Tropical Agricultural Research & Extension 13(4): 2010

Improved culture medium for micropropagation of *Aloe vera* L

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Accepted : 27th September 2010

ABSTRACT

An efficient micropropagation protocol was developed using lateral shoot explants of *Aloe vera*. Both shoot induction and elongation were better on MS medium supplemented with 4mg/l BAP + 0.2mg/l NAA + 1g/l PVP. All cultures showed shoot regeneration in this medium with 16 shoots/explant. Addition of 1g/l PVP was ineffective for controlling phenolic browning of explants and culture discoloration. However, more adventitious buds (21.5 shoots/explant) developed on MS medium supplemented with 4mg/l BAP + 0.2mg/l NAA + 1g/l PVP+10mg/l citric acid+0.5g/l activated charcoal. Browning of explants was minimized in this medium and elongation of micro-shoots and the growth of the plantlets were also better. Further elongation and rooting of micro-shoots were obtained when sub-cultured on to MS basal medium containing 0.5g/l activated charcoal and 100% of the survival of rooted plantlets was observed after acclimatization. Therefore, the above protocol could be effectively used in rapid micropropagation of elite plants of *Aloe vera*.

Key words: *Aloe vera*, Phenolic browning, Micropropagation, Culture media, Growth hormones

INTRODUCTION

Aloe vera (Liliaceae), is a succulent plant indigenous to Northern Africa and Mediterranean countries and has become naturalized almost in all parts of India (Klein *et al.* 1988). The plant has stiff gray -green lance-shaped leaves containing clear gel in a central mucilaginous pulp. *A. vera* has been used for several thousands of years in folk medicine in many cultures from ancient Egypt, Greece, and Rome to China and India (Kemper and Chiou 1999).

Some of the most important pharmacological activities of *A. vera* are antiseptic (Capasso *et al.* 1998), anti- tumor (Winter *et al.* 1981), anti-inflammatory (Yagi *et al.* 1998), wound and burn healing effect (Heggers *et al.* 1993), anti diabetic (Rajasekaran *et al.* 2006) and as an adjunct to current AIDS therapy (Mc Daniel *et al.* 1990).

A. vera propagates vegetatively in its natural state. However, propagation rate is very slow because a single plant can produce only three to four lateral shoots in a year. Moreover, the production of *Aloe* leaves is insufficient to meet the industry demand in India (Aggarwal and Barna 2004) and the production of cosmetics, foods and pharmaceuticals containing *A. vera* has experienced a slow increase due to limited availability of raw material with high quality (Campestrin *et al.* 2006). Therefore, there is a need to develop suitable, an alternative method for traditional propagation of *A. vera.*

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In vitro techniques using micropropagation and tissue culture offer a great possibility to overcome this problem. Micropropagation using stem and lateral shoot pieces of *A. vera* has already been proved successful (Natali *et al.* 1990; Roy and Sarkar 1991; Mayer and Staden 1991; Aggarwal and Barna 2004). However, source of explants, their sterilization procedure, media composition, culture conditions, phenolic browning of explants and media discoloration greatly affect shoot regeneration from different genotypes of the same species. *A. vera* exudes lot of phenolic substances into the culture media which could decrease the survival of explants (Roy and Sarkar 1991). Concentration of phenolic compounds may vary in different genotypes of the same species (Glynn *et al.* 2004), and also those were grown under different climatic conditions (Kjaer *et al.* 2001). Hence culture conditions are needed to be modified accordingly to achieve the desirable targets. Thus, present study aimed to develop a rapid and high frequency shoot regeneration protocol for elite plants of *A. vera* suitable for mass propagation by improving culture media while controlling phenolic browning of explants.

MATERIALS AND METHODS

Source of explant

Lateral shoots (suckers) of *A. vera* (one month old) were harvested from green house grown plants in the Department of Molecular Biology and Biotechnology of the GB Pant University of Agriculture

and Technology, Pantnagar, India during the winter season, i.e. from December-January and they were used as the explant source. Explants were prepared by removing roots and brown colored tissues and extending leaf portions to give an average size of 3 -4cm. They were washed thoroughly with running tap water for about 10 minutes till all soil and other foreign materials washed off. Sets of twenty explants were then washed with tap water containing a few drops of Tween 20 and rinsed in 70% ethanol for 30 seconds followed by initial soaking in sodium hypochlorite containing approximately 4% available chlorine for 10 minutes and then in freshly prepared mercuric chloride solution (0.1 %) for 10 minutes. Finally they were washed 3-4 times with sterile distilled water before culturing.

