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Plantlet Regeneration from Callus Cultures of Selected Genotype of *Aloe vera* L.—An Ancient Plant for Modern Herbal Industries

Mangal S. Rathore · J. Chikara · N. S. Shekhawat

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Abstract *Aloe vera* L., a member of Liliaceae, is a medicinal plant and has a number of curative properties. We describe here the development of tissue culture method for high-frequency plantlet regeneration from inflorescence axis-derived callus cultures of sweet aloe genotype. Competent callus cultures were established on 0.8% agar-gelled Murashige and Skoog's (MS) basal medium supplemented with 6.0 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) and 100.0 mg l^{-1} of activated charcoal and additives (100 mg l^{-1} of ascorbic acid, 50.0 mg l^{-1} each of citric acid and polyvinylpyrrolidone, and 25.0 mg l^{-1} each of L-arginine and adenine sulfate). The callus cultures were cultured on MS medium containing 1.5 mg l^{-1} of 2,4-D, 0.25 mg l^{-1} of Kinetin (Kin), and additives with 4% carbohydrate source for multiplication and long-term maintenance of regenerative callus cultures. Callus cultures organized, differentiated, and produced globular embryogenic structures on MS medium with 1.0 mg l^{-1} of 2,4-D, 0.25 mg l^{-1} of Kin, and additives (50.0 mg l^{-1} of ascorbic acid and 25.0 mg l^{-1} each of citric acid, L-arginine, and adenine sulfate). These globular structures subsequently produced shoot buds and then complete plantlets on MS medium containing 1.0 mg l^{-1} of 6-benzylaminopurine and additives. A hundred percent regenerated plantlets were hardened in the greenhouse and stored under an agro-net house/nursery. The regeneration system defined could be a useful tool not only for mass-scale propagation of selected genotype of *A. vera*, but also for genetic improvement of plant species through genetic transformation.

M. S. Rathore (✉) · N. S. Shekhawat
Biotechnology Center, Department of Botany, Jai Narain Vyas University, Jodhpur, Rajasthan 342005, India
e-mail: mangalrathore@gmail.com

M. S. Rathore
e-mail: mangalrathore@csmcri.org

J. Chikara
Discipline of Wasteland Research, Central Salt and Marine Chemicals Research Institute,
Council of Scientific and Industrial Research, G.B. Badheka Marg, Bhavnagar, Gujarat 364002, India

Present Address:
M. S. Rathore
Discipline of Wasteland Research, Central Salt and Marine Chemicals Research Institute,
Council of Scientific and Industrial Research, G.B. Badheka Marg, Bhavnagar, Gujarat 364002, India

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Introduction

Aloe vera L. (syn. *Aloe barbadensis* Mill.) is known as *Ghee-Kanwar/Gwar-patha* in Hindi is a member of Liliaceae. This plant is considered to be a xerophytic medicinal herb and grows on nutritionally poor soils. The leaves and roots of aloe species are the storehouse of more than 130 interesting secondary metabolites of different classes, viz, alkaloids, anthraquinones, anthrones, bianthraquinoids, chromones, flavonoids, coumarins, and pyrones [1]. *A. vera* is a well-known herbal plant with a long history of uses, which are reported in the earliest Ayurvedic medicinal texts. The clear gel contained within the leaf makes an excellent treatment for wounds, burns, and skin disorders due to the presence of aloectin B, which is reported to stimulate the immune system. Despite the reports for external uses on the skin, some genotypes of aloe (the bitter aloes) can also be taken internally to treat chronic constipation, poor appetite, and to cure digestion-related disorders. *A. vera* is also used as a digestive stimulant and as a strong laxative (http://www.diet-and-health.net/Naturopathy/AloeVera.html; http://www.nationalnutrition.co.uk/acatalog/Aloe_Vera.html) due to the presence of anthraquinones as active ingredients. The leaves are cut transversally at the base to yield a yellow exudate that contains anthraquinones, the bitter compounds responsible for the medicinal effects. The plant species also yields anti-cancer, anti-inflammatory, anti-viral, anti-bacterial, immune-enhancing and parasite-killing [2], and antifungal substances [3].

