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MEDICINAL PLANTS

In vitro culture in combination with aeroponics is an efficient means of mass propagation of *Sarcostemma acidum*: a rare medicinal plant of Indian arid zone

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Abstract

In vitro culture in combination with aeroponics is observed to be an efficient means for mass propagation of *Sarcostemma acidum* in the present investigation. *S. acidum* is a rare leafless xerophytic shrub of the family Apocynaceae, commonly known as Som-lata or khir-kheep, a source of medicines and the religious drink “Somaras.” Aeroponic technique was used for the induction of adventitious roots from stem cuttings of field-grown plants for the development of quality plants in the greenhouse as a source of explants for micropropagation. Among various concentrations of IBA, NAA and NOA used for induction of adventitious rooting from stem cuttings 3.0 g L^{-1} NAA were found to be most suitable with average 90% induction response and 14 adventitious roots. Surface-sterilized nodal shoot segments of 6 to 7 cm length were inoculated on basic and modified Murashige and Skoog’s medium containing 3% (*w/v*) sucrose, 0.8% (*w/v*) agar, and various concentrations of BAP and kinetin for activation of axillary shoot buds. Shoots were mass multiplied on a modified MS (MMS) medium containing 3.5 mg L^{-1} BAP and 0.01 mg L^{-1} IAA. *In vitro* multiplied shoots were rooted *in vitro* on half-strength MS medium containing 0.25 mg L^{-1} NAA. Ninety percent of *in vitro* regenerated plantlets were hardened successfully on Soilrite in the greenhouse and survived on garden and field soils. This protocol will be useful for *in vitro* propagation of *S. acidum* for mass plantations in the desert ecosystem for prospects.

Keywords Adventitious roots · Aeroponics · Propagation · Plant growth regulators · *In vitro* rooting · Acclimatization

Introduction

Sarcostemma acidum (Roxb) Voigt is one of the rare medicinal plants of the Indian Thar Desert. It is a leafless, straggling, xerophytic succulent shrub of the family Apocynaceae, commonly known as Som-lata and Khir-kheep. The plant grows naturally in the rocky sites in Rajasthan, Bihar, West Bengal, and southern India (Bhandari 1990). The plant was used to prepare “Somaras,” a religious drink as recorded in Vedic literature. The rural and tribal people have been using the herb in order to cure various disorders. Pandey *et al.* (2017) in their survey reported that indigenous communities in various states of India have been using this whole plant as traditional medicine (TM) for treating 40 different kinds of ailments including snake bites and rheumatism (Senthilkumar *et al.* 2006). Whole-plant extracts have shown antimicrobial activities against gram-positive bacteria as well as an anti-inflammatory (Shailesh *et al*. 2011), antifertility (Verma *et al*. 2002), anti-ulcer (Gulshan *et al*. 2017), anxiolytic, anti-psychotic, and central nervous system (CNS) inhibitory properties were already reported by Ittiyavirah and Rahees (2013). Dhivya and Kalaichelvi (2017) have reported the presence of about forty bioactive compounds in the GC–MS analysis of ethanolic shoot extract of *S. acidum* such as alkaloids, glycosides, flavonoids, phenols, steroids, amino acids, tannins, terpenoids, quinines, and coumarin. Mass propagation of *S. acidum* is needed for conservation and fulfillment of the increasing medicinal need. Plant tissue culture is a major technique of mass propagation of rare and threatened plants having lower seed setting, viability, and germination ability in nature. The plants can be regenerated using somatic cells, tissues, and organs of selected genotypes of this plant to meet the increasing demand of the

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herbal industry due to its immense medicinal uses. Although Rathore and Shekhawat (2013) reported *in vitro* regeneration of *Sarcostemma acidum* through callus differentiation, this is the first report of mass multiplication of *S. acidum* through mature nodal explants achieved by using a combination of plant tissue culture with aeroponic technique, in which *in vitro* micropropagation of *S. acidum* from nodal shoots was efficiently performed by using aeroponically developed plants. Plant development through adventitious root induction from stem cuttings is a common practice in plant breeding for many horticultural and forestry plant species. Aeroponics is an efficient means of induction of roots from shoot segments in the air by a regular supply of nutrients and water in the form of mist. The potential of aeroponics for the mass propagation of threatened medicinal plants such as *Caralluma edulis*, *Leptadenia reticulata*, and *Tylophora indica* has also been reported by Mehandru *et al*. (2014). Due to the rare availability of *S. acidum* plants in natural habitats, the aeroponic technique can be a useful means for the development of rare and threatened plants in the greenhouse as a source of explants for mass propagation by tissue culture mechanism. It minimizes the dependency on rare and threatened plants growing in natural habitats.

The present investigation was aimed at (i) induction of adventitious roots from stem cutting through the aeroponic technique; (ii) establishment of rooted stem cutting (developed through aeroponic technique) in the greenhouse to raise quality source plants for *in vitro* plantlet regeneration; (iii) standardization of basal culture medium and establishment of *in vitro* culture of shoot segments from plants maintained in the greenhouse; (iv) induction of *in vitro* rooting from *in vitro* produced shoots; (v) hardening of *in vitro* regenerated plantlets. This is the first report on the use of the aeroponic technique in combination with plant tissue culture for micropropagation of *S. acidum* from Indian arid zone.

Materials and methods

Establishment of source plants in greenhouse through aeroponics Mature stem cuttings of 25 to 30 cm length were collected from the rocky areas of the Machia Safari Park, Jodhpur, during September 2017 (Fig. 1*a* and *b*). Half of the explants from these were used directly for *in vitro* culture establishment and the rest of the shoot cuttings were treated with various concentrations (0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 gL^{-1}) of 1-naphthoxyacetic acid (NOA), 1-naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA) (Sigma-Aldrich®, St. Louis MO) for 5 min and control (without treated cuttings) were inserted vertically in the holes of the styrofoam sheet of the aeroponic system