Shoot amplification, elongation and culture conditions

The basal medium consisted of MS salts and vitamins (Murashige and Skoog 1962) supplemented with 3% sucrose and solidified with 6.5g/l agar. The pH of the medium was adjusted to 5.7 with 0.5N NaOH before autoclaving at 121^0C for 20 min. 50ml of nutrient medium was dispensed into 300ml glass jars that were capped with polycarbonate caps. Lateral shoot explants were initially cultured in MS basal medium supplemented with 1 g/l Polyvinylpyrollidone (PVP), and devoid of growth hormones. Cultures were incubated at 27± ^{0}C, under cool white fluorescent tubes (20-30 $E.m^{-2}s^{-2}$), with 16h photoperiod. Explants devoid of contaminations were then incubated on the basal medium supplemented with different concentrations of BAP (2, 4, 6 & 8mg/l) alone or in combination with NAA either 0.1mg/l or 0.2mg/l and with 1g/l PVP for shoot amplification. Cluster of shoots amplified from initial lateral shoot explants were sub-cultured as it is without separation from the explants on the same regeneration media after one month from initial establishment stage. Shoots amplified from lateral shoot explants in shoot induction media, measuring 1.5-2cm tall and of one month old from first stage of sub-culturing were detached from explants and transferred on to a shoot elongation medium containing 0.5g/l activated charcoal.

Additional Treatments for controlling phenolic browning

Since incorporation of 1g/l PVP into the culture media did not satisfactorily reduce the exudation of phenolic substances during initial experiments, five additional treatments were employed to arrest phenolic browning along with 1g/l PVP in the culture media. In the 1st treatment, lateral shoot explants were soaked in 0.5% PVP solution for 30 minutes followed by transferring them on to the shoot induction medium which induced maximum number of shoots (4mg/l BAP + 0.2 mg/l NAA) in initial experiments. In the 2nd treatment, explants were transferred into the shoot induction medium containing 4mg/l BAP + 0.2 mg/l NAA and incorporated with 0.5g/l activated charcoal. In the 3rd treatment explants were transferred on to shoot induction medium containing 4mg/l BAP + 0.2 mg/l NAA and 10mg/l citric acid. In the 4th treatment explants were cultured in shoot induction medium containing 4mg/l BAP + 0.2 mg/l NAA, 10mg/l citric acid and 0.5g/l activated charcoal. In the 5th treatment, explants were serially transferred after 24 hours in to fresh shoot induction media containing 4mg/l BAP + 0.2 mg/l NAA for up to three days.

Rooting of microshoots

Shoots which did not develop roots in shoot elongation medium containing MS basal medium + 0.5g/ l activated charcoal, measuring 4-5cm tall and of two months old after sub culturing on to shoot elongation medium were transferred on to two different rooting media *viz.* MS medium containing 0.2mg /l NAA and MS medium containing no growth hormones respectively.

Acclimatization

About 5-6cm tall, well rooted plantlets were taken out from the culture vessels after one month from root induction media and the root system was washed carefully to remove residues of agar attached to them. Plants were then dipped in (1%) fungicide solution (Bavistin) for 10 minutes and were planted in pots of 7cm X 8cm containing sterilized sand and top soil (1:1) and kept in shade in a plant house under intermittent mist for 10 days. Hoagland solution (25ml per each plant) was supplied during acclimatization and then the plantlets were transferred to plastic containers (1l internal volume) containing a mixture of soil, sand and organic manure (2:1:1) and grown in a plant house at 28-30^{0}C with 85-90% Relative Humidity

RESULTS

Effect of plant growth regulators on organogenesis

Emergence of shoots from lateral shoot explants (suckers) was observed after 2 weeks of culture in all the hormone combinations tested except in the control medium devoid of growth hormones (Table 1 & Plate 1). Shoot buds initiated were light green to yellowish in colour and arisen either as single, or in clusters. Callus formation did not occur at the cut end of explants. Treatment difference for the percentage of cultures showing shoot proliferation and the number of shoots per explant was noticed within four weeks of culture and there was a significant difference ($P \leq 0.05$) in number of shoots per explant among many of the hormone combina-

Table 1: Effect of BAP and NAA on shoot proliferation, number of shoots per explant and shoot elongation. Data were recorded after one month of culture and values represent Means ± SE of 20 explants tested.

