VOL. 10, NO. 1, JANUARY 2015 ISSN 1990-6145
ARPN Journal of Agricultural and Biological Science

www.arpnjournals.com

A TISSUE CULTURE STRATEGY TOWARDS THE RESCUE OF ENDANGERED MASCARENE *ALOES*

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ABSTRACT

The present study aimed at developing an efficient protocol for the micropropagation and restoration of four endemic threatened Masacrene *Aloes* namely *Aloe lomatophylloides, A. macra, A. purpurea* and *A. tormentorii*. Explants consisting of 2cm long hypocotyls with radicles were cultured on Murashige and Skoog's (MS) basal medium supplemented different plant growth regulators Thidiazuron, Benzyl amino purine and Naphthalene acetic acid (TDZ, BAP, NAA). The diameter of protocorm-like bodies (PLBs) and the length of developing shoots were calculated using digital photography. All explants produced significantly ($p<0.05$) higher number of PLBs on MS containing 0.01 NAA mg/l except for *A. tormentorii* explants whereby more PLBs were obtained in MS with NAA and TDZ. Over 95% of rooted plantlets survived acclimatisation. The genetic integrity of the regenerated and acclimatised plantlets were evaluated by employing RAPD, ISSR and IRAP marker assays and results indicated that all the tissue culture-derived plants are true-to-type. Preliminary secondary metabolite screening from the in vitro regenerated *Aloe* plantlets revealed the presence of numerous bioactive components such as anthraquinones, phenols and flavonoids, and possible presence of alkaloids, coumarins, tannins, saponins and terpenes which are also detected in the crude extracts of their field counterparts. This study paves the way towards a rapid propagation and restoration strategy for endangered medicinal Mascarene *Aloes*.

Keywords: aloes, micropropagation, protocorm like bodies, Mauritius, Reunion.

INTRODUCTION

Plant biodiversity is threatened worldwide at an unprecedented rate due to environmental perturbations, habitat loss and increased extinction rates. Numerous species are listed as rare or critically endangered, and integrated programs are required to safeguard many of these species. A significant portion of all plant species worldwide is restricted to islands, [1]. Together with Madagascar, the Western Indian Ocean Islands are considered as one of the 35 global biodiversity hotspots [2, 3] and are well known for their unique biodiversity and high level of endemism [4].

The succulent-leaved genus *Aloe* L. (Asphodelaceae) which now includes the former genus *Lomatoplyllum* comprises over 500 species occurring throughout Africa, the Arabian Peninsula, Madagascar and Mascarene Islands [5, 6]. *Aloes,* particularly the reputed *Aloe vera (Aloe barbadensis* Miller*)* have been used in traditional medicine for centuries and are found in many pharmacopoeias. The Aloe gel contains many constituents which are known for their antimicrobial and anti-inflammatory actions wound healing properties [7, 8], antidiabetic effects [9], anticancer and antiviral properties [10, 11], while antioxidant effects are becoming of interest [12]. Anthraquinones present in most Aloes are well reputed for their biological properties, which include anti-inflammatory, anticancer, antiprotozoal and antioxidant activity [13, 14, 15]. The exclusive presence of 12 phenolic compounds in the plant sap with the important ones being aloin and aloe emodin, are associated with pharmacological properties such as antibacterial and antifungal.

Today, *Aloes* are a world-renowned source of natural products derived from the leaf exudate and the gel-like leaf mesophyll. Species such as *Aloe ferox* Mill. in South Africa and *Aloe secundiflora* Engl are wild-harvested to cater for the international trade in natural products from the bitter exudate and gel of *Aloes*, but *A. vera* still remains the principal source of these products [16]. Due to their reputable medicinal properties, Aloe-derived compounds are currently used for the manufacture of tropical products such as ointments and gel preparations, as well as in the production of tablets and capsules. In the food industry, they are employed as functional food and as an ingredient in other food products, for the production of gel-containing health drinks, beverages and yogurt.

The Mascarene *Aloes* which are part of the former section *Lomatophyllum* comprise *A. tormentorii* (Marais) Newton and Rowley and *A. purpurea* Lam. which are endemic to Mauritius [17, 18], *A. macra* Haw. and *A. lomatophylloid*es Balf.f endemic to Réunion Island and Rodrigues Islands respectively [19, 20]. *A. tormentorii* grows only on two small rocky islands (Round Island and Gunner's coin) in the north of Mauritius. *A. macra*, *A. purpurea* and *A. lomatophylloid*es are nowadays only found inwards in the mountains, since the natural coastal habitats of islands have been severely degraded. In the Mascarene Islands, the crushed leaves of these *Aloe* species are applied as poultice on muscular pains, wounds, boils and to treat cutaneous bacterial infections. The plants are also used internally as antispasmodic and to increase menstrual flow [17]. The leaf sap is applied to the breast to encourage weaning [21]. Mascarene *Aloes* have been reported to possess phytochemical and antimicrobial

attributes [15], which validate the use of Mascarene *Aloes* in the Mauritian and Réunion folk medicine. The bioactive components detected include anthraquinones, phenols and flavonoids, and possibly alkaloids, coumarins, tannins, saponins and terpenes [15]. As the Mascarene *Aloes* are being drastically invaded in their habitat by exotic flora, there is an urge to develop propagation strategies for conservation.