Pharmaceutical and cosmetic industries have a great demand for *A. vera* material. *A. vera* is an ancient, traditional medicine, and in modern herbal industries, it occupies a key position, as it is used in almost all kinds of herbal preparations; therefore, it is considered as an ancient plant for modern herbal industries. Although this plant is used in the herbal industry, some of the genotypes of *A. vera* (sweet aloes) are edible and are consumed as a vegetable and to make curry and desserts. Due to wide use of aloe products as alternative medicines and dietary supplements and with the rapid expansion of the aloe product industries (herbal, pharmaceutical, and cosmetic), there is an increased demand for its leaves as raw material. Current production of leaves is insufficient to meet the industry demand. Thus, there is a need to undertake large-scale cultivation of selected genotype(s). Propagation of *A. vera* by conventional methods or by means of offshoots is slow; a single plant produces three to four offshoots every year. The presence of male sterility is also a barrier in rapid propagation [4]. The vegetative propagation of aloe is insufficient to meet the increasing demand. Thus, there is limited availability of standard clonal propagules for commercial planting of elite/selected genotype(s). Tissue culture and *in vitro* plant regeneration systems provide alternative production systems for mass propagation of the desired plant species. One of the most extensive uses of plant tissue culture techniques is rapid and large-scale multiplication of plants, where the natural propagation rate is not meeting the demand.

Various workers have attempted to develop tissue culture systems of *Aloe* species. Roy and Sarkar [5] established callus cultures of *A. vera* on nutrient medium with 2,4-dichlorophenoxyacetic acid (2,4-D) and were successful at inducing multiple shoots. Meyer and van Staden [6] induced multiple shoots *A. barbadensis* through adventitious bud formation using decapitated shoots. Abrie and van Staden [7] used seedling-derived explants for micropropagation of *Aloe polyphylla*, which is an endangered aloe species. Liao et al. [8] developed a micropropagation technique of *A. vera* var. *chinensis*, which is

another endangered taxa. Aggarwal and Barna [9] described the tissue culture propagation of an elite line of *A. vera* selected for its higher yield of leaf biomass. Velcheva et al. [10] regenerated *Aloe arborescens* through somatic embryogenesis. Bairu et al. [11] incorporated *meta*-topolin [6-(3-hydroxybenzylamino) purine] and its derivatives as alternatives to the benzyladenine and zeatin for optimizing the micropropagation protocol for *A. polyphylla*. Singh et al. [12] defined micropropagation system for mass propagation of selected genotypes of *A. vera* using axillary meristems. Reports on plant regeneration via callus or somatic embryogenesis of *A. vera* are limited. Since the efficient and reproducible regeneration protocols are required to carry out somaclonal variation and any genetic transformation studies. Our aim is to standardize protocol for high-frequency plantlet regeneration, which is useful for somaclonal variation and genetic engineering experiments. The present study describes the development of tissue culture method for high-frequency plantlet regeneration from callus cultures derived from soft base of immature inflorescence axis of selected germplasm (sweet aloe) of *A. vera* L., used as a vegetable and a source of food.