Hormone concentration (mg/L)		% of Cultures showing shoot proliferation	No. of shoots per explants from initial culturing	Length of the shoots (cm)
BAP	**NAA**			
0	0	0	0	0
2.0	0	55	0.7±0.82[a*]	0.2±0.4[a*]
4.0	0	80	2.1±0.61[c]	0.3±0.4[b]
6.0	0	50	0.6±0.52[a]	0.3±0.4[b]
8.0	0	45	0.7±0.43[a]	0.2±0.5[a]
2.0	0.1	90	1.7±0.58[b]	0.4±0.3[c]
4.0	0.1	100	2.9±0.43[c]	0.5±0.3[d]
6.0	0.1	95	2.3±0.52[c]	0.3±0.4[b]
8.0	0.1	75	2.7±1.08[c]	0.2±0.4[b]
2.0	0.2	100	14.2±0.35[f]	0.7±0.3[e]
4.0	0.2	100	16.0±0.33[g]	1.0±0.3[g]
6.0	0.2	100	10.3±0.34[e]	0.8±0.3[f]
8.0	0.2	85	4.8±0.4[d]	0.4±0.4[c]

*Means having different letters in superscript in each column are significantly different from each other (P≤0.05) according to Duncan's Multiple Range Test.

Table 2: Effect of activated charcoal and citric acid on shoot proliferation and elongation. Data were recorded after one month of culture and values represent Means ± SE of 10 explants.

Treatment	No. of Shoot/ explants from initial culturing	Shoot length
MS† + 1g/l PVP	15.1±1.2[a*]	0.9±0.3[a]
MS + 1g/l PVP + 0.5g/l activated charcoal	18.5±0.9[b]	1.4±0.6[b]
MS + 1g/l PVP + 10mg/l citric acid	18.7±1.2[b]	1.3±0.9[b]
MS + 1g/*L* PVP + 0.5g/l activated charcoal + 10mg/l citric acid	21.5±1.3[c]	1.6±0.7[c]
Soaking of explants in 0.5% PVP solution for 30 min. and culture in MS + 1g/l PVP	15.3±1.4[a]	1.0 ±0.4[a]
Serial transfer of explants in to fresh media within 24 hours for 3 days and culture in MS + 1g/l PVP	16.3±1.0[a]	1.1±0.5[a]

*Means having different letters s.s in each column are significantly different from each other (P≤0.05) according to Duncan's Multiple Range Test.
† MS medium with 4mg/*L* BAP + 0.2mg/l NAA.

The maximum number (16.0) of shoot bud per explant was observed in the presence of 4.0 mg/l BAP and 0.2mg/l NAA within four weeks of culture (Table 1). Only few shoot buds were initiated in media containing BAP alone and majority of the shoot buds did not develop further into shoots and remained arrested even at eight week of culture. Although some shoots were formed in media containing either higher BAP levels as higher as 8.0 mg/l or 8.0 mg/l BAP + 0.1 mg/l NAA, they produced abnormally large, broad and wrinkled, light yellowish leaves and shoots were remained stunted.

Effect of growth regulators on shoot multiplication and elongation

Shoot buds proliferated on medium containing 4 mg/l BAP and 0.2 mg/l NAA multiplied into clusters of small buds and elongated when subcultured on the same regeneration media (Plate 1). The maximum shoot length (1.0 cm) was obtained in the above mentioned medium within four weeks of culture (Table 1). However, media containing 6 mg/l BAP + 0.2 mg/l NAA and 2 mg/l BAP + 0.2 mg/l NAA produced significantly ($P \leq 0.05$ level) a lower shoot length (0.89 cm and 0.78 cm) compared to medium supplemented with 4 mg/l BAP and 0.2 mg/l NAA.

Effect of pretreatments and addition of activated charcoal and citric acid into culture media on phenolic browning and shoot regeneration efficacy

Since incorporation of 1.0 g/l PVP into culture media did not markedly reduce phenolic exudation as reported by Roy and Sarkar (1991), five additional treatments were employed in order to arrest phenolic browning.

Incorporation of 0.5g/l activated charcoal and 10mg/l citric into culture media either alone or in combination significantly increased the multiple shoot production and elongation (Table 2). A significant ($P \leq 0.05$ level) higher shoot number (21.5) per explant was obtained in the presence of 0.5g/l activated charcoal + 10mg/l citric acid after one month of culture compared with the other treatments (Table 2). Media containing either 0.5g/l activated charcoal or 10mg/l citric acid alone were resulted in a significantly lower shoot numbers (18.5 and 18.7) compared to medium supplemented with a combination of 10mg/l citric acid and 0.5g/l activated charcoal. The maximum shoot length (1.6cm) was observed in the medium containing both 0.5g/l activated charcoal and 10mg/l citric acid. In this medium about 12-15 shoots elongated above 0.5cm from a cluster of shoot buds within one month of culture.