The *Aloe* genetic diversity has been significantly reduced due to cytoplasmic male sterililty [22, 23], long vegetative regeneration, low recombination potential [24] and abortion of some interspecific hybrids [25]. As the natural rate of reproduction by seeds is low, plant tissue culture is proposed as an alternative for mass-propagation of endemic Mascarene *Aloes*, either to meet the increasing demands of the medicinal *Aloes* or for conservation purposes. Although *Aloe* species are difficult to culture owing to the fact that the *Aloe* explants secrete phenolics that lead to browning and often death of the explants, some *Aloe* species have successfully been cultured in vitro by various researchers. In vitro plant regeneration has been reported in *A. vera* from young and strong underground stems [26, 27], meristem segments [28] and inflorescence axis-derived callus cultures [29]. *Aloe polyphylla*, one of the rarest and most unique of all *Aloe* species was regenerated using seeds as explants for conservation purposes [30]. *A. arborescens,* globally rated as one of the most important species in the *Aloe* genus has been successfully regenerated in vitro using seeds and young inflorescence as explants [25, 31]. Cytokinin types and concentrations have been reported to influence in vitro secondary metabolite production in *A. arborescens* with variable amounts of total iridoids, phenolics, flavonoids and condensed tannins produced in regenerated shoots of this species [31]. Nevertheless, to date no report has been published on the in vitro culture of endemic threatened medicinal Mascarene *Aloes.*

Plant tissue culture techniques have been widely employed in the propagation of a large number of threatened plants and the management of botanical collections. The maintenance of genetic fidelity is critical when using plant tissue techniques for conservation purposes. However, plant cell cultures are known to induce somaclonal variation. Molecular techniques represent powerful and valuable tools used in the analysis of clonal fidelity of in vitro regenerated plants. Polymerase chain reaction (PCR) techniques, randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers have been favoured because of their simplicity, cost-effectiveness, stability, sensitivity, high reproducibility and reliability [29, 32]. IRAP (inter-retrotransposon amplified polymorphism) is a potential marker due to the abundance of retroelements and their ability to create new copies. Furthermore it is easy to assess and could be established at low cost [33]. RAPD markers have been used to assess the genetic fidelity of in vitro generated *Aloe vera* plantlets and detected an overall of 86% variations in the regenerants produced via an intermediate callus phase, as compared to regenerants produced following direct regeneration of axillary buds, where no variation was observed [29].

In view of the previous reports on the phytochemistry, bioactivity and endangered status of the Mascarene *Aloes* [15, 34, 35], this work proposes an efficient protocol for the in vitro regeneration of endemic Mascarene *Aloes* towards conservation and restoration purposes. Likewise, preliminary comparative phytochemical screening from wild and in vitro propagated *Aloe* species is reported. IRAP and ISSR markers have been used to check for genetic fidelity of the regenerated Mascarene *Aloe* plantlets.

EXPERIMENTAL

Tissue culture

Plant material and explant preparation

Immature berry fruits of *A. pupurea*, *A. tormentorii*, *A. lomatophylloides* and *A. macra* were harvested from field grown plants (National Parks and Conservation Service (Mauritius) Mauritius Herbarium garden, MSIRI and Conservatoire National Botanique de Mascarin (Réunion). It is assumed that all plants had been cultivated under the same agroclimatic conditions in view of the similar geography and ecology of these three Mascarene Islands. The fruits were washed with detergent and surface sterilised with 1.5 % Benlate and few drops of Tween 20 (wetting agent), for 30 min. In the laminar flow cabinet, the fruits were treated with 1.5 % Sodium hypochlorite for 10 min, rinsed with sterile water and longitudinally cut to remove the seeds. The seeds were then sterilised with 0.5% sodium hypochlorite for 10min, followed by 70% ethanol for 1 min. To remove any traces of sterilant, the seeds were washed with at least five changes of sterile distilled water for 10 min. The seed coat was then scratched using a sharp blade and the seeds were used for in vitro germination.