Materials and Methods

Chemicals, Glassware, and Media Preparations

During the present course of study, pure and analytical grade chemicals of E. Merck (India) Ltd., E. Merck, Germany; British Drug House, Mumbai; Hi Media, Mumbai; Qualigens Fine Chemicals, Mumbai (India); and Sigma Chemical Company, USA were used. Culture tubes (25×150 mm) and 380–400-ml culture bottles/jar (70×135 mm) served as culture vessels for raising, multiplication, and regeneration of cultures and hardening of *in vitro* regenerated plantlets in greenhouse. Murashige and Skoog's (MS) basal medium [13] supplemented with different concentrations of growth regulators, carbohydrate source (glucose and maltose), and other organic supplements was used during the present study. The pH of the media was adjusted to 5.8 using either 0.1 N NaOH or 0.1 N HCl prior to adding 0.8% (*w*/*v*) agar (Qualigen, India). Medium was dispensed into culture vessels. Culture tubes were plugged with non-absorbent cotton wrapped in one layer of cheese cloth, while bottles were capped with polycarbonate caps. Media were steam sterilized at 121 °C and 1.05 kg cm^{-2} for 15 min in an autoclave (NAT Steel Equipment Pvt. Ltd., Mumbai). The cultures were incubated under controlled culture conditions in a culture room. Soilrite (a mixture of horticultural grade perlite with Iris Peat moss and exfoliated vermiculite, supplied by Kel Perlite, Banglore, India) served as supporting medium during hardening of plantlets in a greenhouse.

Plant Material and Surface Sterilization

Plants of the sweet aloe (*A. vera*) genotype (2.5–3.0 years old) were provided by the Botanical Survey of India (BSI), Jodhpur. These were maintained in the greenhouse of the Department of Botany, Jai Nrain Vyas University. Axillary buds, segments of roots, root tips, plant bases, and inflorescence (segments and soft basal part of inflorescence) were evaluated as explants to establish callus culture. The soft basal portion of inflorescence axis was found to be the suitable explants (Fig. 1a) for culture establishment. The explants were harvested and immediately kept in a chilled sterile anti-oxidant solution (200.0 mg l^{-1} of ascorbic acid, 50.0 mg l^{-1} of citric acid and 25.0 mg l^{-1} of polyvinylpyrrolidone (PVP)). These were pre-treated with 0.1% aqueous solution of each of bavistin (a systemic fungicide) and streptomycin (an antibiotic) for 10.0–15.0 min and surface-sterilized with 0.05–0.1%