No significant increase in shoot number or shoot length was observed when explants were either presoaked in PVP or serially transferred on to fresh media although there was a slight decrease in the media discoloration compared with the medium containing 1g/l PVP. However, degree of media discoloration was not assessed in the present study since addition of activated charcoal was already resulted in a black colored medium. There was no browning of explants in the media containing activated charcoal irrespective of citric acid after two weeks of culture. However, a slight discoloration in the medium as well as on explants was observed in media containing only 1g/l PVP. Explants that were presoaked in PVP solutions and also those were serially transferred on to fresh media also showed a slight browning on explants.

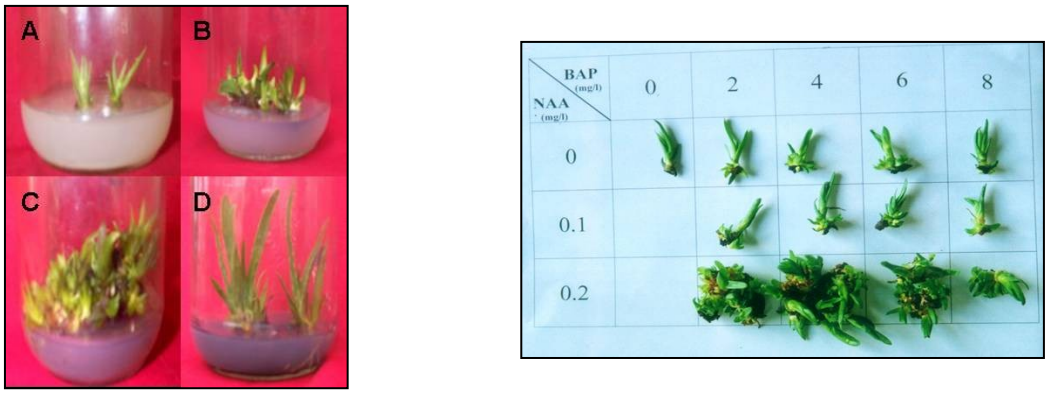

Plate 1: Developmental stages of *Aloe vera in vitro* (Left side); A: initial establishment of lateral shoot explants on MS medium devoid of growth hormones, B & C: shoot regeneration and multiplication in MS medium containing 4 mg/*L* BAP + 0.2 mg/*L* NAA (see the media discoloration due to phenolic exudation), D: shoot elongation in MS basal medium containing activated charcoal. Regeneration of shoots from lateral shoot explants in various combinations of BAP and NAA after one month of culture (Right side).

Plate 2: Rooting of *Aloe vera* after one month in three different rooting media (Left side); 1: MS basal medium with 0.5 g / *L* activated charcoal, 2: MS medium devoid of growth hormones and 3: MS medium containing 0.2 mg/*L* NA. Acclimatization of rooted plantlets (Right side).

Rooting of regenerated shoots

Shoots in the initial regeneration media containing BAP alone or in combination with NAA did not produce roots. However, media containing activated charcoal irrespective of presence of citric acid induced roots after one month of culture. However, the roots initiated in these media were thin and delicate. Therefore, rooting potential in two other media; one devoid of growth hormones and other containing 0.2 mg/l NAA was also evaluated.

Table 3: Effect of three different media on root induction and elongation. Data were recorded after 2 weeks of culture and values represent Means ± SE of 10

Treatment	% of Shoots inducing roots	No. of roots	Length of roots
MS basal medium	80%	1.5±0.22[a*]	0.6±0.43[a]
MS medium + 0.5 g/*L* activated charcoal	90%	1.4±0.56[a]	3.5±0.52[b]
MS medium + 0.5 g/*L* activated charcoal + 10mg/L citric acid	90%	1.4±0.72[a]	3.1±0.52[b]
MS medium + 0.2mg/L NAA	100%	1.3±0.34[a]	4.1±0.55[c]

* Means having different letters in superscript in each column are significantly different from each other (P≤ 0.05) according to Duncan's Multiple Range Test.

Rooting occurred within two weeks in all rooting media (Plate 2). 100% rooting was observed in media containing 0.2 mg/l NAA while 90% rooting was observed in media containing 0.5 g/l activated charcoal irrespective of presence of citric acid and lacking hormones within two weeks of culture (Table 3).