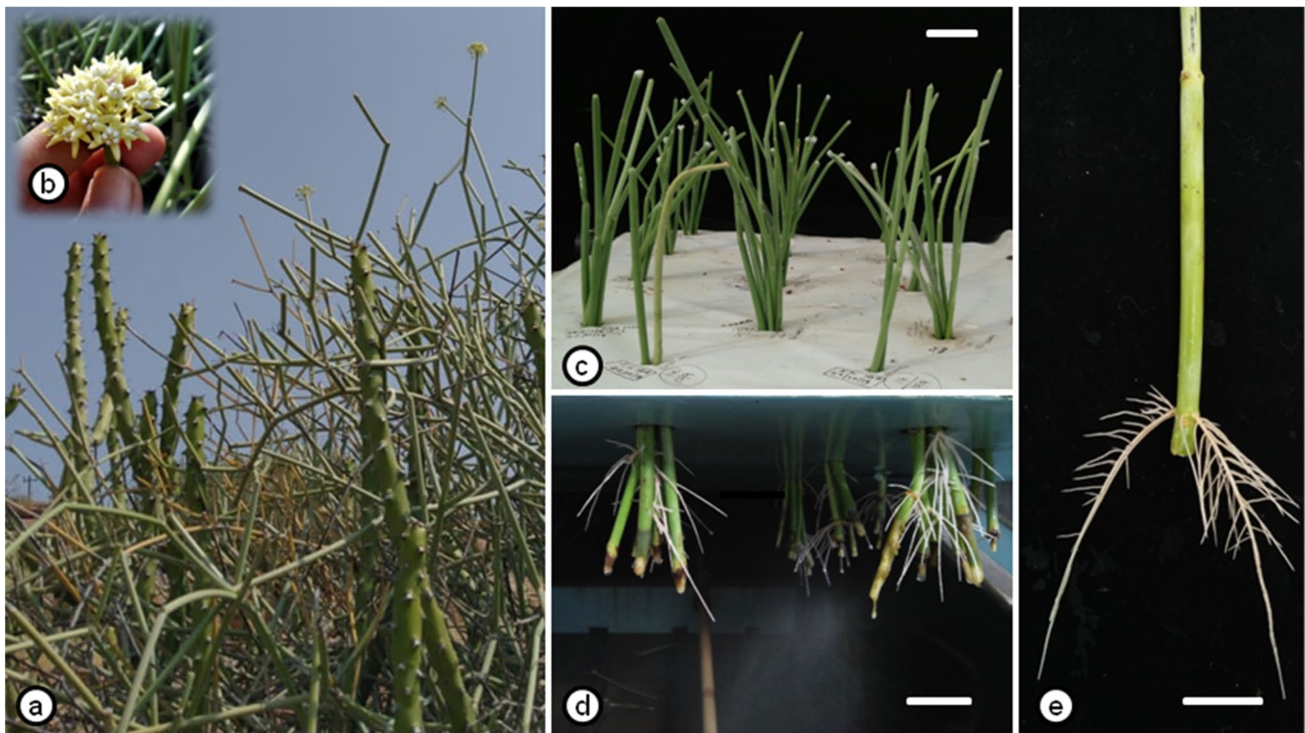

Fig. 1 *Sarcostemma acidum* (Roxb) Voigt. (*a*) In a natural habitat. (*b*) Inflorescence. (*c*) Stem cuttings treated with 3.0 g L^{-1} NAA and inserted vertically in an aeroponic chamber. (*d*) Adventitious root induction from stem cuttings in an aeroponic chamber. (*e*) Shoot cuttings with adventitious roots induced (scale bar, 2.5 cm) (*NAA* 1-naphthaleneacetic acid)

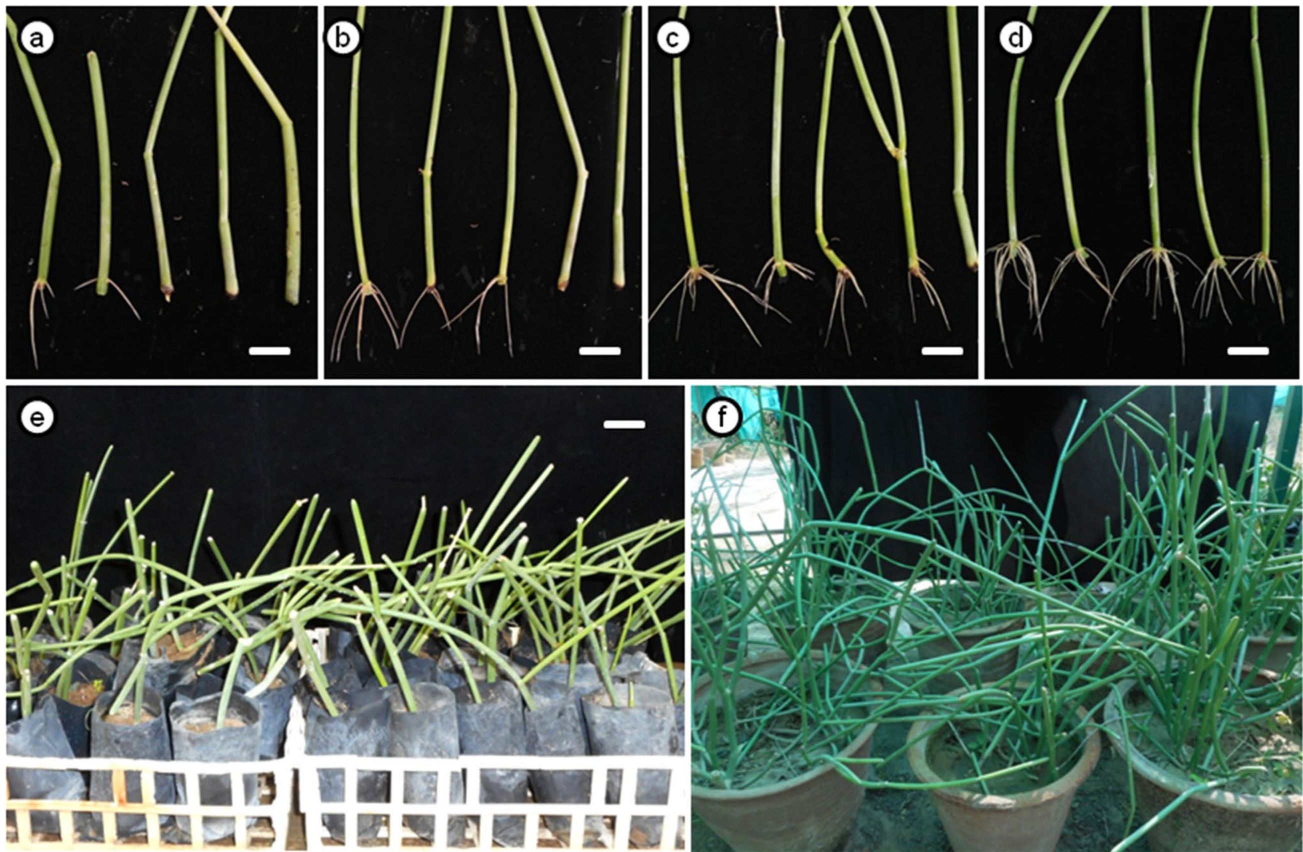

Fig. 2 Induction of adventitious root from stem cuttings of *Sarcostemma acidum* (Roxb) Voigt through aeroponics, stem cuttings pretreated with 3.0 g L^{-1} auxins: (*a*) control, (*b*) NOA, (*c*) IBA, (*d*) NAA, (*e*) rooted shoots in polybags containing garden soil, (*f*) plants developed through rooted stem cuttings in greenhouse (scale bar, 2.5 cm) (*NOA* 1- naphthoxyacetic acid, *IBA* indole-3-butyric acid, *NAA* 1-naphthaleneacetic acid)