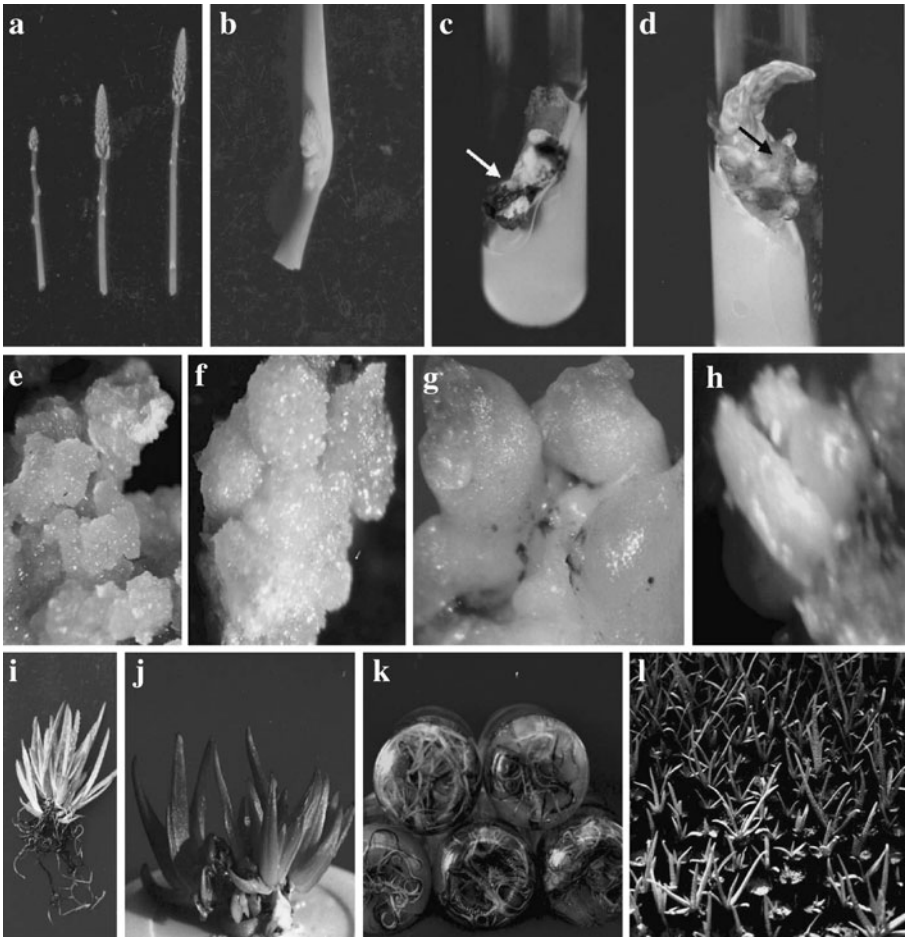

Fig. 1 Culture establishment and regeneration: **a** Young inflorescences and **b** soft base of young inflorescence in leaf axil. **c**, **d** induction of callus cultures of aloe on culture medium with 2,4-D. **e** Microphotograph of callus cultures at multiplication stage and **f** differentiation of globular structures. **g**, **h** Microphotographs of cultures at different stages of culture regeneration. **i** A clump of *in vitro* regenerated plantlets of *Aloe*. **j** *In vitro* regenerated shoots of *Aloe* on medium with higher concentration of BAP. **k** Bottom of culture vessels showing roots in regenerated plantlets and **l** soil transferred plants of aloe produced through tissue culture

aqueous solution of mercuric chloride ($HgCl_2$) for 2.0–5.0 min. These surface-sterilized explants were washed extensively and sectioned carefully into segments of 1.0–1.5 cm (0.5–0.8 cm thickness) and then again kept in chilled and sterile anti-oxidant solution for 2.0–5.0 min until further inoculation on callus induction medium. All the work with the plant material and the tissue culture system were carried out in laminar air flow bench.

Culture Initiation, Multiplication, and Maintenance

The surface-sterilized explants were cultured on 0.8% agar-gelled MS basal medium supplemented with 2,4-D or α-naphthoxyacetic acid (NAA), 100 mg l^{-1} of activated charcoal (AC), and additives (100.0 mg l^{-1} of ascorbic acid, 50.0 mg l^{-1} each of citric acid and PVP, and 25.0 mg l^{-1} each of L-arginine and adenine sulfate) in 25×150-mm culture

tubes for culture induction. Experiments were carried out to evaluate the effects of various concentrations (0.0–10.0 mg l^{-1}) and types (2,4-D and NAA) of auxins on callus induction from explants. The cultures were incubated at 30±2 °C temperature, 60–70% relative humidity (RH) under 12 h per day (h d^{-1}) of photoperiod with a light intensity (provided by cool, white florescent tubes, Philips, India) of 15–20 μmol m^{-2} s^{-1} spectral flux photon (SFP) of photo-synthetically active (460–700 nm) radiation for activation of meristem(s) to proliferate as callus cultures. Subsequently, the callus cultures were transferred on the agar-gelled MS medium containing 3.0% sucrose; 0.5% each of glucose and maltose; and different concentrations and combinations of 2,4-D, cytokinin [6-benzylaminopurine (BAP) and Kinetin (Kin)], and additive (50.0 mg l^{-1} of ascorbic acid and 25.0 mg l^{-1} each of citric acid, L-arginine, and adenine sulfate) for further multiplication and maintenance of cultures. These cultures were incubated in growth chamber at 28±2 °C temperature, 60–70% RH under 12 h d^{-1} of photoperiod with a light intensity of 25–30 μmol m^{-2} s^{-1} SFP.

