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MICROPROPAGATION OF *ALOE JUCUNDA* L. (ASPHODELACEAE) – A MEDICINAL AND ORNAMENTAL PLANT

HUMA RAO[1], SAIFULLAH KHAN[1], SAIMA SALEEM ANSARI[2] AND ZARREEN BADAR[3]

[1]*Biotechnology Wing, H.E.J Research Institute of Chemistry, International center of Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan*

[2]*Dr. A. Q. Khan Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, Karachi-75270, Pakistan*

[3]*Research Institute for Science and Engineering (RISE), University of Sharjah, Sharjah P.O. Box 27272, United Arab Emirates*

[*]*Corresponding author's e-mail: drsaif65@gmail.com*

Abstract

Aloe jucunda L. is a valuable medicinal and ornamental plant of the family Asphodelaceae. The way Aloes have been known and used; specific threats are there across the genus. *Aloe jucunda* is a narrow endemic to Northern Somalia. However, it is fairly common in cultivation. It contains lignin, which has therapeutic qualities such as antioxidant and antibacterial activity, used to treat wounds, burns and digestive problems. In its native area of Northern Somalia, is excessively grazed by cattle, also ruthlessly chopped for wood and charcoal burning, are major threats to this plant. *A. jucunda* is enlisted as endangered plant in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). However, *A. jucunda* is cultivated in various countries for its horticultural and to some extent medicinal importance also, therefore, the present study was undertaken. The primary objective of this study was to design a reliable, large-scale micropropagation methodology for developing whole plantlets of *A. jucunda*. A good quality callus was established by culturing stem nodal explants in Murashige and Skoog (MS) medium supplemented with 2.0 mg/L BAP (6-Benzyl Aminopurine) and 3.0 mg/L NAA (α-naphthaleneacetic acid). After four weeks, the proliferated calli were transferred to the multiplication medium. Within 14-25 days, highest (mean 7.6 ± 1.5) mean number of shoots and (3.2 ± 0.7) mean length of shoots were observed with 2 mg/L BAP (6-Benzyl Aminopurine) and 0.1 mg/L IBA (Indole-3-Butyric Acid). While only one shoot was observed in the control media. Shoots rooted successfully when 0.5 mg/L of IBA was supplemented and resulted in higher number of roots, (10.3 ± 1.5) and length (3.7 ± 1.1cm), within 14-20 days. It was observed that growth hormones played a significant role in the development of the plant at different growth stages. Consequently, this *In vitro* propagation protocol could be applied to conserve critically endangered and threatened *A. jucunda* from extinction.

Key words: *Aloe jucunda*, Threatened, CITES, Micropropagation, Murashige and Skoog.

Introduction

The leaf-succulent genus *Aloe* comprises a total of ±548 accepted species (Grace *et al.*, 2009). *Aloe* species vary in size ranging from very small shrubs of few centimetres to large trees (Eshun & He, 2004; Grace, 2011). Succulent leaves of *Aloe* contain transparent mucilaginous pulp called as Mannan (Ni *et al.*, 2004). Generally, medical research recommends *Aloe* for two main areas: the epithelium and mucilage gel. A viscous exudate or sap called 'bitter aloes' and 'drug aloes' that consists of pericyclic cells beneath 'the epidermis. Whereas the gel obtained from mucilaginous cells (middle region of the leaf) is mainly used in preparation of skin related medicines and cosmetics (Bachman *et al.*, 2020).

According to IUCN evaluation of 134 *Aloe* species, the major danger to the family is loss in habitat induced by anthropogenic activities such as, cultivation, mining, city growth, clearance of vegetation for agricultural or other purposes, over grazing by farm animals and soil erosion. Trading of ornamental plant has impacted many *Aloe* species compared to wild species (Grace, 2011). Endemic and rare taxa of *Aloe* are the major focus for conservation. Risk of misidentification (or substitution) are higher in East Africa where various species are victimised, and probability of wrong identification represents a substantial threat to taxa. Generally, conventional harvesting practises hardly effect plant death compared to wild harvesting or uncultivated land, fire, and pests (Melin, 2009; Grace, 2011; Clark *et al.*, 2022). Preservation is a primary goal of succulent plant collectors and societies. However, some groups ensure the legal and ethical plant collecting practices as for the Convention on Biological Diversity (CBD) and national legal requirements (Grace, 2011; Martin, 2022).