Culture media

The basal culture medium consisted of Murashige and Skoog's (Murashige and Skoog 1962) used singly or supplemented with auxin; α- naphthaleneacetic acid (NAA) and cytokinin; 6- benzylaminopurine (BAP) or thidiazuron (TDZ). 3% Sucrose was added as the carbon source and the medium was solidified with phytagel (2.4g/l). The pH of the medium was adjusted to 5.86 with 1 N NaOH or HCl prior to autoclaving at 121ºC and 105kPa for 20 min. The media were poured in 50 ml culture tubes (20ml/culture tube) and slants were prepared to give a maximum surface for the tissues to grow.

Culture conditions and culture establishment

The seeds were aseptically germinated in hormone free culture media. After germination, approximately 1.5 cm long hypocotyls with the radicle were aseptically inoculated on MS medium supplemented with plant growth regulators (TDZ, BAP and NAA).

The cultures were maintained at a temperature of 24 ±2°C and exposed for 16hr per day to an illumination

1200 ±100 lux provided by cooled fluorescent lamps. After 2 weeks, culture-tubes showing uniform growth of protocorm - like bodies (PLBs) were selected and were used for digital photography. A minimal of 15 culture tubes were inoculated for each different treatments and readings were taken at fortnightly intervals.

Digital photography and growth assessment

The method of digital photography for growth assessment [7] was used to study in vitro development of explants. Briefly, after 2 weeks photographs were taken using a Samsung TL110 Digital Camera at 2 weeks intervals. A small piece of graph paper (graduated paper) was stuck on the culture tubes as a scale to estimate the growth/increase in size (mm) of the PLBs, prior to taking photographs. Using the software Scion Image (Alpha 4.0.3.2), the diameter of the PLBs was calculated by measuring the vertical length of the PLBs and length of the longest leaf to its base, for plantlets.

Statistical analysis

The data were collected at 2 week intervals after explant inoculation on MS medium supplemented with plant growth regulators. The statistical analysis was carried out by using SPSS 16.0 and the results are expressed as mean ± standard error and the significance of differences among mean values was analyzed by using t-test with $P < 0.05$.

Acclimatisation

Prior to first phase of acclimatization, the well-developed and rooted in vitro regenerated plantlets were taken out of the media, separated individually by hand and washed carefully under running tap water to remove traces of media from the roots. The basal parts of the plantlets were then dipped in 0.1% solution of Benlate (fungicide) for 2 min and the elongated roots were pruned. The plantlets were then transferred in plastic trays containing a mixture of sand and soil in a ratio of 1:1 and kept in a shade house at an average temperature of 29 ±1.8°C and light intensity of 6000 to 10 000 lux. In the second phase of acclimatization (after 1 month), the partially acclimatised plants were transferred in plastic bags containing soil and shifted to a net house where the average temperature was 31.4 ± 0.97°C and light intensity varied between 45 500 to 54 400 lux.

Assessment of genetic fidelity by RAPD, ISSR and IRAP

Total DNA was extracted from in vitro leaflets regenerated Mascarene *Aloe* plantlets (5th Subculture) and the mother plant [15]. PCR reactions were performed in a volume of 25 µL containing 1X Taq PCR Buffer, 2.5mM $MgCl_2$, 0.20 mM dNTP Mix, 0.5µM primer, 1 U Taq polymerase, 25 ng DNA template and made up to final volume with sterile Millipore water using the Applied Biosystems 2720 Thermal Cycler. For RAPD-PCR, 30 random primers of operon series OPA and OBA were used for screening for indexing polymorphism in tissue culture-raised clones. The thermal profile for RAPD was as follows: 90 s denaturation at 95°C, annealing for 40 cycles for 30 s at 94°C, 1min at 35°C, 3 min at 72°C, extension for 10 min at 72°C and 5 min at 15°C. The ISSR - PCR reaction program consisted of: initial denaturation of 94ºC for 7 mins; 35 cycles of 94ºC for 30 s; 53ºC for 1 min (depending on optimized annealing temperature); 72 ºC for 2 min and a final extension of 72ºC for 10 minutes. The thermal profile for IRAP was similar to ISSR, but the annealing temperature used was 50°C. 30 ISSR primers and 30 IRAP primers were screened to detected polymorphism.

Phytochemical studies

Metabolite extraction

Lyophilised leaflets of in vitro regenerated and leaves of mother plants from wild Mascarene *Aloes* were used. *A. vera* was used as a standard and the in vitro regenerated *A.vera* was supplied by Food and Agriculture Research Council (Réduit, Mauritius). 0.1 g of dried sample was extracted in 10 ml solvent (Dichloromethane: methanol. 1:1 v/v) by sonication for 48hr. The crude extract was filtered and concentrated to dryness using the in vacuo at 50°C. Residues were re suspended in 10 ml of Dichloromethane: methanol.