Plantlet Regeneration, Hardening, and Soil Transfer of Regenerated Plantlets

For regeneration, the calli were subcultured on growth regulator-free one-fourth, one-half, and full strength of MS salts with 3.0% sucrose. Effects of various concentrations (0.0–5.0 mg l^{-1}) and types (BAP and Kin) of cytokinins were also tested for conversion of callus cultures into plantlets. Cultures were incubated under full light intensity (35–40 μmol m^{-2} s^{-1} SFP) at 28± 2 °C temperature, 60–70% RH for 12 h d^{-1} of photoperiod. For hardening, the culture bottles containing regenerated plantlets were kept under greenhouse conditions and natural light intensities for 10–15 days. The *in vitro* regenerated plantlets were taken out of culture vessels carefully and washed with water to remove adhered nutrient agar and transferred to microbe-free soilrite and moistened with one-fourth strength of MS macro-salts solution in 70×135-mm glass bottles. The polycarbonate-capped bottles containing *in vitro* regenerated plantlets were kept in the greenhouse near a cellulose pad to maintain 70% RH and 25–27 °C through evaporative cooling. After 10–12 days, the polycarbonate caps of the bottles were loosened to allow free gaseous exchange with the external environment and to expose the plantlets to *ex vitro* conditions. The bottles were slowly moved from the pad section to the fan section (30% RH and 32±2 °C) in the greenhouse. The hardened plantlets were transferred to soil in black polybags for complete acclimatization. These were kept in a nursery covered with agro-net. The plants were then transferred to the field under the environmental conditions of Rajasthan. All the experiments were set up in completely randomized block design and repeated three times, each treatment having at least ten replicates. The observation on initiation, multiplication, maintenance, and regeneration of cultures was scored after a regular time interval. The rate of culture multiplication was measured by measuring fresh weight of callus at the time of subculture. The data were subjected to one-way ANOVA using SPSS 7.5 software.

Results and Discussion

The development of biotechnological approach to medicinal plant production systems has been the subject of intense research and reviews [14–17]. The application of plant tissue culture for the improvement of *Aloe* species has received little attentions from researchers. In addition to supplying planting propagules, a successful *A. vera* improvement program requires the development of an efficient, comprehensive *in vitro* culture system so that explants can be mass-produced. The present study describes the development of an efficient and reliable system for the plantlet regeneration of a selected genotype of aloe (sweet *A.*

vera genotype) from inflorescence axis-derived callus cultures. Among the different explant materials which were evaluated for the establishment of the callus cultures, the soft base of young inflorescences axis (Fig. 1b) was determined to be the most productive explant source [10]. Older plant bases did not respond to the culture system, while segments of mature inflorescence produced watery, glossy, and non-regenerative callus cultures. Other explants, i.e., axillary buds, roots, and root tips responded also very poorly. Moreover, pre-treatment with a combination of bavistin and streptomycin (0.1% aqueous solution of each) for 15 min and subsequent surface sterilization with 0.1% mercuric chloride for 3 min sterilized the explants and prevented fungal and bacterial growth in cultures. The microbe-free cultures have been maintained in laboratory for more than 3 years. Treatment of explants before and after surface sterilization with chilled and sterile anti-oxidant solution prevented the leaching of phenolic substances from cut ends of the explants thus prevented browning of medium and tissues in the culture system.

Results presented in this work suggest that the process of initiation of regenerative callus cultures is strongly dependent on the nature and concentrations of the growth regulator(s) and on the media supplements (additives and AC) incorporated to the culture initiation medium. Explants responded in cultures after 30–35 days and produced callus from cut ends of explants (Fig. 1c, d). More than 75% of the explants responded, and an efficient callus culture initiation was observed on 0.8% agar-gelled MS medium supplemented with 6.0 mg l^{-1} of 2,4-D, 100 mg l^{-1} of AC, and additives. Fewer explants responded on MS medium with lower (less than 6.0 mg l^{-1}) concentrations of 2,4-D. MS medium containing higher (more than 7.0 mg l^{-1}) concentrations of 2,4-D yielded watery, glossy, and non-regenerative callus; this material turned brown or black within 6–8 days of initiation. The percentage explant response decreased with increasing concentration of 2,4-D in the culture medium (up to 10.0 mg l^{-1}), where the response reached less than 50.0% (Fig. 2). Addition of anti-oxidants and AC in the culture medium reduced the leaching of phenolic compounds and prevented the browning of the tissues and of the culture medium. The 2,4-D was determined to be more performing growth regulator when compared to the addition of NAA for the induction of callus cultures. On MS medium supplemented with 6.0 mg l^{-1} NAA and additives, approximately 65.0% of the explants responded; however, the response was delayed as compared to that with similar concentrations of 2,4-D. An increase in NAA concentration in culture medium had a similar effect as that of 2,4-D. Incubation of cultured tissues under diffused light at 30.0±2.0 °C promoted early meristem(s) activation and proliferation of callus cultures.