Universally, there is an increasing trend to transfer assets from allopathic to antiquated healthcare system (Shinwari, 2010; Shaheen *et al.*, 2023). *Aloe* species are well known for miracle cure, and they are used as a folk medicine for numerous diseases. *Aloe barbadensis* and *Aloe ferox* are considered to have great commercial importance (Van Vuuren & Naidoo, 2010). Some communities use these species to cure burns, skin cancer, eczema, blood pressure and diabetes (Loots *et al.*, 2007; GolMohammadi, 2022). Aloes are both highly collectable and decorative species. They are also commonly used in horticultural trade mainly in the region where they grow naturally. Today, *Aloe* has entered decorative plant trade along with the succulent plants (Reynolds, 2004; Grace, 2011; Margulies *et al.*, 2023).

A. jucunda L. a native of Northern Somalia, is a narrow endemic, restricted to Northern Somalia. However, it is fairly common in cultivation in the various parts of the world. In Pakistan its cultivation is rather rare, where it is cultivated only for ornamental purpose rather than for medicinal use.

Specific epithet of *A. jucunda* is derived from the word "jucundus" that means 'pleasant', that was explained by Reynolds (1953). It was noted and collected as charming and very distinctive little Aloe by Mr. P.R.O. Bally, botanist at Coryndon Museum, Nairobi, on 2nd May 1949, at Gaan Libah, Somaliland Protectorate (Walker, 2022). In view of *A. jucunda* importance it was thought to grow the plant on large side through micropropagation.

Miniature *Aloe jucunda* has been seen in the form of tiny, flat rosettes leaves that are intense green in colour and have tiny spines on the outer edges. *A. jucunda* can reach a height of about 35 cm. It has shiny, glossy, triangular, dark green leaves, usually about twelve, flecked with pale green spots, up to 4 cm long and 2–5 cm wide, with triangular teeth on the margins. Flowers are pale pink to coral pink. *A. jucunda* leaf juice includes lignin, a substance that assists in the penetration of ferments and hold moisture, making the juice an efficient moisturizer. The juice contains biologically active ingredients and vitamin complexes that enhance the metabolism and promote cell regeneration (Kvitkovska *et al.*, 2019).

The IUCN Red List of Threatened Species last updated its assessment of *A. jucunda* in 2011. According to B1ab (iii) criteria (Weber, 2013), *A. jucunda* is classified as 'Critically Endangered'. *A. jucunda* environment started to degrade in 1981, according to records. "Intensive animal grazing, timber cutting, and charcoal burning" are the main factors which attributed to the decline (Carter *et al.*, 2011; Walker, 2022).

The *In vitro* plant tissue culture technology is an effective tool for the multiplication, conservation, and management of endangered species (Deb *et al.*, 2018; Avhad *et al.*, 2023). A single explant is a small piece of tissue that can be continuously used to grow hundreds and thousands of plants. Endangered, threatened, and rare species have been successfully grown and conserved using micropropagation. By this technique threatened *Aloe* species or those in high demand can be maintained and effectively employed without depleting their own inherent populations. The use of biotechnological strategy secures the genetic diversity of plant species, which is essential for their sustained existence (Hlatshwayo *et al.*, 2020). Additionally, plant tissue culture is believed to be the most effective technology for crop improvement by the production of somaclonal and gametoclonal variants (Oseni *et al.*, 2018), Therefore, the purpose of the current study was to establish a convenient, quick, and efficient micropropagation procedure for *A. jucunda*.

Material and Methods

The *In vitro* micropropagation of *Aloe jucunda* was performed in the Plant Tissue Culture Laboratory, Biotechnology Wing, Industrial Centre for Chemical and Biological Sciences (I.C.C.B.S.), University of Karachi.

Plant collection and sterilization: Plant material of *A. jucunda* was collected from Karachi University Botanical Garden, Centre for Plant Conservation, University of Karachi. The Voucher specimen (Voucher number: SINDH020034) was deposited in the herbarium of Institute of Plant Sciences, University of Sindh, Jamshoro. Laminar flow cabinet was used to work in sterilized environment. To prevent contamination, 70% ethanol was applied for 1 minute to the plant material. Explants were kept in 0.1% Tween 20 with constant shaking for 1-2 minutes and washed with freshly prepared 0.12% mercuric chloride for 10 minutes. Washing steps were repeated several times using autoclaved distilled water.

