA_3/BAP [29]. The combination of two cytokines (BAP and Kin) with an auxin (NAA) produced the best results in terms of number of leaves and shoot length, compared to the combination of a cytokine and an auxin or the use of a single hormone. Authors reported that the combination of two different cytokines induced a higher number of shoots and higher shoot lengths compared to the singular effects of the individual hormones [29,51]. The particular death rate (6.67%) of induced shoots on MS/2 media supplemented with BAP, Kin and NAA is attributed to the size of the initial explants. Indeed, explants shorter than 1.5 cm were converted to callus.

To our knowledge, the shea tree is a recalcitrant species in tissue culture. In fact, adventitious root formation is very difficult to achieve, while it is critical for successful vegetative propagation of this species [17]. The development of healthy and vigorous roots is essential for successful acclimatization to ex vitro conditions.

The high percentage (100%) of rooting was obtained when young new axillary shoots (4–6 weeks) were transferred to media containing 3 mg/L IBA, 0.1 mg/L *m*T and 40 mg/L putrescine, while no signs of rooting were observed in the same media with old axillary shoots (7 weeks and older), can be explained that *V. paradoxa* rooting is efficient when the explant is young. However, older axillary shoots could be used as explants for subculture because they had a high average shoot length. This result is consistent with that obtained in a previous study of *V. paradoxa* cuttings rooting using juvenile and mature shea trees treated with IBA [17]. The authors observed a rooting rate of 41% in juvenile shea trees, while only 28% was observed in mature shea trees. The use of plant growth regulators such as putrescine has been shown to have a positive effect on adventitious root formation in *Argania spinosa* (Sapotaceae) [24] and other plant species [52]. In our study, no signs of rooting were observed on media containing half and quarter-strength MS media. However, authors observed 8% rooting of *V. paradoxa* [10]. *In vitro* rooting was reported when a combination of BAP/NAA was used for an axillary shoot induction study on shea tree [22].

Plantlet acclimatization is a critical step that determines the efficiency of the entire micropropagation process. During acclimation, the regenerants are exposed to new growth conditions that cause biotic and abiotic stresses. These stresses include water loss, tissue dehydration, and reduction in synthesis processes [53]. The substrate used during acclimation has a significant effect on the survival and growth of plantlets regenerated by *in vitro* culture [24,54,55]. Other authors noted the positive effect of *meta*-topolin on plantlet acclimatization when used in shoot induction media [56,57,58]. The findings of this study show that *V. paradoxa* plantlets transferred in potting soil-perlite mixture showed efficient adaptation to ex vitro conditions, as well as good growth and development. It could be postulated that a low concentration of *meta*-topolin (0.1 mg/L) in the shea tree rooting medium could improve rooting and the acclimation of *in vitro* rooted plantlets. However, further studies are needed to confirm this claim. Mixture substrate, such as the combination of potting soil and perlite, has demonstrated its efficiency in ex vitro acclimation of plant species of the Sapotaceae family [24,33].

Few studies have been conducted on micropropagation systems of Sapotaceae family plant species, and none of them has evaluated the genetic homogeneity of plants grown *in vitro*. ISSR markers have been widely used to assess genetic fidelity in various plant species [36,38,40,41,59,60].

In this study, the polymorphic level of 5 ISSR primers was primarily evaluated in shea tree. The polymorphic and monomorphic percentages were 65% and 35%, respectively, while the polymorphism information content was 0.22. This indicated a moderate genetic diversity among the shea trees using the ISSR markers [42]. Regarding the polymorphism information found in this study, the ISSR primers demonstrated the advantages of ISSR

markers in detecting genetic variation in *V. paradoxa*. Therefore, the five ISSR primers were then used to evaluate the genetic fidelity of *V. paradoxa* plantlets regenerated by our *in vitro* regeneration protocol. The use of molecular markers to investigate genetic fidelity in plants with long juvenile periods (10–15 years) such as *V. paradoxa* is primordial. Regarding the genetic fidelity analysis, the five ISSR primers produced 34 bands ranging from 200 to 2000 bp. These results were consistent with previous genetic fidelity assessment studies in *Muehlenbeckia platyclada* micropropagation [40], in *Crinum brachynema* [61], *Lilium* spp [62], *Curcuma angustifolia* [59], and *Acacia auriculiformis* [60]. The plantlets produced *in vitro* did not show any polymorphisms as the DNA bands amplified with the ISSR primers were monomorphic to the donor plant (Fig. 6).

5. Conclusions

The efficiency of micropropagation of *V. paradoxa* depends on the Murashige and Skoog medium composition, the type and concentration of plant growth regulators. This study showed differences in the response of *V. paradoxa* tissue cultures to different concentrations and different regimes (single or combined) of plant growth regulator application. The best shoot regeneration response was observed on half-strength MS medium supplemented with 3:1.2:1 mg/L BAP:Kin:NAA. The presence of the cytokinin Kin in the hormone combinations resulted in better shoot growth and leaf development, although the cytokinin Kin alone did not induce vigorous shoot growth. Rooting was induced only on half strength of modified MS medium with 3:0.1:40 mg/L IBA:*m*T:Putrescine. Plantlets were successfully acclimated on a universal compost-perlite mixture substrate. No genetic alterations were found in the regenerated plants based on ISSR analysis. The developed micropropagation protocol is the most advanced in *V. paradoxa* micropropagation, and genetic fidelity analysis is helpful for mass multiplication, conservation, and *in vitro* culture-based biotechnological experiments. The micropropagation protocol presented in this study can be used for routine *in vitro* regeneration of *V. paradoxa*. However, further studies are required for the induction of multiple microshoots.

CRediT authorship contribution statement

Affi Jean Paul Attikora: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Formal analysis, Conceptualization. **Souleymane Silué:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Mongomaké Kone:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **Napkalo Silue:** Writing – review & editing, Methodology. **Yves Kwibuka:** Writing – review & editing. **Saraka Didier Martial Yao:** Writing – review & editing, Resources, Methodology. **Caroline De Clerck:** Writing – review & editing. **Sok Lay Him:** Writing – review & editing, Methodology. **Nafan Diarrassouba:** Writing – review & editing, Supervision, Resources, Methodology. **Taofic Alabi:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization. **Ludivine Lassois:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

Data availability

All data supporting the findings of this study are included within this manuscript.

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Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2025.01.007.

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