(Fig. 1*c*) in the greenhouse for induction of adventitious roots (AR). Basal-treated part of the vertically inverted shoots was kept moist by misting of water. Misting was uninterruptedly applied (60 s of regular misting phase with the pause of 600 s between two misting phases) by nine 50-μm high-pressure nozzles connected to 50-mm diameter polyvinyl chloride (PVC) pipes (Prince Piping System, Mumbai, India) through which 125 mL of water per nozzle was pumped by a 0.373-kW electric motor (Barupal *et al.* 2018). Data were observed after 3 to 4 wk of insertion of basal-treated part of shoots in the holes of aeroponic system. The parameters used for observation were based on percentage of adventitious rooting, number of adventitious roots per shoot segment, and average length of adventitious roots. All the aeroponically rooted plantlets were transferred into the polybags containing garden soil and kept for 25 to 30 d in greenhouse and then transferred in nursery. These established plants were used as a source of explants after 5 to 6 mo of aeroponic rooting (7 to 8 mo of shoot segment treatment with auxins) when these were fully matured for *in vitro* multiplication of shoots and regeneration of plantlets.

Establishment of *in vitro* culture and shoot activation Plants that were collected directly from the field and aeroponically raised in the greenhouse were selected as a source of explants. The medium consisted of Murashige and Skoog (MS) (Murashige and Skoog 1962) basal salts and modified MS with high nitrates (5.325 g L^{-1} NH_4NO_3 and KNO_3 supplement (Hi-Media®, Mumbai, India)), reduced concentration of calcium chloride (220 mg L^{-1} $CaCl_2$ (Hi-Media®)) and 200 mg L^{-1} meso-inositol (Loba Chemie Pvt.Ltd., Mumbai, India), 3% (*w/v*) sucrose, and 0.8% (*w/v*) agar–agar (Qualigens Fine Chemicals, Mumbai, India). The medium was supplemented with various concentrations (0.0, 0.5, 2.0, 4.0, 6.0, 8.0, 10.0 mg L^{-1}) of 6-benzylaminopurine (BAP) and 6-furfurylaminopurine (kinetin; Kin) (Hi-Media®) for axillary shoot bud activation. The pH of medium was adjusted to 5.8 using 1 M KOH or 0.1 N HCl (Loba Chemie Pvt.Ltd., Mumbai, India) before autoclaving at 121°C temperature and 1.1 kg cm^{-2} pressure for 15 min. Nodal shoot cuttings of 6 to 7 cm length were taken from aeroponically developed plants (maintained in the nursery) and used for activation of axillary shoot buds *in vitro* for large-scale

shoot multiplication. Excised nodal shoot segments were pretreated first with 70% ethanol for 10 to 15 s and then surface sterilization of the nodal shoots was done by treating with 0.1% (*w/v*) Bavistin® (a fungicide containing 50% (*w/w*) Carbendazim, BASF India Limited, Mumbai, India) for 5 min followed by 0.1% (*w/v*) $HgCl_2$ (Sigma-Aldrich®) for 4 min followed by thorough rinses of shoot cuttings with autoclaved distilled water for 4 to 5 times. Both the ends of these surface-sterilized shoots were trimmed with sharp scissors and inoculated on a culture medium. The cultures were kept in a growth room at 28° ± 2°C, 10-h photoperiod of 40 to 50 μmol m^{-2} s^{-1} photon flux density (PFD) per day, and 50 to 60% relative humidity (RH). Shoots were regularly transferred on fresh culture medium after 25 to 30 d of inoculation.

Shoot multiplication *In vitro* produced axillary shoots of 3 to 4 wk were excised from mother explants and used same as mother explant (nodal part of the previously activated axillary shoot with trimmed tip) for further mass multiplication of shoots. Various concentrations (0.00, 0.01, 0.25, and 0.5 mg L^{-1}) of indole-3-acetic acid (IAA; Sigma-Aldrich®) and indole-3-butyric acid (IBA; Sigma-Aldrich®) were used along with BAP for optimization shoot multiplication. Different types and sizes of culture vessels were used for mass multiplication of shoots, which are borosilicate (Borosil Glass Works Ltd., Mumbai, Maharashtra, India) culture tubes (60 mL; 25 mm × 150 mm), and Erlenmeyer conical flasks (150 mL, 72 × 124 mm; and 250 mL, 85 mm × 145 mm).

***In vitro* root induction** *In vitro* multiplied shoots were excised from mother explants and inoculated on one-half and one-fourth strength basal and slightly modified (half strength of nitrates and double strength of calcium chloride of full-strength MS medium) MS medium containing 100 mg L^{-1} activated charcoal (AC; Sigma-Aldrich®), and 3% sucrose and 0.8% agar–agar, supplemented with NOA, IBA, and NAA (0.00, 0.01, 0.025, 0.05, 0.10, 0.25, and 0.50 mg L^{-1}) for induction of *in vitro* rooting from shoots. Cultures were kept in dark initially until the roots were initiated and then shifted in the light.

Hardening and acclimatization *in vitro* regenerated plantlets *In vitro* rooted shoots (plantlets) of *S. acidum* were taken out from the medium and washed thoroughly with autoclaved water to remove agar from roots. Rooted parts of plantlets were inserted in autoclaved Soilrite (horticulture-grade perlite and Irish peat moss mixture) and exfoliated vermiculite (Kel Perlite, Bangalore, India) in glass bottles and moistened with one-fourth strength of MS basal salts. Bottles were tightly kept covered with polycarbonate caps for an initial 1 wk, after that caps were loosened and finally removed. Simultaneously, for the acclimatization of the *in vitro* generated plantlet to the external environment, bottles with plantlets were gradually shifted from high to low relative humidity and low to high temperature (from cooling pad to fan sections) in the greenhouse. Hardened plantlets were further transferred in a mixture of Soilrite and garden soil (1:1) in polybags (Lodha Plastic Industries, Jodhpur, India) for field transfer.