Preparation of culture media: MS media (Murashige & Skoog, 1962) was prepared by mixing stock solutions of macro and micronutrients, vitamins and iron solution. For callus induction and plant organogenesis, basal agar medium was supplemented with different ratios and combinations of plant growth regulators. The pH of the media was adjusted to 5.7 using 1 M HCl, 1 M NaOH, and was solidified with 8.0 mg/L w/v agar. Then 25 ml of media was poured into the 250 ml glass jars. The medium was sterilized by autoclaving at 121ºC for 15 minutes while at 15 psi of pressure.

Callus induction: For induction of callus, different compositions of MS media containing phytohormones were utilized. A total of 16 calluses induction media formulations were prepared using MS medium supplemented with different concentrations (1.0, 2.0 and 3.0 mg/L) of BAP (6-Benzyl amino purine) alone. Other media compositions included the combinations of BAP and NAA (α-Naphthaleneacetic acid) (Table 1). Each sterilized stem nodal explants were cut down in aseptic conditions into small pieces of about 1 cm x 1 cm which were transferred into MS basal agar medium. The basal medium, without any plant growth regulator (PGR), the MS medium, was used as the control. The cultures were incubated at 24 ± 2ºC in illumination white, fluorescent tube lights. The cultures were incubated for a period of 4 weeks.

Effect of cytokinins on shoot multiplication: After 1 month of incubation, calli that formed shoots were cultured on MS medium, containing different combinations of BAP (0-2.0 mg/L) and IBA (0-0.5 mg/L), to investigate their potential for organogenesis. Cultures were incubated at 22 ± 2 ºC and alternating 16:8 h light/dark photoperiod at photon flux density of 75 µmol m-2 s-1. During 4th week the length and quantity of shoots were noted.

Effect of auxins on root induction: *In vitro* cultured plantlets were shifted on the rooting medium, comprising different concentration of hormones. MS basal medium was supplemented with indole butyric acid (IBA), naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) in concentration ranging from 0.5 to 2 mg/L, to determine the best hormone concentration.

Acclimatization: The cultivated *In vitro* plantlets were carefully taken out from the rooting media jars after 5 weeks. Roots were carefully washed under tap water to remove traces of media. The plants were transferred in pots containing only cocopeat, shifted to cultivating areas and watered regularly. Later plantlets were moved to a greenhouse.

Statistical analyses: Using SPSS statistical software (version 16.0), the acquired data were submitted to two-way analysis of variance (ANOVA). Duncan's Multiple Range Test was used to match the mean values and demonstrate significant differences at $p = 0.05$.

Results

Results of ANOVA indicated that the mean (±SD) number of days to shooting, shoot number, and shoot length were statistically significantly different among the treatments ($p \leq 0.05$).

Based on current studies, the type and concentration of plant growth regulators seemed to have a bigger impact on the *In vitro* response than the makeup of the culture medium. In the present study, different types, concentrations of auxins and cytokinins in varied combinations were supplemented to the basal MS medium. Cultured explants followed the indirect plant regeneration pathway. *Aloe* stem nodal explants were placed on MS medium supplemented with different concentrations and combination of BAP and NAA ranging from 1.0 mg/L to 3.0 mg/L (Table 1).

The first response of stem nodal explant was observed after 4 weeks, when they started to show the first signs of callus and then the explants were subcultured on the same nutrient media. In addition to having a texture that was compact, loosely compact and friable. Moreover, various media combinations formed Calli that appeared to be off-white, green and greenish brown in colour. The high-quality and compact off white-green callus was formed on the media comprising of 3.0 mg/L NAA and 2.0 mg/L BAP (Fig. 1). For callus induction, nodal explants in various combinations of media showed 100% response.

After four weeks, the proliferated calli from stem nodal explant were cultivated in the multiplication medium containing various concentrations and combinations of BAP and IBA (Table 2) to facilitate the regeneration of multiple shoots. When compared to the control (PGR-free) treatment, the production of shoots was considerably boosted by all cytokinin types and concentrations, except for 1.0 mg/L BAP and the combination of 2.0 mg/L BAP and 0.5 mg/L IBA. Increases in cytokinin concentration were accompanied by a reduction in shoot proliferation. In indirect organogenesis, shootlets formed from calli that appeared on the callus' surface. The effectivity of the media was evaluated in terms of elongation of multiple shoot buds. The potential of the media was evaluated in terms of enhancing multiple shoot buds. The average 7.6 number of shoots and 3.2 ± 0.7 cm of shoot length were developed in the medium containing 2 mg/L BAP with 0.1 mg/L IBA (Fig. 2). However, an increase in cytokinin/auxin concentrations did not lead to exponential plantlet regeneration.