Phytochemical screening

Phytochemical screening of the major constituents was done using qualitative methods [36, 37], through a series of test tube tests for coumarins, tannins, anthraquinones, leucoanthocyanins and flavonoids. Phenols, alkaloids, steroids and terpenes were identified by thin layer chromatography (TLC) techniques and UV techniques.

RESULTS

PLB induction and proliferation

Germination of inoculated seeds occurred between the 3rd and 4th week of culture. Upon the culture of explant (hypocotyl containing radicle) in medium supplemented with the different PGRs, formation of a massive structure originating from the base of the explants was observed as from week 2 (Figure-1a). This structure lengthened and subsequently developed into protocorm - like structure (PLS), which appeared in large clusters (PLBs). Interestingly, based on the recorded parameters, PLB induction was also observed on MS medium devoid of any PGRs for all the *Aloe* species under study, but the rate of induction was relatively slow. The PLBs after 3-4 of culture became green in colour and appeared to be globular or granular in shape in week 4 (Table-1) in all the media except for MS 5 and MS 6, which appeared to be green in week 8 (Table-1).

As per Table-2, significantly maximum growth ($p<0.05$) of PLBs was obtained on MS 2 (0.01 TDZ mg/l + 0.01 NAA mg/l) followed by MS 1 (2.0 BAP mg/l + 0.01 NAA mg/l) for *A. purpurea*: 4.72 mm; *A. lomatophylloides*: 4.67 mm; *A. macra*: 6.35 mm) except for *A. tormentorii* whereby maximum growth (5.82 mm)

of PLBs was obtained in MS 2 (0.01 TDZ mg/l + 0.01 NAA mg/l) followed by MS 3 (2.0 mg/l BAP). Highest PLB development was observed in *A. macra.* (6.35 mm) followed by *A. tormentorii* (5.82 mm) (Table-2). The globular structures of plantlets developed shoot primordia at week 9 (Figure-1b) and adventitious shoot proliferation was observed as from week 10 (Figure-1c) for all the species except *A. tormentorii*. Root formation started as from week 12 (Figure-1d). Maximum and fastest plantlets regeneration were observed in *A. macra*, while for *A. tormentorii,* the rate and time for plantlets formation was relatively slow (as from week 11). *A. purpurea* and *A. tormentorii* cultures did not show PLB maturation on MS 1, MS 2, MS 3 and MS 4 (Table-1) after week 12 and, the PLBs were transferred to basal medium where they matured and developed into plantlets.

Acclimatisation of in vitro regenerated plantlets

In the first phase of acclimatisation, 30 days after transplanting, over 95 % of the *Aloe* plantlets under study survived and had newly emerged leaves indicating that the media was suitable in providing favourable anchorage for the plant as well for the development of root (Figure-2a). All the partially acclimatised plantlets survived following secondary acclimatisation (40 days), when the plants were kept in a green house in plastic bags containing soil (Figure-2b). There was no visible variation among micropropagated plantlets on their vegetative growth. *A. purpurea*, and *A. tormentorii* started to show characteristics similar to their field counterparts (for example: leaf, margin and prickles colour) at a very early stage as soon they were acclimatised.

Assessment of genetic fidelity

Out of the several primers screened used for preliminary screening, only 8 RAPD, 5 ISSR primers and 3 IRAP primers resulted in scorable bands and were selected for further study. These primers produced distinct amplification profiles and displayed monomorphic banding pattern in all the in vitro generated plantlets for all the treatment and the mother plant. The representative profile of the in vitro raised plants and the mother plant with primer ISSR and IRAP 2 are shown in Figure 3 and 4.