No significant difference in root number was observed after two weeks of culture among three rooting media as well as in the control. However, the root length was different significantly and the maximum root elongation was observed in the medium containing 0.2mg/l NAA (Table 3). Roots regenerated in this medium were relatively thick and strong compared to other media even during initial stage of development. However, after one month of culture, plants that were grown in the medium containing activated charcoal or in combination with citric acid also had very healthy and thick roots and growth of the plants was also better in those media compared to other two rooting media (Plate 2). Hence an economic point of view, an additional rooting medium would not be required since the shoot elongation medium containing 0.5g/l activated charcoal irrespective of presence of citric acid could be used for rooting as well.

Acclimatization of rooted plantlets

Well developed rooted plantlets (4.5 cm – 5 cm long) were obtained after two months of culture on MS basal medium containing 0.5g/l activated charcoal and were transferred to pots containing soil and sand of 1:1 ratio. Hundred percent of the explants was survived during and after the acclimatization in the pots in the plant house (Plate 2). Plants looked healthy and they were morphologically similar to that of mother plants.

DISCUSSION

A number of factors such as genotype, culture medium (including growth regulators and their combinations), physical environment, explant developmental stage, etc affect adventitious shoot regeneration from tissue cultured explants (Qu *et al.* 2000). Therefore, present study attempted to optimize the growth regulator and their concentration for efficient direct shoot regeneration from lateral shoot explants of *Aloe vera* while controlling the phenolic browning. Moreover, the *in vitro* regeneration of direct adventitious shoots is an essential component to produce plants from elite materials as to avoid formation of somaclones.

BAP and NAA were selected for shoot regeneration/multiplication in the present study as they are among the growth regulators used most often for the shoot organogenesis (Datta *et al.* 2006). Although shoot amplification occurred in almost all the hormone combinations, BAP alone was less favorable for shoot induction. Nevertheless, it had been favorable in previous studies (Aggarwal and Barna, 2004) with shoot tip explants. Nevertheless, BAP was not detrimental to expalnts as reported by Mayer and Staden (1991) and Natali *et al.* (1990) with decapitated shoot explants since about 50% of shoot frequency and 0.5 number of shoots per explants were observed in the present study with BAP . However, a combination of BAP and NAA was crucial for direct shoot regeneration.

According to Chaudhuri and Mukundan (2001), the best medium for shoot induction from shoot tips of *Aloe vera* was MS supplemented with 10 mg/l BAP + 160 mg/l Adenine sulphate and 0.1 mg/l IBA However, this is in contrast to the results obtained in the present investigation as when BAP at a higher concentration (8 mg/l) either alone or in combination with NAA was added, it reduced the shoot production and increased the number of abnormal shoots. When there is high cytokinin level present in the medium, it causes cytogenetic instability (Qu *et al.* 2000) thus unsuitable for clonal propagation. In order to obtain desirable clonal fidelity, cytokinins must be used at levels that stimulate adventitious shoots thereby avoiding potential somaclonal variants (Peddaboina *et al.* 2006).

Aggarwal and Barna (2004) proved that *Aloe vera* could be cultured *in vitro* using axillary buds and MS medium containing 1 mg/l BAP and 0.2 mg/l IBA. Citric acid at 10 mg/l improved the shoot multiplication. However, they could obtain maximum of 5 shoots per explant after 4 weeks of culture. In the present study, a medium containing 4mg/l BAP+0.2mg/l NAA+ 1g/l PVP+10mg/l citric acid increased the number of shoots per explants to 18.7 and when 0.5g/l activated charcoal was incorporated, number of shoots per explant was increased to 21.55 and their elongation was also better. This is the highest number of shoots per explants reported from *Aloe vera* compared to previous studies with different explant sources and media compositions.

The enhancing effect of citric acid and activated charcoal in controlling phenolic browning and accelerating organogenic potential is crucial. Explant browning and media staining were major impediments in the culture establishment phase. Browning is usually attributed to the production of phenolic compounds released from the cut surfaces of the explants. Phenolic compounds include polyphenols and tannins and they may not only prevent explant development, but also lead to death of the explant (Bhat and Chandel 1991). The presence of 1g/l PVP in the culture medium reduced secretion of phenolic substances from stem segment explants of *Aloe vera* (Roy and Sarkar, 1991). However, in the present study, incorporation of 1g/l PVP was not sufficient to control phenolic browning as evident by increase in media discoloration and explant blackening from first week of culture.