Statistical analysis Present experiments were conducted using the randomized block design method for single-factor experiments (Compton and Mize 1999) and repeated three times. Each value of induction of adventitious roots from stem cuttings, activation of axillary bud from nodal shoot segments, mass multiplication of shoots, and *in vitro* rooting represents an average number of five replicates using 35, 35, 20, and 35 explants, respectively. The observed data were analyzed statistically by analysis of variance test (Gomez and Gomez 1984) through SPSS v.17 software (SPSS, Chicago, IL). The significance among mean differences was calculated by Duncan's multiple range test (DMRT) (Duncan 1955) at $P < 0.05$.

Results and Discussion

Induction of adventitious roots from mature shoot segments through aeroponic system Adventitious roots were induced from all stem segments after 1 wk of insertion in the aeroponic chamber (Fig. 1*d* and *e*) including the control (Fig. 2*a*). In plants, growth and development processes are directly or indirectly regulated by both environmental and endogenous factors (Gehlot *et al*. 2014). Endogenous factors like plant growth regulators (PGRs) play an important role in adventitious root development. The endogenous auxin concentration is responsible for the developmental stages in the root. High endogenous auxin concentration is directly proportional to the high rate of root initiation. (Heloir *et al*. 1996; Blazkova *et al*. 1997; Caboni *et al*. 1997). Endogenous level of auxin increases in exogenously treated shoot cuttings with auxin, which is corresponding to the root initiation at the treated zone (Gaspar *et al*. 1996; Gatineau *et al*. 1997). Auxin interacts with other signaling pathways such as cytokinin, ethylene, nutrient, and stress to regulate branching and growth of roots (Murphy *et al*. 2016; Veloccia *et al*. 2016; Qu *et al*. 2017).

Among all the auxin treated shoots used for inductions of adventitious roots, NAA (3.00 g L^{-1}) was the most suitable for *S. acidum* with an average 90% induction response and average 14.27 ± 1.01 ARs per cutting (Table 1; Fig. 2*d*) followed by IBA (Fig. 2*c*), NOA (Fig. 2*b*), and control (Fig. 2*a*). NAA has been reported the most suitable auxin for the induction of adventitious roots from the stem segments

Table 1 Induction of adventitious roots by aeroponic technique from stem cuttings of *Sarcostemma acidum* (Roxb) Voigt using various concentrations of NOA, NAA, and IBA

Auxin (g L^{-1})			Percentage of AR Mean ± SD	Number of AR Mean ± SD	Length of AR(cm) Mean ± SD
NOA	NAA	IBA			
0.00			13.33 ± 5.80 ^{h}	1.67 ± 0.76 ^{j}	1.15 ± 0.30 ^{l}
0.50			33.33 ± 11.55 ^{fg}	2.73 ± 0.12^{ghi}	4.27 ± 0.25^{ghi}
1.00			50.00 ± 10.00^{de}	5.80 ± 0.20^{e}	4.90 ± 0.36 ^{fg}
2.00			71.67 ± 10.41^{bc}	12.20 ± 0.35^{b}	6.50 ± 0.50 ^{cd}
3.00			58.33 ± 2.89 ^{cd}	8.53 ± 0.23^{c}	3.50 ± 0.30^{jk}
4.00			33.33 ± 11.55 ^{fg}	3.13 ± 0.31 ^{g}	3.83 ± 0.29^{ij}
5.00			16.67 ± 5.77^{gh}	2.07 ± 0.31^{hij}	2.93 ± 0.40 ^{k}
	0.50		16.67 ± 5.77^{gh}	2.00 ± 0.87^{ij}	3.63 ± 0.15^{ijk}
	1.00		38.33 ± 2.87^{ef}	2.93 ± 0.12^{gh}	4.17 ± 0.29^{hij}
	2.00		55.00 ± 5.00^{cde}	3.53 ± 0.42 ^{g}	5.60 ± 0.53^{ef}
	3.00		90.00 ± 10.00^{a}	14.27 ± 1.01^{a}	7.17 ± 0.35^{bc}
	4.00		71.67 ± 10.41^{bc}	9.07 ± 0.12^{c}	7.67 ± 0.58^{b}
	5.00		53.33 ± 7.64^{de}	7.00 ± 0.20^{d}	6.17 ± 0.29^{de}
		0.50	26.67 ± 11.54^{fgh}	4.40 ± 0.20^{f}	3.53 ± 0.11^{ijk}
		1.00	31.67 ± 10.40 ^{fg}	5.93 ± 0.50^{d}	4.63 ± 0.07^{gh}
		2.00	52.67 ± 11.01^{de}	8.33 ± 0.30^{c}	7.10 ± 0.33^{bc}
		3.00	80.00 ± 20.00^{ab}	12.53 ± 0.99^{b}	8.85 ± 1.04^{a}
		4.00	58.33 ± 2.89 ^{cd}	7.53 ± 0.31^{d}	7.16 ± 0.32^{bc}
		5.00	55.00 ± 3.00^{cde}	5.87 ± 0.31^{e}	5.52 ± 0.12^{ef}

Observations are based on qualitative characteristics, AR initiation, number, and length after 1 wk. Cuttings were pulse treated with PGRs (IBA, NOA, and NAA). Each value of mass multiplication of shoots represents an average number of five replicates using 35 explants. The Duncan multiple range test at $p < 0.05$ was used for calculation of significance among mean differences. *IBA* indole-3-butyric acid, *NAA* 1-naphthaleneacetic acid, *NOA* 1-naphthoxyacetic acid, *AR* adventitious root, *SD* standard deviation

of *Passiflora pahlii* on semisolid medium (Simão *et al.* 2016). All the rooted shoots were successfully established on garden soil in polybags (Fig. 2*e*) and pots (Fig. 2*f*) and