Maintenance of callus cultures on culture initiation medium (MS+6.0 mg l^{-1} of 2,4-D) for longer duration (more than three to four passages) leads to the production of watery, glossy, and

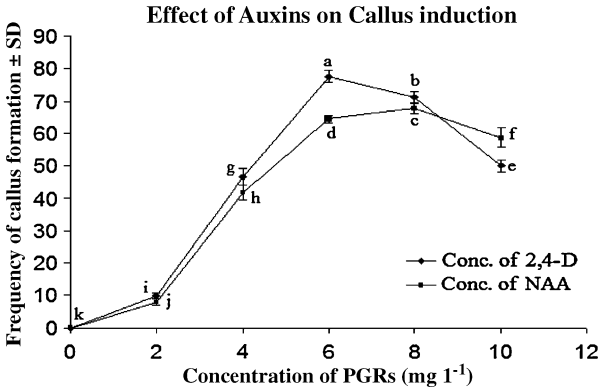

Fig. 2 Effect of auxins on callus induction from explants cultured on full strength of MS medium. Each treatment contains ten explants with three replicates. *Vertical bar* represents standard deviation

non-regenerative cultures, i.e., hyper-hydration of cultures. Hyper-hydration is a physiological disorder of *in vitro* cultures, which adversely affects growth and regeneration ability of cultures which leads to material that cannot be maintained or propagated. It is suggested that hyper-hydration of cultures generally occurs due to the higher concentration of plant growth regulators and ensuing increased water potential of the culture medium [18, 19]. It is therefore important that once the cultures are established, medium, growth regulators, and additives should be optimized so as to maintain the regenerative capabilities of the cultures. Reducing the level of 2,4-D in culture medium and raising osmotic concentration of the medium could reduce the hyper-hydration of cultures. It is also suggested that once meristematic activity is achieved then cultures could be maintained or grown on medium containing lower concentration of plant growth regulators [12]. Therefore cultures were transferred on MS medium containing lower concentration (1.5 mg l^{-1}) of 2,4-D along with 0.25 mg l^{-1} of Kin, 4% carbohydrate source (3.0% sucrose and 0.5% each of maltose and glucose), and additives for multiplication and long-term maintenance of regenerative callus cultures. The cultures produced on this medium were cream-colored and regenerative (Fig. 1e). A further decrease in the concentration (1.0 mg l^{-1}) of 2,4-D in the culture medium leads to the differentiation of callus cultures and produced regenerative, cream-colored, globular structures (Fig. 1f). These cultures maintained on MS medium containing 1.0 mg l^{-1} of 2,4-D, 0.25 mg l^{-1} of Kin, 4% carbohydrate source, and additives were further used for regeneration of cultures.

Regeneration of callus cultures is desirable for uniformity, genetic stability, and fidelity. The globular structures produced shoot buds on growth regulator free and cytokinin supplemented full strength of MS salts. On one-fourth and one-half strengths of MS salts, the response was slow and poor. Incorporation of cytokinins (BAP and Kin) up to a concentration (3.0 mg l^{-1}) in the regeneration medium promoted the conversion of callus cultures into plantlets as compared to growth regulator-free MS medium. Initially, the globular structures differentiated into buds-like structures (Fig. 1g, h) and then produced complete plantlets. On regeneration medium with 1.0 mg l^{-1} of BAP, response was early and the plantlets produced were healthy, sturdy, and dark green with well-developed root system (Fig. 1i). Further increase in concentration (more than 5.0 mg l^{-1}) of BAP in the regeneration medium produced only shoots without roots (Table 1; Fig. 1j). Less than 10% of regenerated shoots rooted on MS medium with 5.0 mg l^{-1} of BAP. However, these