Rooting: The *In vitro* grown multiple shoots were separated and transferred to rooting medium supplemented with different ratios and combinations of NAA, IBA and IAA to determine how auxins affect *In vitro* root generation. In our study, the optimal medium for rooting had 0.5 mg/L IBA on which mean number of the roots 10.333 ± 1.527, with mean length of 3.700 ± 1.135 cm were observed within 14 to 20 days (Fig. 3). No more root growth was seen after 25 days.

It means fresh rooting media needed to be used instead of the old one. Root induction was quick in every auxin that was tested throughout this study. and within two weeks, root primordia were visible. However, the same concentration of NAA and IAA (Figs. 4 & 5, Table 3) did not respond as IBA because at the same concentration of NAA the average number of roots was 2.66 and average length of root was 0.50 cm. While, at the same concentration of IAA the average number of roots was 7.33 and average length of root was 0.66 cm.

Table 1. Effects of different concentrations of phytohormones for callus formation from explant of *Aloe jucunda*.

Media Codes	BAP (mg/L)	NAA (mg/L)	Description of callus	
			Colour	Texture
CD1	0.0	0.0	-	-
CD2	1.0	0.0	Off-white	Compact
CD3	2.0	0.0	Light green	Friable
CD4	3.0	0.0	Greenish	Friable
CD5	0.0	1.0	Off-white	Compact
CD6	1.0	1.0	White	Loosely Compact
CD7	2.0	1.0	Light green	Compact
CD8	3.0	1.0	White	Loosely Compact
CD9	0.0	2.0	Green	Friable
CD10	1.0	2.0	Off-white	Compact
CD11	2.0	2.0	Greenish brown	Compact
CD12	3.0	2.0	Off-white	Compact
CD13	0.0	3.0	Off-white	Compact
CD14	1.0	3.0	White	Compact
CD15	2.0	3.0	Off-white green	Friable
CD16	3.0	3.0	Greenish brown	Compact

Table 2. Effects of BAP and IBA on plantlet regeneration from calli in *Aloe jucunda*.

Media codes	BAP (mg/L)	IBA (mg/L)	Number of shoots	Shoot length (cm)
Control	0.0	0.0	1.333 ± 0.577a	1.166 ± 0.251a
SJ2	1.0	0.0	2.666 ± 0.577b	1.433 ± 0.152ab
SJ3	1.0	0.1	5.00 ± 1.00c	1.80 ± 0.20ab
SJ4	1.0	0.5	5.666 ± 1.154cd	2.166 ± 0.251b
SJ5	2.0	0.0	4.666 ± 0.577bc	2.033 ± 0.757b
SJ6	2.0	0.1	7.666 ± 1.527d	3.233 ± 0.723bc
SJ7	2.0	0.5	2.666 ± 0.577b	1.366 ± 0.152a

Table 3. Effect of Auxins on root formation from regenerated plantlets of *Aloe jucunda*.

Hormone	Concentration (mg/L)	No. of roots	Length of roots (cm)
MS	-	1.333 ± 0.577a	0.566 ± 0.208a
NAA	0.5	2.666 ± 1.154b	0.500 ± 0.200a
	1.0	5.333 ± 1.527c	1.066 ± 0.416a
	1.5	6.666 ± 1.527d	1.266 ± 0.251a
	2.0	4.333 ± 1.154c	1.533 ± 0.416a
IBA	0.5	10.333 ± 1.527g	3.700 ± 1.135cd
	1.0	5.666 ± 2.081c	3.366 ± 0.472b
	1.5	3.333 ± 0.577b	3.500 ± 1.252c
	2.0	7.00 ± 2.645e	3.433 ± 0.513b
I AA	0.5	7.333 ± 2.081e	0.666 ± 0.152a
	1.0	7.666 ± 2.516ef	3.966 ± 0.550bc
	1.5	6.00 ± 2.645cd	2.766 ± 0.602b
	2.0	6.666 ± 3.055d	3.633 ± 0.550bc