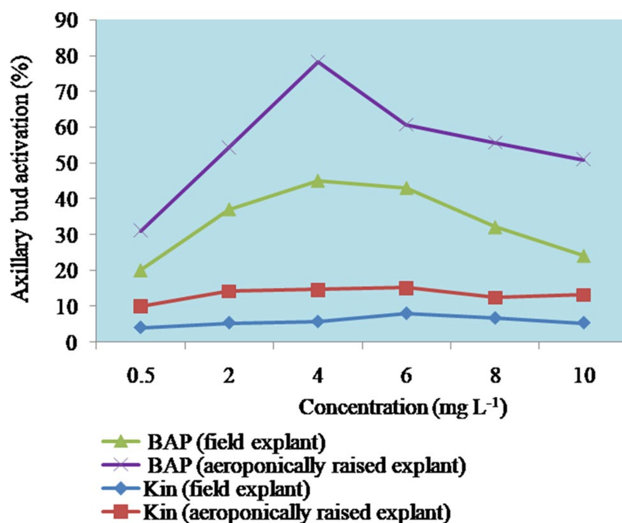

Fig. 3 Comparative effect on axillary bud activation due to explant type on normal Murashige and Skoog basal media (field collected and aeroponically raised) in *Sarcostemma acidum* (Roxb) Voigt. Highest 78.33 ± 2.89% axillary buds activated in aeroponically raised explant, whereas 45.00 ± 3.00% axillary buds activated in field explants at 4.0 mg L^{-1} BAP (*6-benzylaminopurine)*

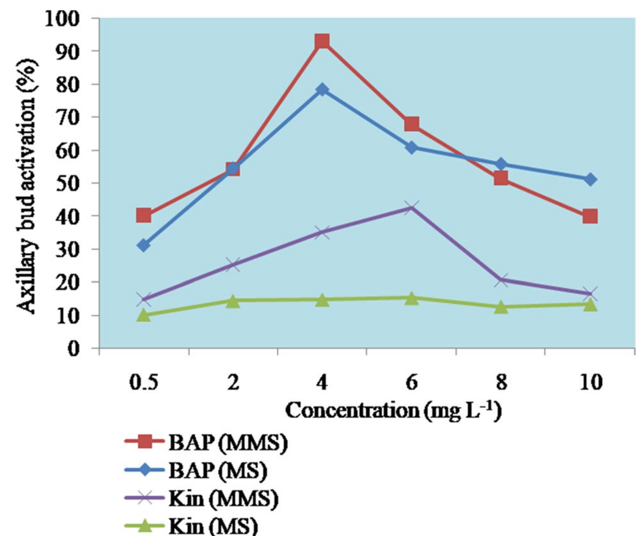

Fig. 4 Comparative effect of basal medium on axillary bud activation in mature explants of *Sarcostemma acidum* (Roxb) Voigt. Normal Murashige and Skoog medium (MS) and modified MS (MMS) medium. Maximum axillary bud activation percentage recorded at MS basal medium was 78.33 ± 2.89 whereas on MMS medium it was 93.00 ± 3.00% (BAP 6-benzylaminopurine, Kin 6-furfuryle aminopurine)

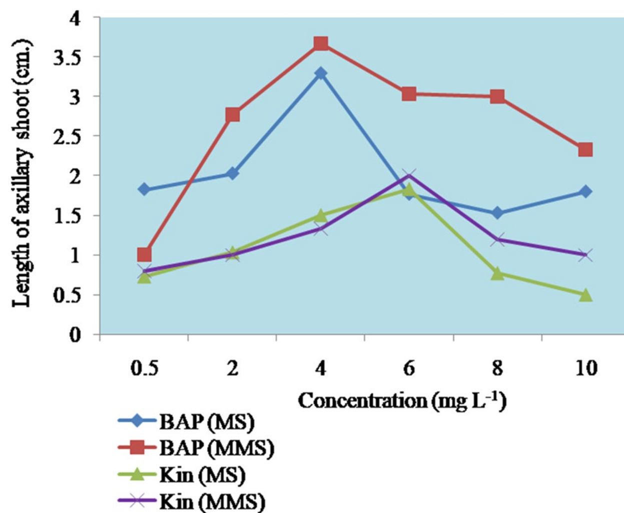

Fig. 5 Comparative effect of BAP and Kin and basal medium (MS and MMS) on length of axillary bud of *Sarcostemma acidum.* Maximum length of axillary shoot observed was 3.67 cm at 4.0 mg L^{-1} BAP in aeroponically raised explant (*BAP* 6-benzylaminopurine, *Kin* 6-furfuryle aminopurine, *MS* Murashige and Skoog medium, *MMS* modified Murashige and Skoog medium)

maintained in the nursery.

***In vitro* shoot bud activation** Between explants collected directly from the field and aeroponically raised in the nursery, those from aeroponically raised plants were found more responsive for the *in vitro* culture establishment. Furthermore, a modified MS medium with high nitrates (1.5X of MS medium) and low calcium chloride (half of MS medium) was found to be the most appropriate basal media for the activation of *S. acidum* axillary shoot buds from mature shoot segments and shoot multiplication in comparison to unmodified basal MS medium. Axillary buds were induced after 1 wk after inoculation of nodal shoots, Among various concentrations of BAP and Kin used for activation of axillary buds, BAP (4.0 mg L^{-1}) was observed to be more suitable than Kin in both types of explants (Fig. 3; and Fig. 6*a*). BAP has also been proved to be more efficient for shoot proliferation of medicinal plants in *Pongamia pinnata* (Sugla *et al*. 2007), *Andrographis paniculata* (Purkayastha *et al.* 2008), *Rhodiola imbricata* (Bhardwaj *et al*. 2018), and *Salvia sclarae* (Grigoriadou *et al*. 2020) and in ornamental shrub *Physocarpus opulifolius* L. maxim cultivar (Jagiello-Kubiec *et al.* 2021). In field-collected explants, 45.00 ± 3.00% of axillary shoot buds were activated on a basal MS medium, whereas approximately 84.00 ± 2.00% bud activation was observed on modified MS (MMS) medium containing 4.0 mg L^{-1} BAP (Fig. 4). However, in the aeroponically raised explants, 78.33 ± 2.89% of axillary shoot buds were activated on basal MS medium and approximately 93.00 ± 3.00% bud activation was observed with 3.67-cm length of axillary shoots on modified MS (MMS) medium containing 4.0 mg L^{-1} BAP (Figs. 3 and 5). Some difficulties were faced during the *in vitro* culture establishment of this plant. The first problem observed was the high contamination rate and the lesser response of axillary bud activation, which was overcome by the use of aeroponically raised plants as a source for *in vitro* culture establishment.