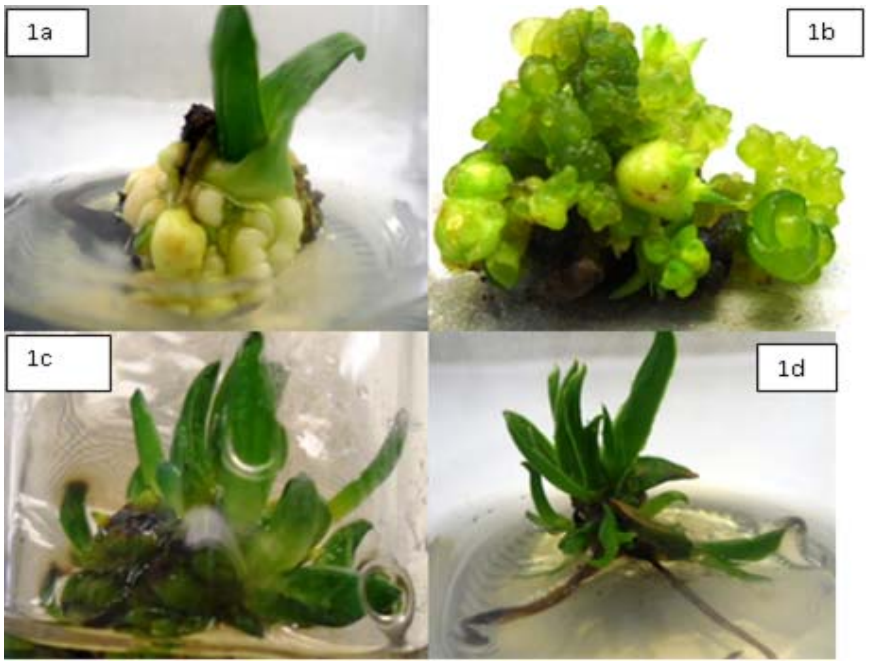

Figure-1. (a) PLS formation at the base (arrow) of *A. purpurea* explants on MS 1 **(b)** Proliferating PLBs of *A. macra,* with multiple shoot primordia) in Week 9. **(c)** Adventitious shoot proliferation in *A. macra* as from Week 10. **(d)** Adventitious shoots proliferation and root formation in *A. lomatophylloides* on MS 2.

Figure-2. (a) Acclimatized plantlets of *A. purpurea*in mixture of sand and soil, in the shade house **(b)** Well-acclimatized plant of *A. tormentorii* grown in soil in green house.

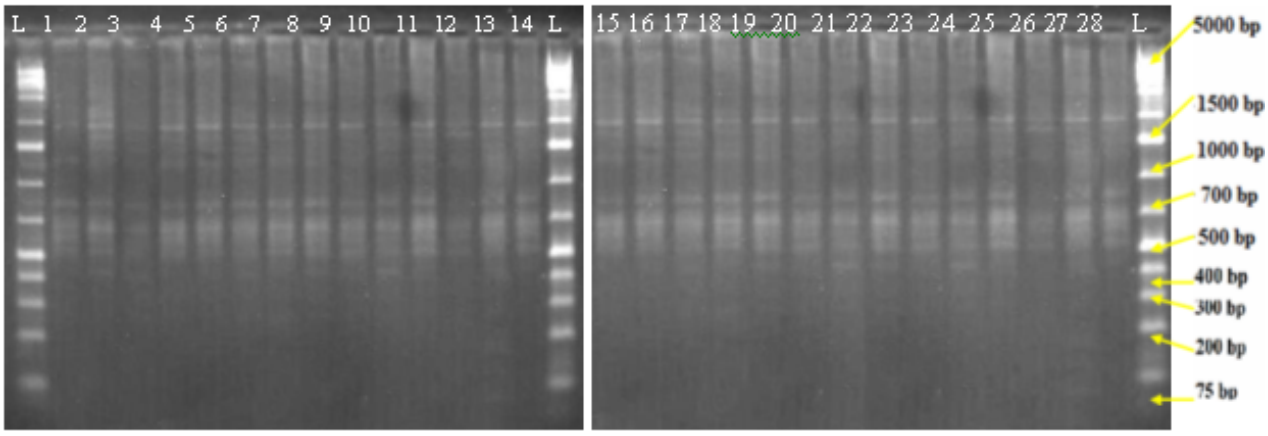

Figure-3. ISSR pattern produced from DNA amplification using primer ISSR 2. Lanes L: O Gene Ruler 1kb Plus DNA ladder. Lanes 1-7*: A.purpurea* (Mother plant and clones from each treatment MS 01 -06). Lanes 8-14*: A. tormentorii* (Mother plant and clones from each treatment MS 01 -06); Lanes 15-21: *A. lomatophylloides* (Mother plant and clones from each treatment MS 01 -06); Lanes 21-28*: A. macra* (Mother plant and clones from each treatment MS 01 -06).