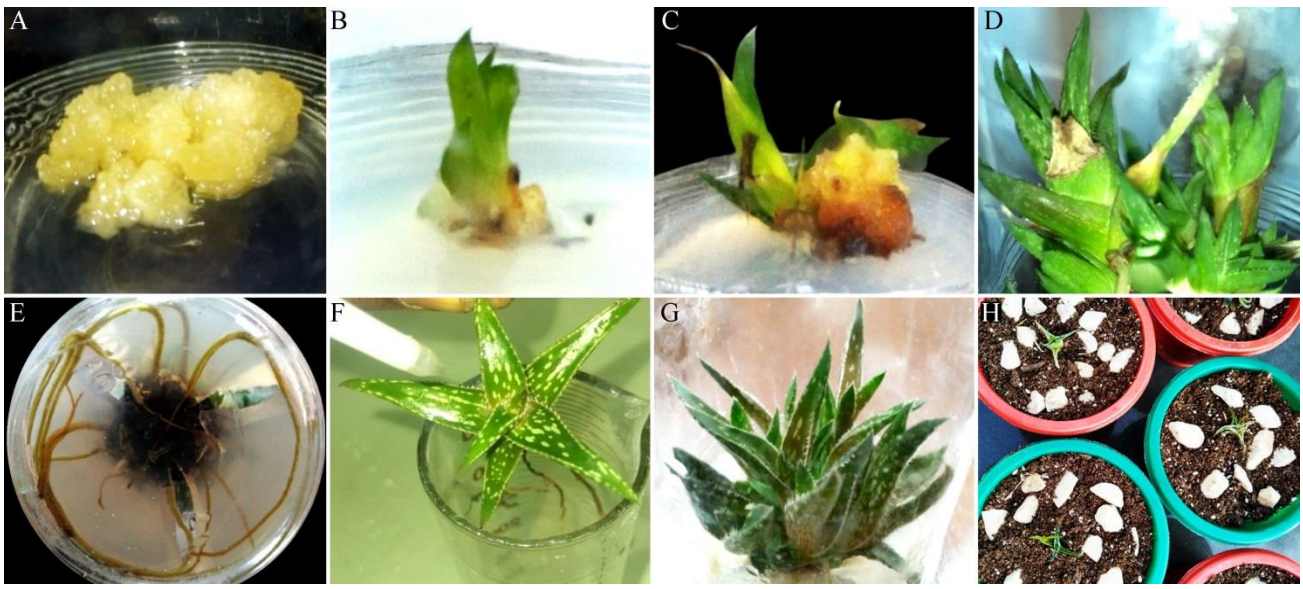

Fig. 1. Plant regeneration from stem nodal explants: A, callus formation in *Aloe jucunda*; B, initiation of single shoot from callus; C, proliferation of shoots from callus; D, proliferation of multiple shoots; E, formation of roots; F, washing of roots; G, regeneration of multiple shoots; H, plantlets transferred in pots and kept under illumination, plantlets shifted in the field after 40 days.

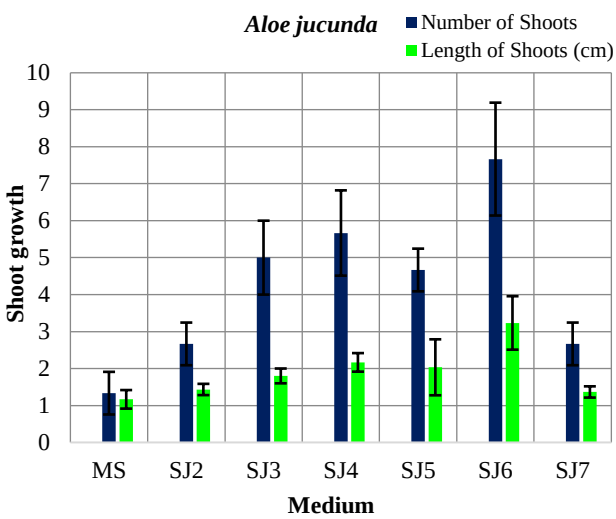

Fig. 2. Effect of BAP and IBA on shoot length of *Aloe jucunda*.