The establishment of mother plants in the greenhouse ensures better growth and development of mother plants and helps in the reduction of contaminations during the establishment of aseptic culture (Debergh and Maene 1981). Another problem encountered was the appearance of huge callus formation at the basal part of the explant

Table 2 Multiplication of shoots of *Sarcostemma acidum* (Roxb) Voigt on modified MS medium containing various concentrations of BAP in combination with IAA and IBA

PGRs (mg L^{-1})			Number of shoots Mean ± SD	Length of shoots (cm) Mean ± SD
BAP	IAA	IBA		
0.0	0.0	0.0	0.00 ± 0.00[j]	0.00 ± 0.00[k]
3.0			1.67 ± 0.58[i]	1.27 ± 0.25[h]
3.5			2.33 ± 0.50[hi]	1.77 ± 0.25[g]
4.0			2.67 ± 0.50[ghi]	2.23 ± 0.25[fg]
3.0	0.01		3.33 ± 0.58[fgh]	2.77 ± 0.25[def]
3.0	0.25		5.69 ± 0.60[cd]	1.77 ± 0.25[g]
3.0	0.50		4.65 ± 0.56[de]	2.03 ± 0.21[g]
3.5	0.01		12.00 ± 1.00[a]	3.67 ± 0.39[ab]
3.5	0.25		7.00 ± 1.00[b]	3.27 ± 0.25[bcd]
3.5	0.50		4.33 ± 0.58[ef]	3.03 ± 0.25[cde]
4.0	0.01		4.67 ± 0.58[de]	2.67 ± 0.29[ef]
4.0	0.25		5.64 ± 0.55[cd]	2.27 ± 0.25[fg]
4.0	0.50		3.66 ± 0.59[efg]	2.10 ± 0.17[g]
3.0		0.01	4.69 ± 0.57[de]	3.27 ± 0.25[bcd]
3.0		0.25	3.67 ± 0.58[efg]	3.43 ± 0.40[abc]
3.0		0.50	2.33 ± 0.54[hi]	3.27 ± 0.25[bcd]
3.5		0.01	6.00 ± 1.00[bc]	3.03 ± 0.25[cde]
3.5		0.25	3.67 ± 0.58[efg]	3.87 ± 0.12[a]
3.5		0.50	2.62 ± 0.54[ghi]	3.70 ± 0.46[ab]
4.0		0.01	1.67 ± 0.58[i]	3.20 ± 0.20[bcd]
4.0		0.25	2.68 ± 0.57[ghi]	2.77 ± 0.25[de]
4.0		0.50	2.00 ± 0.60[i]	2.63 ± 0.15[ef]

Modified Murashige and Skoog (*MS*) medium (5.325 g L^{-1} NH_4NO_3 and KNO_3 supplement and 220 mg L^{-1} $CaCl_2$) along with 3% (*w/v*) sugar, 0.8% (*w/v*) agar. Data were recorded after 25 to 30 d of subculture. Each value of mass multiplication of shoots represents an average number of five replicates using 20 explants. The Duncan multiple range test at $p < 0.05$ was used for calculation of significance among mean differences. *BAP* 6-benzylaminopurine, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *MS* Murashige and Skoog, *PGRs* plant growth regulators