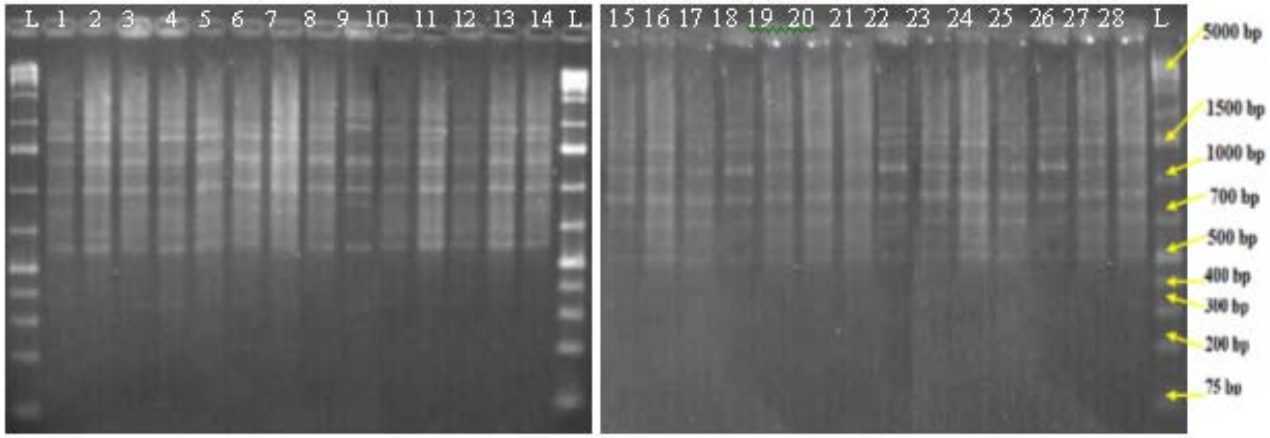

Figure-4. IRAP pattern produced from DNA amplification using primer IRAP 6. Lanes L: O Gene Ruler 1kb Plus DNA ladder. Lanes 1-7*: A.purpurea* (Mother plant and clones from each treatment MS 01 -06) . Lanes 8-14: *A. tormentorii* (Mother plant and clones from each treatment MS 01 -06); Lanes 15-21*: A. lomatophylloides* (Mother plant and clones from each treatment MS 01 -06); Lanes 21-28: *A. macra* (Mother plant and clones from each treatment MS 01 -06);

Phytochemical analysis

Using the standard phytochemical screening protocol, the compounds detected in the in vitro regenerated plants appear to be similar as in their wild counterparts (Table-3). The classes of compounds detected were anthraquinones, phenols and flavonoids, and possibly alkaloids, coumarins, tannins, saponins and terpenes.

DISCUSSIONS

In the present study, a system for the in vitro plant regeneration of Mascarene *Aloes* was developed using hypocotyls including radicle as explants. Induction of somatic embryogenesis from aseptically germinated seedling explants of *A. pretoriensis* [39], *A. ferox* [40] and *A. polyphylla* [30] has been reported. The use of seeds for the establishment of primary cultures can prevent most of the decontamination problems that are often associated with explants establishment [30]. In this experiment, the use of immature seeds as starting materials has resulted in efficient establishment of sterile cultures. Upon the culture of the hypocotyl containing the radicle on MS medium supplemented with NAA, BAP and TDZ, the formation of protocorm-like bodies was observed. Direct PLB induction was first reported in a *Cymbidium* sp from in vitro culture of the shoot apex used for producing virus-free plants [41]. Initially, the term PLBs was used for orchids only, but later on, PLBs have been reported in a wide range of other plant genera including *Heliconia* [42], *Musa* [43], *Pinellia* [44], *Rosa* [45], and *Syngonium* [46]. However, to our best knowledge, there is no report on the induction of PLBs in the genus *Aloe.* Micropropagation through PLB induction without intervening callus phase is a preferred method by most researchers because it is rapid and efficient, and ensures genetic stability in clones [47].

The type and concentration of PGRs have been shown to influence the direct induction and the proliferation of PLB in tissue cultures [47]. In this study, the remarkable finding is that PLBs formation was observed in all the treatments for the all Mascarene *Aloes*. However, maximum growth of PLBs was obtained on medium containing both TDZ and NAA for all the species. Among cytokinins, TDZ is known to exhibit a higher activity than BAP and other cytokinins like zeatin and kinetin [48, 44]. Even at a very low dose (0.01mg/l), TDZ treatment showed highest PLB formation. However, prolonged culture of PLBs in medium supplemented with TDZ gave rise to necrotic tissues implying that after a certain time, it is advisable to transfer the PLBs to basal media. Conversion of PLBs into plantlets was reported to occur without the influence of PGRs [49, 50]. In the present work, *A. macra* and *A. lomatophylloides* successfully showed PLB maturation and plantlets formation for all the treatments, except for *A. tormentorii* and *A. purpurea* where no regeneration was obtained (Table-1) after week 8. Therefore these PLBs were transferred to basal medium (MS) where they matured and developed in plantlets after 4 weeks. The differences in PLB maturation and plantlets formation between the different Mascarene *Aloes* account for the fact that different genotypes respond differently to in vitro culture. Genotypic differences have been demonstrated in many plant tissue culture studies [51, 52, 53].