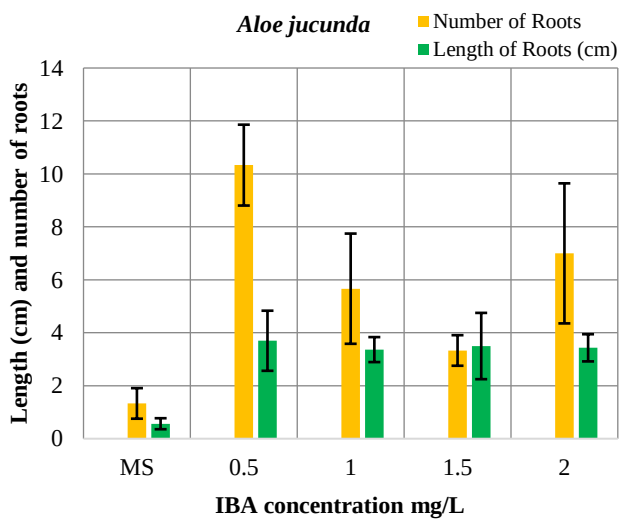

Fig. 3. Effect of IBA on roots of *Aloe jucunda*.

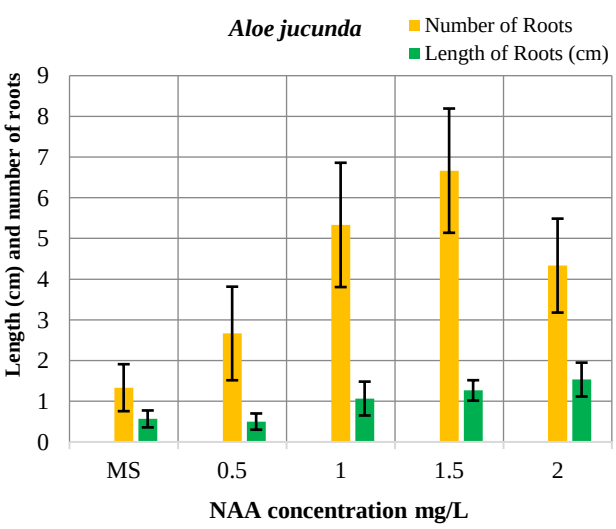

Fig. 4. Effect of NAA on roots of *Aloe jucunda*.

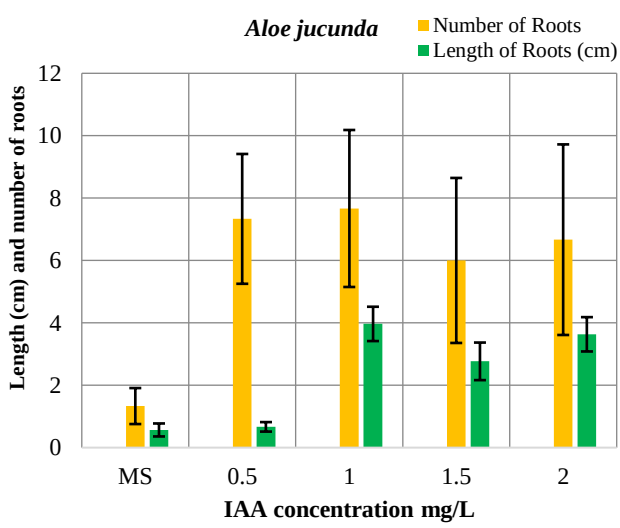

Fig. 5. Effect of IAA on roots of *Aloe jucunda*.

Discussion

Tissue culture for various species of Aloe has been established for its potential medicinal applications. It is beneficial for plants having slow growth rate, (Maryam *et al.*, 2014; Haque *et al.*, 2022) and large scale propagation of medicinal plants, conservation of germplasm and production of bioactive compounds (Nalawade & Tsay, 2004). It is an effective technique for propagation and regeneration of more than 1000 different plant species in addition to many rare and endangered species (Abrie & Staden, 2001; Cruz-Cruz *et al.*, 2013; Desai *et al.*, 2022). Micropropagation of *A. arborescens* (Amoo *et al.*, 2012), *A. barbadensis* (Roy & Sarkar, 1991), *A. polyphylla* (Abrie & Staden, 2001) and *A. vera* (Singh & Sood, 2009) was already established by plant tissue culture techniques. Several *Aloes* are threatened because of over collection for cultivation, harvesting of leaf exudates and destruction of natural habitats (Reynolds, 2004). However, limited information is available regarding *A. jucunda*. Tissue culture technology can be used not only for conservation of *A. jucunda* but also for the cultivation on larger scale. Habitat destruction and the illegal trade in wild plants are the main factors affecting almost all succulent species with the exception of *A. barbadensis* (Malda *et al.*, 1999).