Media fortification either with ascorbic acid or PVP did not markedly influence reduction in severity of oxidative browning and phenolic exudation of explants of apple (*Malus domestica*) in culture medium (Dalal *et al.* 2004). Moreover, the percentage of explants exudating phenolic compounds from *Quercuss suber* varied according to the sterilization process and time of sampling and maximum browning was observed in December, in winter period. Since the explants were harvested from December-January i.e. during winter in the present study, it might have been contributed to increase level of phenolic substances on explants which would have affected on culture establishment and regeneration.

Media incorporated with 10mg/l citric acid improved the shoot regeneration frequency and number of shoots per explant. Citric acid has a dual role as a potential antioxidant and a growth enhancer (act as vitamins) in plant tissue culture systems.

Application of citric acid at 1.0 mM in MS media improved the shoot development from microshoots of *Prunus avium* (Vasar, 2003) and addition of 118.1μM citric acid in to culture medium increased the multiple shoot formation from stem node explants of *Santalum album* **(**Sanjaya *et al*. 2006). Furthermore, the antioxidant property of citric acid in reducing phenolic browning in tissue culture systems has been well documented (Bhatt and Dhar, 2004).

A higher number of shoots per explant, their multiplication and elongation were observed on MS medium containing 0.5 g/l activated charcoal irrespective of presence of citric acid after one month of culture. It has been reported that addition of activated charcoal was found to be beneficial for explant survival of Mango and reducing phenolic exudation into medium (Thomas and Ravindra 1997). Moreover, enhancing effects of activated charcoal on microshoot elongation are well documented in other plants such as *Juglans regia* L (Saadat and Hennerty 2001), *Taxus wallichiana* (Datta *et al.* 2006) and *Jovellana punctata (*Ronse 1995). Activated charcoal adsorbs excess growth regulators found on plants and also toxic phenolic compounds produced during culture regeneration and stimulate growth and differentiation. It darkens the culture medium and thus avoid formation of free radicals by oxidation of poly phenolic compounds (Mohamed-Yaseen *et al.* 1995). A lower phenolic content was associated with a higher explant survival rate (Dalal *et al.* 1993). Phenolic compounds are involved in growth regulation process and a group of them are auxin protectors. The auxin protectors should be kept within the tissue to stimulate growth and their release to the culture medium should be reduced to alleviate the phytotoxic effect (Debergh and Read 1991).

Pretreatment with PVP was found to be effective in controlling phenol exudation from mature explants of *Myrica esculenta* (Bhatt and Dhar 2004). However, in the present study there was no significant reduction in the media discoloration and increase in number of shoots per explant when they were presoaked with 0.5% PVP. Serial transfer of *Aloe vera* shoot explants in to fresh culture media after every 24 hour intervals up to about three days did not significantly result in the increase of shoot number or decrease in media discoloration when compared to other treatments. Serial transfer of explants is beneficial in reducing phenolic browning in *Myrica esculenta* (Bhatt and Dhar 2004) and Mango explants (Thomas and Ravindra 1997).

Root induction and elongation were more or less similar in media containing 0.5g/l activated charcoal and 0.2mg/l NAA after one month of culture. This suggests that external application of auxins are not necessary for root induction of *Aloe vera* and these results are consistent with the findings of Aggarwal and Barna (2004) and Roy and Sarkar (1991). Hormone free root induction has been reported in other plants such as Garden phlox, *Phlox paniculata* (Fraga 2004).

CONCLUSION

The present study described an efficient protocol for shoot proliferation of *Aloe vera* with 100% development of shoots per explants with better quality plantlets in terms of growth. Higher shoot number reported in the present study in the shoot induction media could be attributed to the beneficial effect of activated charcoal and citric acid in culture media. Rooting was also possible in shoot elongation medium containing activated charcoal. Therefore, an additional rooting medium was not required and it is important in terms of cost of production. Moreover, 100% survival of plants after acclimatization could also be achieved. Hence, the shoot regeneration procedure described in the present study could be ideal for rapid micropropagation of elite plants of *Aloe vera.*

ACKNOWLEDGEMENTS

Authors wish to acknowledge the Council for Agricultural Research Policy, Sri Lanka for funding for the research. Thanks are due to Dr V Sundaresan of the Central Institute of Medicinal and Aromatic Plants at Nagla Sub Station, India for providing plant materials of *Aloe vera.*

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