Significant efforts have been made to optimise the conditions for in vitro stages of micropropagation, but the process of acclimatisation of many micropropagated plants to the soil environment has not been fully studied. Consequently the acclimatisation phase continues to remain a major bottleneck in the propagation of many plants [54]. In this study, various successful protocols reported earlier were considered, for example washing, pruning and treating the roots micropropagated plantlets with a fungicide [47, 55]. Washing thoroughly and treating the roots with fungicide avoided microbial contamination. Similarly, root-pruning is known to largely facilitate the ex vitro establishment of in vitro regenerated plants, as it enhances regeneration of new roots [47]. In the present work, successful acclimatisation of the tissue cultured Mascarene *Aloes* was observed with a survival rate of over 95%. Furthermore, the micropropagated *Aloes* plants did not show any detectable variation in morphological or growth characteristics when compared with their respective field donor counterparts.

Genetic fidelity testing of the micropropagated plantlets needs to be authenticated for conservation purpose and large scale application of any developed micropropagation protocol especially in the case of endemic medicinal species. PCR-based molecular markers have emerged as simple, fast, reliable and labour-effective tools for testing the genetic fidelity of in vitro raised plantlets. In the present investigation, none of the primers showed any discrepancy in the banding pattern (Figures 3 and 4) between the micropropagated and source plants, indicating that the micropropagated plants were genetically identical to the field plant and no variation was induced during in vitro propagation. Many investigators have reported genetic stability of several micropropagated plants: *Citrus* [33]; *Cymbopogon pendulus* [56]; *Clivia miniata* [57]; *Ceropegia bulbosa* [58]; *Gloriosa superba* [32] using RAPD, ISSR and IRAP analysis. As observed, the results of this study indicate the genetic integrity and true-to-type nature of the micropropagared endemic Aloe plants.

The capacity for plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of in vitro technology. The strong and growing demand in today's marketplace for natural, renewable products has refocused attention on in vitro plant materials as potential factories for secondary phytochemical products [59]. In vitro plant cultures often produce secondary metabolites in quantities equal to those produce by the plants in nature [60]. Since production of secondary metabolites is generally higher in differentiated tissues, there are many attempts to culture shoots and roots in vitro for the production of medicinally important compounds. However, as compared to cell cultures and root cultures, shoot cultures remain a preferred method for the production of secondary metabolites due to their genetic stability and better capacities for secondary metabolite production, especially

where metabolite synthesis is specially confined to the shoots [30, 61]. In *Aloe* species, most of the bioactive secondary metabolites (e.g. the anthraquinones aloin and aloe-emodin) are found mainly in the succulent leaves [16, 62, 63]. However, in vitro secondary metabolite production in plants may be influenced by the choice of the PGR [31]. In the present experiment, a preliminary detection of secondary metabolite compositions from the leaves of in vitro regenerated *Aloes* plantlets revealed the presence of the same classes of compounds, including anthraquinones, phenols and flavonoids, and possibly alkaloids, coumarins, tannins, saponins and terpenes that have been reported in their wild counterparts [15], irrespective to the PGRs used (Table-3).

Table-1. Status of *Aloe* explants tissues cultured on MS media with BAP, TDZ and NAA monitored during 12 weeks.

Qualitative and quantitative parameters	Species	MS 1 (2.0 BAP + 0.01 NAA)	MS 2 (0.01 TDZ + 0.01 NAA)	MS 3 (2.0 BAP)	MS 4 (0.01 TDZ)	MS 5 (0.01 NAA)	MS 6 Control
Week 2							
Tissue colour	*A. purpurea*	White	White	White	White	White	White
	A. tormentorii						
	A. macra						
	A. lomatophylloides						
Week 4							
Tissue colour	*A. purpurea*	Green/ Swollen	Green/ Swollen	Green/ Swollen	Green/ Swollen	White	White
	A. tormentorii						
	A. macra						
	A. lomatophylloides						
Week 8							
Tissue colour	*A. purpurea*	Green	Green	Green	Green	Green/ Swollen	Green/ Swollen
	A. tormentorii						
Tissue appearance	*A. macra*						
	A. lomatophylloides	Globular	Globular	Globular	Globular	Granular	Granular
Week 12							
Developmental stage	*A. purpurea*	PLB/ necrosis	PLB	PLB/ necrosis	PLB	Plantlets	Plantlets
	A. tormentorii	PLB/ necrosis	PLB	PLB necrosis	PLB/ necrosis	Plantlets	Plantlets
	A. macra	Plantlets	Plantlets	Plantlets	Plantlets	Plantlets	Plantlets
	A. lomatophylloides	Plantlets	Plantlets	Plantlets	Plantlets	Plantlets	Plantlets

Table-2. Diameter (mm) of PLBs from Mascarene *Aloe* tissue cultures at week 8 under different PGR treatments.