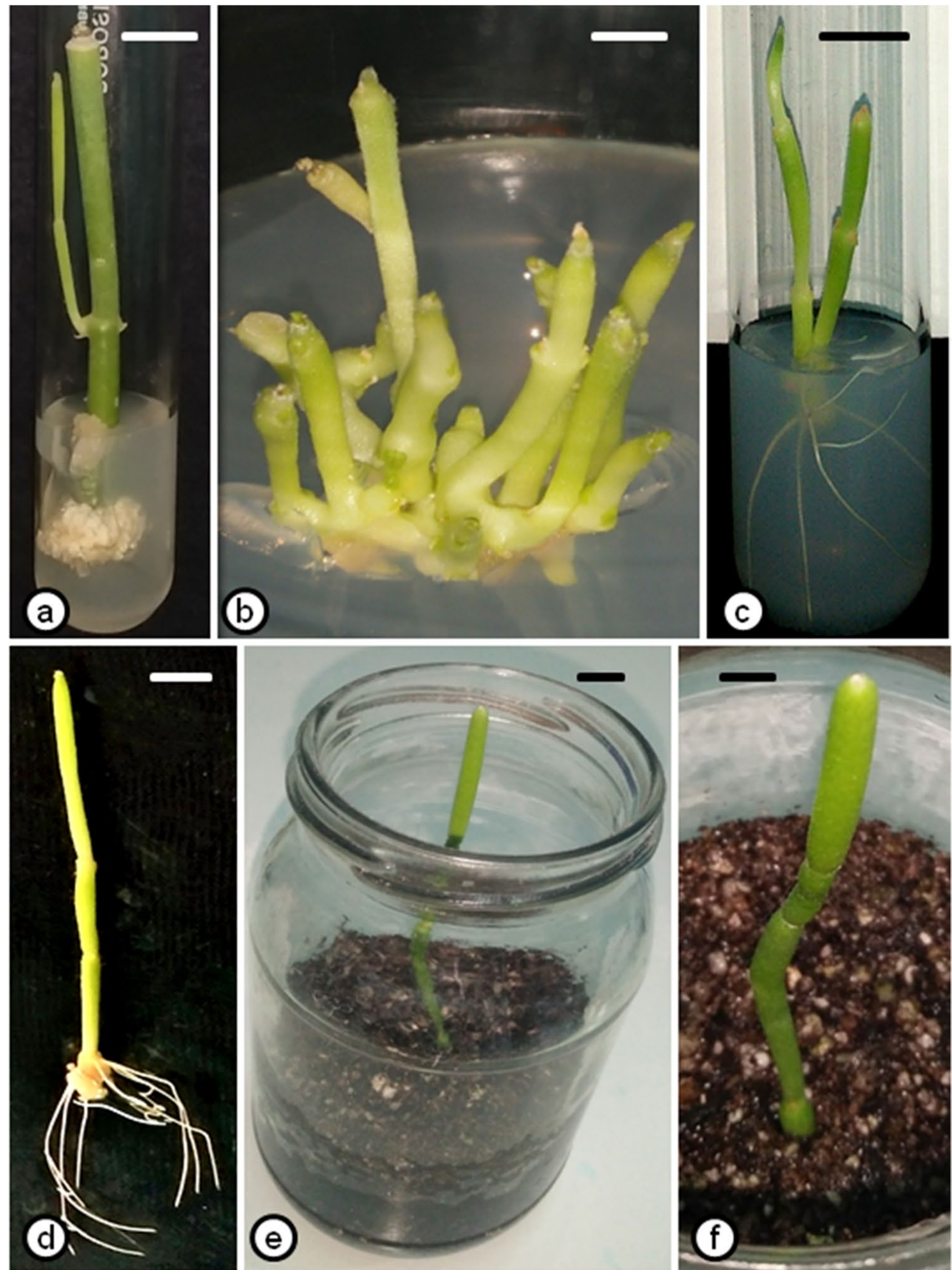

Fig. 6 *In vitro* regeneration of *Sarcostemma acidum* (Roxb) Voigt. (*a*) Axillary bud activation from nodal shoot segments on MMS (modified Murashige and Skoog) medium containing 4.0 mg L^{-1}BAP. (*b*) Shoot multiplication on MMS medium containing 3.5 mg L^{-1} BAP and 0.01 mg L^{-1} IAA. (*c*) *In vitro* root induction from *in vitro* multiplied shoots on half-strength MS medium containing 0.25 mg L^{-1} NAA and 100 mg L^{-1} activated charcoal. (*d*) *In vitro* rooted plantlet. (*e* and *f*) Acclimatized plant on Soilrite in greenhouse (*scale bar*, 1 cm) (*BAP* 6-benzylaminopurine, *NAA* 1-naphthaleneacetic acid)

during axillary bud activation; this was reduced by doing some modifications in macronutrients of basal MS media. Callusing at basal part of explants was minimized by decreasing the calcium concentration in unmodified MS media because calcium enhances the cell division and higher nitrogen level was found to be more appropriate for activation of axillary shoot buds from mature shoot segments of *S. acidum*, in comparison to basal MS medium in the present investigation. Kang and McMahan (2014) also reported a moderate (150 to 300 mg L^{-1}) level of calcium for emergence of single, elongated shoots in guayule tissue culture. Although MS medium has high inorganic nitrogen concentrations and is being used widely in many plant species, in some plant cultures the concentration of inorganic nitrogen in MS medium is far more the amount required for the normal growth of plantlets *in vitro* (Zhang *et al*. 2019). Induction of callus during axillary bud activation from nodal shoot segments was observed to be the major hindrance for clonal propagation of *S. acidum* from field-grown mature plants. Although callus induced from nodal shoots can be an alternative source of *in vitro* plant regeneration as reported by Rathore and Shekhawat (2013), there may be chances of genetic variability during de-differentiation and re-differentiation of cells. Hence,

Table 3 *In vitro* rooting of *in vitro* multiplied shoots of *Sarcostemma acidum* (Roxb) Voigt on half and one-fourth strength MS medium containing various auxins and 100 mg L^{-1} activated charcoal

Auxin (mg L^{-1})			Rooting (%) mean ± SD		Number of roots Mean ± SD		Length of roots Mean ± SD	
NOA	IBA	NAA	½ MS salts	¼ MS salts	½ MS salts	¼ MS salts	½ MS salts	**¼ MS salts**
0.00	0.00	0.00	0.00 ± 0.00^{i}	0.00 ± 0.00^{i}	0.00 ± 0.00^{r}	0.00 ± 0.00^{r}	0.00 ± 0.00^{n}	0.00 ± 0.00^{n}
0.01			6.67 ± 5.77^{gh}	13.33 ± 11.55^{gh}	0.13 ± 0.11^{pq}	0.13 ± 0.11^{pq}	0.07 ± 0.15^{m}	0.07 ± 0.15^{m}
0.025			13.33 ± 11.55^{gh}	13.33 ± 5.77^{gh}	0.20 ± 0.10^{opq}	0.20 ± 0.20^{opq}	0.01 ± 0.01^{m}	0.02 ± 0.02^{m}
0.05			13.33 ± 11.54^{gh}	20.00 ± 10.00^{fgh}	0.13 ± 0.11^{pq}	0.27 ± 0.12^{opq}	0.02 ± 0.02^{m}	0.15 ± 0.03^{klm}
0.10			26.67 ± 15.28^{fgh}	26.657 ± 11.55^{fgh}	0.60 ± 0.20^{mnop}	1.20 ± 0.20^{jk}	0.06 ± 0.02^{lm}	0.18 ± 0.50^{klm}
0.25			33.33 ± 5.78^{efgh}	26.67 ± 5.77^{fgh}	0.60 ± 0.31^{lmnop}	1.13 ± 0.12^{jkl}	0.08 ± 0.02^{lm}	0.24 ± 0.04^{klm}
0.50			26.67 ± 15.28^{fgh}	20.00 ± 10.00^{fgh}	0.40 ± 0.20^{nopq}	0.80 ± 0.20^{klmn}	0.03 ± 0.0^{m}	0.09 ± 0.01^{lm}
	0.01		33.33 ± 5.77^{efgh}	26.67 ± 5.77^{fgh}	1.00 ± 0.20^{jklm}	0.60 ± 0.20^{mnop}	0.12 ± 0.03^{lm}	0.27 ± 0.03^{kl}
	0.025		20.00 ± 10.00^{fgh}	33.33 ± 15.28^{efgh}	0.53 ± 0.12^{mnopq}	0.80 ± 0.20^{klmn}	1.00 ± 0.20^{ij}	2.76 ± 0.25^{lm}
	0.05		40.00 ± 20.00^{efg}	46.67 ± 23.09^{def}	1.33 ± 0.23^{jkl}	2.00 ± 0.20^{i}	1.37 ± 0.16^{i}	2.09 ± 0.25^{fg}
	0.10		60.00 ± 10.00^{bcde}	80.67 ± 20.00^{ab}	4.13 ± 0.64^{cd}	5.40 ± 0.35^{b}	2.17 ± 0.03^{f}	3.38 ± 0.02^{c}
	0.25		46.67 ± 11.58^{def}	66.67 ± 23.09^{abcd}	2.07 ± 0.31^{i}	3.80 ± 0.20^{de}	1.50 ± 0.27^{i}	1.72 ± 0.06^{h}
	0.50		46.67 ± 11.58^{def}	46.67 ± 11.54^{def}	1.87 ± 0.31^{i}	3.30 ± 0.26^{fg}	0.70 ± 0.17^{j}	1.35 ± 0.13^{i}
		0.01	60.00 ± 5.00^{bcde}	60.00 ± 5.00^{bcde}	1.87 ± 0.12^{i}	3.53 ± 0.12^{ef}	2.16 ± 0.05^{f}	3.37 ± 0.07^{c}
		0.025	60.00 ± 20.00^{bcde}	73.33 ± 11.55^{abc}	1.93 ± 0.31^{i}	5.20 ± 0.40^{b}	2.97 ± 0.23^{d}	4.04 ± 0.25^{b}
		0.05	73.33 ± 23.09^{abc}	60.00 ± 18.02^{bcde}	2.80 ± 0.20^{h}	3.87 ± 0.31^{de}	2.64 ± 0.27^{e}	3.02 ± 0.26^{d}
		0.10	73.33 ± 11.58^{abc}	53.33 ± 7.64^{cde}	4.40 ± 0.20^{c}	2.93 ± 0.31^{gh}	1.89 ± 0.15^{gh}	3.23 ± 0.14^{c}
		0.25	86.67 ± 11.55^{a}	46.67 ± 13.58^{def}	$5..93 \pm 0.31^{a}$	2.07 ± 0.50^{i}	4.95 ± 0.05^{a}	0.81 ± 0.05^{j}
		0.50	80.00 ± 20.00^{ab}	33.33 ± 11.55^{efgh}	5.07 ± 0.12^{b}	1.27 ± 0.23^{jk}	2.89 ± 0.06^{d}	0.36 ± 0.02^{k}