In this study nodal explants were used from *A. jucunda* to establish a simple protocol for *In vitro* growth development and multiplication. Studies have shown that stem nodal explants and shoot tips were used for micropropagation of *Aloe vera L* (Singh & Sood, 2009). In other study long hypocotyls with radicles served as reliable explant for regeneration of four different *Aloe* species (Lobine *et al.,* 2015), while young axillary buds were used to induce multiple shoots in *A. barbadensis* (Badar, 2013).

The growth regulator(s) type and concentration have a major influence on callus initiation. Explants responded in cultures after 4 weeks and produced a callus on their sliced ends. In this study, for callus formation, 15 different concentrations and combination of cytokinin (BAP) with auxin (NAA) ranges from 1.0 mg/L to 3.0 mg/L were supplemented with different media. However, explant showed regeneration potential in all media. The good quality callus of *A. jucunda* was achieved on MS medium containing 3.0 mg/L NAA and 2.0 mg/L BAP. It was off-white greenish in colour and friable in texture. In addition, Sahoo and Rout, (2014) also developed callus of *A. barbadensis* Mill. by adding 1 mg/L (BAP) and 0.5 mg/L (NAA) in MS medium, our results are in accordance with findings of Cenkci *et al.*, (2008), for *Thermopsis turcica* a critically endangered species. It is concluded from our observations that for callus growth in *A. jucunda* 3.0 mg/L NAA and 2.0 mg/L BAP supplementation is sufficient.

Tissue culture technique provides excellent means to support/save the endemic threatened *Aloe* species namely *A. lomatophylloides, A. macra, A. purpurea* and *A. tormentorii.* (Seran & Ahmad, 2018). In present study the stem nodal explant and phytohormones were used for rapid and large-scale *In vitro* propagation of critically threatened *A. jucunda*. Different combinations of cytokinin and auxin, were used to enhance the yield of *A. jucunda* production. The addition of 2 mg/L BAP and 0.1 mg/L IBA in the media produced multiplication of shoots *i.e.,* 7.6 ± 1.5 number of shoots, 3.2 ± 0.7 mean shoot length on media SJ6 when compared to control. BAP proved unable to stimulate shoot growth alone, but when combined with IBA, it showed rapid growth in shoots proliferation. This research found that a combination of auxins and cytokinins was required for optimal shoot induction. Other researchers also demonstrated that a concentration of both auxin and cytokinin was required for shoot proliferation *In vitro* development of *A. vera* (Liao *et al.*, 2004; Bhandari *et al.*, 2010; Jayakrishna *et al.*, 2011).

The kind and concentration of growth regulators used on medium had a substantial influence on the course of rooting on small shoots in *A. Jucunda*. Roots in the *A. jucunda* were produced by supplementing of the MS medium with 0.5 mg/L IBA *i.e.,* 10.3 ± 1.5 number of roots, 3.7 ± 1.1 an average root length. There were no apparent variations in the number and length of roots at different IBA concentrations. While at the same amount of IBA, NAA and IAA failed to respond in the same manner as IBA. It has been demonstrated by other researchers that plant growth regulators require being present on a medium to allow for shoots to root formation (Velcheva *et al.*, 2005). The results agreed with Chae *et al.*, (2013), as similar findings were also authenticated in highly endangered species *A. polyphylla* (Abrie & Staden, 2001).

This study significantly states that tissue culture can be very successful tool for conservation of threatened and endangered *Aloe* species and growth hormones can successfully help to develop plants at different stages. To sum up, this work offers the first account of a very effective approach for *A. jucunda In vitro* micropropagation, which may pave the way for *Aloe* genetic modification.

Conclusions

Tissue culture technique is an important tool for the conservation of germplasm and the large-scale cultivation of ornamental and medicinally important plant resources, such as *Aloe* species. This work is the first to provide an outstanding approach of rapid and *In vitro* regeneration strategy for the large-scale multiplication and commercial establishment of *A. jucunda*, as a species of plant that is critically endangered. Based on the research mentioned above, it is concluded that both the type and concentration of plant growth regulators (PGRs) significantly influence plant regeneration during callus induction, shoot initiation, proliferation and plant rooting. Considering *A. jucunda* is a critically endangered *Aloe* species, the *In vitro* regeneration mechanism described herein might be useful for its large-scale conservation initiatives.

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