Treatments	Growth in mm measured by average diameter of PLB (Week 8) (Mean ± SE)			
	A. purpurea	***A. tormentorii***	***A. lomatophylloides***	***A. macra***
MS 1	$4.67 \pm 1.06^{*}$	$4.54 \pm 1.21^{*a}$	$4.58 \pm 1.18^{*a}$	$5.81 \pm 3.45^{*}$
MS 2	$4.72 \pm 1.10^{*}$	$5.82 \pm 1.73^{*abcde}$	$4.67 \pm 1.77^{*bcd}$	$6.35 \pm 4.34^{*a}$
MS 3	$4.07 \pm 1.12^{*}$	$5.54 \pm 1.24^{*b}$	$3.89 \pm 1.32^{*}$	$4.94 \pm 2.84^{*}$
MS 4	$4.19 \pm 1.18^{*}$	$4.73 \pm 0.96^{*cfg}$	$3.70 \pm 1.78^{*bd}$	$5.06 \pm 2.78^{*}$
MS 5	$4.40 \pm 1.29^{*}$	$4.19 \pm 1.22^{*d}$	$3.52 \pm 0.90^{*ac}$	$4.39 \pm 3.05^{*a}$
MS 6	$4.26 \pm 1.07^{*}$	$3.88 \pm 0.99^{*efg}$	$3.93 \pm 1.07^{*}$	$4.50 \pm 2.58^{*}$

MS 1 (2.0 BAP mg/l + 0.01 NAA mg/l); MS 2 (0.01 TDZ mg/l + 0.01 NAA mg/l); MS 3 (2.0 mg/l BAP); MS 4 (0.01 mg/l TDZ); MS 5 (0.01 mg/l NAA); MS 6 (Control)

Each value represents the mean ± SE, each with sample size=50. * significant at 0.05 level

Mean values within a column followed by the same letters are significantly different at P=0.05 using ANOVA following Tukey's Multiple Comparison Method.

Table-3. Phytochemicals detected in dichloromethane: methanol crude extracts of wild and in vitro regenerated Mascarene *Aloes and A.vera*.

	Type of plant growth regulators																			
	Wild					**BAP** (2.0 mg/l)					**TDZ** (0.01 mg/l)					**NAA** (0.01 mg/l)				
Extracts	Av	Ap	At	Al	Am	Av	Ap	At	Al	Am	Av	Ap	At	Al	Am	Av	Ap	At	Al	Am
Alkaloids	++	+	+	+	+	+	+	+	+	+	++	+	+	+	+	++	+	+	+	+
Anthraquinones	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Terpenes	+	+	+	+	+	+	++	++	++	+	++	+	+	++	+	+	++	+	++	+
Phenols	+	++	++	++	+	+	+	+	++	+	+	+	++	++	+	++	++	+	++	+
Saponins	-	+	++	-	+	-	+	-	-	+	-	+	+	-	+	-	-	++	+	+
Tannins	+	+	++	++	+	+	+	+	++	+	+	+	+	++	+	+	+	++	++	+
Coumarins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Leucoanthocyans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Av: A.vera; Ap: A.purpurea; At: A.tomerntorii; Al: A.lomatophylloides; Am: A.macra;
+ Trace of metabolite; ++ Metabolite present; - Metabolite Absent

CONLCUSIONS

Tissue culture technology offers a vital tool for the conservation of germplasm and mass propagation of medicinally important plant resources like *Aloe* species. The present protocol is viewed as an excellent system for the rapid and large-scale in vitro propagation of endemic Mascarene *Aloes* with minimal risk of genetic variation which is reflected at the morphological and biochemical level as established by preliminary phytochemical screening studies. The use of PLBs for the micropropagation of Aloes is novel and ensures the genetic stability of micropropagated plants. Interestingly, the fact that the same class of medicinally important phytochemicals was accumulated in micropropagated *Aloe* plantlets and source plants provides a basis for further work on the synthesis of bioactive metabolites using in vitro culture systems. However, future work is required for the identification and isolation of the bioactive molecules for pharmaceutical exploitation.

ACKNOWLEDGEMENTS

This project was supported from a grant by the Mauritius Research Council and Tertiary Education Commission (Mauritius). Plant samples have been provided by the National Parks and Conservation Service, the Mauritius Herbarium garden (Mauritius Sugar Industry Research Institute), the Conservatoire Botanique National de Mascarin and Mr Jean-Bernard Castillon (Réunion). The authors gratefully acknowledge the Food and Agriculture Research Council (Mauritius) for the provision of tissue culture facilities.

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