Modified Murashige and Skoog (*MS*) basal medium with 3% (*w/v*) sugar, 0.8% (*w/v*) agar, and PGR (IBA, NAA, and NOA). Data were recorded after 25 to 30 d on subculture on rooting media. Each value of *in vitro* rooting from the shoot segments represents an average number of five replicates using 35 shoots. The Duncan multiple range test at $p < 0.05$ was used for calculation of significance among mean differences. *IBA* indole 3-butyric acid, *NAA* 1-naphthaleneacetic acid, *NOA* 1-naphthoxyaceticacid, *SD* standard deviation

plants regenerated through axillary shoots of nodal shoots are preferred over callus differentiation for efficient mass propagation of mature plants.

***In vitro* shoot multiplication** The highest shoot multiplication was achieved by the addition of IAA to MMS medium containing BAP (Table 2). Shoots were multiplied suitably on a modified MS medium containing 3.5 mg L^{-1} BAP and 0.01 mg L^{-1} IAA. An average of 12.00 ± 1.00 shoots per node with 3.67 ± 0.39 cm length was observed on modified MS medium containing 3.5 mg L^{-1} BAP and 0.01 mg L^{-1} IAA (Table 2; Fig. 6*b*). BAP in combination with auxin has been proved to be best for mass multiplication of shoots in *Thalictrum foliosum* (Mishra *et al*. 2020). Arya et al. (2003) used IAA in combination with BA for *in vitro* multiplication of shoot of *Leptadenia reticulata*. This IAA and BAP combination has also been reported as the best combination by Nayak *et al*. (2007) and Makunga and van Staden (2008) in *Aegle marmelos* and *Salvia africana-lutea* respectively. Comparatively longer shoots were observed in the medium supplemented with BAP along with IBA, and the highest average length (3.87 ± 0.12 cm) of shoots was observed at 3.5 mg L^{-1} BAP and 0.25 mg L^{-1} IBA containing medium (Table 2).

Among all types of culture vessels tested for shoot multiplication responses, 250-mL conical flasks were found to be more suitable than culture tubes and 150-mL flasks for *in* vitro mass multiplication of shoots due to more headspace. It is important to choose the optimal size of culture vessel for any plant species, and ensure the proper gas exchange between the medium and the atmosphere in the vessel for the shoot proliferation (Monette 1983).

***In vitro* rooting and acclimatization of *in vitro* regenerated plantlets** Among various concentrations of NAA, IBA, and NOA, and strengths of MS salts used for induction of *in vitro* rooting, NAA followed by IBA and NOA in half-strength MS medium was found to be more appropriate. NAA was also found to be suitable for induction of rooting in micro-cuttings of *Cornus alba* (Ilczuk and Jacygrad 2016) and *Achyranthes asper* (Pai *et al*. 2018). Low-strength MS salts generally promote root induction in shoots (Bopana and Saxena 2008). Half-strength MS macronutrient was also reported to be suitable for rooting of shoots in *Dendrobium candidum* (Zhao *et al*. 2007). Low-strength MS medium has also been proved to be more effective for induction of roots from shoots of

Dendrocalamus longispathus (Saxena and Bhojwani 1993), *Lavendula vera* (Andrade *et al.* 1999), and *Cicer microphyllum* (Singh *et al.* 2019). Reduced concentration of nitrogen ions in low-strength MS medium generally promotes root formation (Driver and Suttle 1987).

Activated charcoal also plays an important role in *in vitro* root induction. There are many reports proving the increased rooting responses shown by activated charcoal when added to the rooting media. Firoozabady *et al.* (2006) reported enhanced rooting ability in transgenic shoots of pineapple (*Ananas comosus*) by the addition of activated charcoal. Agrawal *et al.* (2002) also reported rooting in nodal segment-derived explants of *Simmondsia chinensis* (jojoba), when MS medium supplemented with activated charcoal; according to the author, activated charcoal not only increased the percentage of rooting response but also reduced the callusing of the explants. *In vitro* rooting was observed after 25 to 30 d of inoculation of shoots. The highest 86.67% rooting and average 5.93 roots per shoot with 4.95-cm length were observed on a half-strength MS medium containing 0.25 mg L^{-1} NAA (Table 3; Fig. 6*c* and *d*) and *in vitro* rooting in half-strength MS medium supplemented with NAA, Kin, and activated charcoal in *Fortunella crasifolia* (Yang *et al*. 2006). Regeneration protocol standardized in the present investigation using the aeroponic technique in combination with plant tissue culture technique can be an efficient means of micropropagation for the prospects of large-scale plantation and conservation of this rare and endangered plant species of the Indian arid zone. Light green shoots turned dark green during hardening (Fig. 6*e* and *f*). Approximately 90% of *in vitro* regenerated plantlets successfully hardened in the greenhouse within 6 to 8 wk and survived on garden and field soils.

Conclusions

The aeroponic technique was found to be a very useful technique for induction of adventitious root and development of quality plants in greenhouse. The plants developed in greenhouse through aeroponics are proved to be a better source of explants for efficient *in vitro* plantlets regeneration and conservation of *S. acidum*.

Author contribution Author VK made conception and designed experiment present investigation and author PC performed experiments, collected data, and analyzed scientifically.

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Declarations

Conflict of interest The authors declare no competing interests.

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