Table 1 Influence of types (BAP and Kin) and their concentrations on callus culture regeneration in sweet aloe

S. No.	BAP concentration (mg l^{-1})	Kin concentration (mg l^{-1})	Average no. of plantlets regenerated±SD	Average % of rooted shoots±SD	Average of % un-rooted shoots±SD
1	0.0	0.0	11.8±1.317 i	94.55±2.65 ab	5.44±2.65 gh
2	1.0	0.0	23.5±1.49 f	95.89±1.22 a	4.10±1.22 h
3	2.0	0.0	36.8±0.757 c	56.09±4.99 d	40.93±4.99 e
4	3.0	0.0	53.73±1.142 b	37.27±2.17 e	62.72±2.17 d
5	5.0	0.0	59.76±1.29 a	7.52±2.26 g	92.47±2.26 a
6	0.0	1.0	16.66±0.889 g	91.29±4.59 b	8.7±4.59 g
7	0.0	2.0	26.4±0.644 e	77.18±5.83 c	22.81±5.83 g
8	0.0	3.0	32.53±0.99 d	13.68±6.66 f	86.32±6.66 c
9	0.0	5.0	36.03±0.77 c	8.87±2.25 g	91.12±2.25 b

Means±SD followed by the same letter in same column are not significantly different by the Duncan's multiple range test at 0.05% probability level

shoots could be rooted on growth regulator-free full-strength MS. The synthetic cytokinin BAP was determined to be a more efficient growth regulator than Kin for the conversion of callus cultures into plantlets. With increasing incubation time, the cultures (shoot buds differentiated) developed a healthy and strong root system (Fig. 1k); however, after 50 days, the shoots became flattened and the tips of the shoots became brown and then dry. Therefore, to avoid this problem, the cultures had to be transferred for hardening.

The different temperature and humidity regimes in the greenhouse and the gradual exposure of plants to *ex vitro* conditions ensured plant adaptation to growth under environmental conditions. One hundred percent of *in vitro* regenerated plantlets were hardened. The hardened plants of *A. vera* were transferred to soil in black polybags containing garden soil, sandy soil, and organic manure in 1:1:1 (*w*/*w*/*w*) ratio. These were stored in an agro-net house (Fig. 1l) until field transfer. These tissue culture-raised plants were field transferred to various sites in Western Rajasthan at (1) Rampura Agriculture Research Station, Government of Rajasthan, Jodhpur, (2) Garden of Botanical Survey of India (BSI), Jodhpur, and (3) Field of Anuraj Farms, Manai, Jodhpur. At all of the sites, the plants are growing well under field/environmental conditions.

Conclusion

In the present investigation, a tissue culture protocol for high-frequency regeneration from callus cultures derived from soft basal portion of inflorescence axis of sweet aloe genotype has been defined. The regenerative callus cultures were established on 0.8% agar-gelled Murashige and Skoog's (MS) basal medium supplemented with 6.0 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) and 100.0 mg l^{-1} of activated charcoal (AC) and additives (100 mg l^{-1} of ascorbic acid, 50.0 mg l^{-1} each of citric acid and PVP, and 25.0 mg l^{-1} each of L-arginine and adenine sulfate). These were multiplied and maintained on MS medium containing 1.5 mg l^{-1} of 2,4-D, 0.25 mg l^{-1} of Kinetin (Kin), and additives with 4% carbohydrate source. The cultures were regenerated on MS medium with 1.0 mg l^{-1} of 6-benzylaminopurine (BAP) and additives. One hundred percent regenerated plantlets were successfully hardened and transplanted in soil.

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