In Vitro Propagation of Aloe vera (*Aloe indica* Royle) through Apical Shoot Tip Culture

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Abstract

An efficient *in vitro* protocol was established for large production of Aloe vera (*Aloe indica* Royle). The shoot tip explants, collected from the axenic cultures were used for the optimization of rapid shoot production on MS medium supplemented with different concentrations (0.5, 1.0, 2.0 and 3.0 mg/L) of BAP and Kn. The maximum shoot number were recorded as 14.33 per culture on medium added with MS + 1.0 mg/L BAP + 4% sucrose + 2.8 g/L gelrite after 30 days of culture. The shoots were rooted under *in vitro* and *in vivo* condition. About 90% shoots produced root on medium, ½ MS + 0.5 mg/L IBA+ 2% sugar after 30 days of culture. Simultaneously *in vitro* grown shoots were inoculated in the sand made propagation bed for *ex vitro* rooting under mist house condition. In the propagation bed 100% of the micro shoots survived and well rooted while taking a longer time (8 weeks) than that of *in vitro* rooting. The rooted seedlings were transferred in polybag containing garden soil, compost and sand with the proportion of 1:1:1 respectively. After hardening 99% seedlings survived in polybag and showed excellent growth.

সারসংক্ষেপ

এ্যালোভেরা উদ্ভিদের কাণ্ডের শীর্ষাগ্র (Apical shoot tip) থেকে অধিক সংখ্যক চারা উৎপাদনের একটি কার্যকরী *In vitro* কৌশল উদ্ভাবন করা হয়েছে। জীবাণুমুক্ত কালচার এ নতুন জন্মানো ক্ষুদ্র বিটপগুলি থেকে explant নিয়ে দ্রুত বিটপ উৎপাদনের মাত্রা নিরূপণে MS মিডিয়ামে বিভিন্ন ঘনত্বে (০.৫, ১.০, ২.০ ও ৩.০ মিলিগ্রাম/লিটার) BAP ও Kn যোগ করা হয়। চার সপ্তাহ পরে ১.০ মিলিগ্রাম/লিটার ঘনত্বে BAP গ্রোথ হরমোন ও ৪% চিনিযুক্ত যুক্ত খাদ্য মিডিয়ামে কালচার প্রতি সর্বোচ্চ গড়ে ১৪.৩৩টি বিটপ পাওয়া যায়। উৎপাদিত বিটপগুলিকে নিয়ন্ত্রিত ও অনিয়ন্ত্রিত উভয় পরিবেশে শিকড় জন্মানো হয়। নিয়ন্ত্রিত পরিবেশে ৩০ দিনের মধ্যে অর্ধ শক্তির MS মিডিয়াম এর সাথে ০.৫ মিলিগ্রাম/লিটার IBA এবং শতকরা ২ ভাগ চিনিযুক্ত খাদ্য মিডিয়ামে শতকরা ৯০% বিটপ এ শিকড় গজায়। একইভাবে নিয়ন্ত্রিত পরিবেশে জন্মানো বিটপগুলিকে মিস্ট হাউজে বালুর বেডে লাগিয়ে শিকড় জন্মানো হয়। বালুর বেডে বিটপগুলির শতকরা ১০০ ভাগ বেঁচে থাকে এবং শিকড় গজায়। এ ক্ষেত্রে বিটপে শিকড় গজাতে কিছুটা সময় বেশি লাগে। বেডে লাগানোর ৮ সপ্তাহ পর শিকড় গজাতে শুরু করে। এভাবে উৎপাদিত অনু চারাগুলিকে পলিব্যাগে স্থানান্তর করা হয় এবং ৯৯% চারা বাইরের পরিবেশে দ্রুত বেড়ে উঠে।

Key words: *Aloe indica*, *In vitro* propagation, Optimization, Shoot tip culture.

Introduction

Aloe indica is one of the important species which has valuable medicinal properties and used commercially in pharmaceutical, cosmetic and food industries. It is an important xerophytic medicinal plant that belongs to the family Asphodelaceae (Liliaceae). Although Aloe vera originated in the warm, dry climates of Africa, the plant is readily adaptable and grows even in rainfall condition worldwide (Steenkamp and Stewart 2007). The plant prefers sunny weather, requires well-drained soil and can grow in nutritionally poor soil. Mexico, followed by the rest of Latin America, China, Thailand and the USA were described as main producing countries for Aloe (Rodriguez *et al.* 2010). Pharmaceutical and cosmetic industry has great demand for Aloe vera. Its therapeutic use was reported earlier by several scientists (Cera *et al.* 1980; Afzal *et al.* 1991; Davis *et al.* 1998). *Aloes* have been used for a variety of purposes in ancestral and modern societies. They are used for preparing food, feed and beverages, formulating ethnomedicinal and ethnoveterinary remedies and preparing traditional and modern cosmetic products. They are also grown for ornamental purposes. *Aloe* gels and latexes are used in treating bacterial, fungal, and viral diseases, healing wounds and skin burns, treating protozoan and helminthic infections and normalizing noninfectious physiological ailments. *Aloe* based cosmetic and healthcare products such as shampoos, moisturizers and skin conditioners are good sources of revenues in many countries (Dwivedi *et al.* 2014; Habtemariam and Medhanie 2017; Yemane and Medhanie 2016; Beyene 2015; Abdi *et al.* 2013; Elsheikh *et al.* 2013; Abadi and Kaviani 2010). Moreover, many *Aloe* species are extensively used in the preparation of cosmetic and toiletry industries. *Aloes* grow nearly in all parts of the world (Dwivedi *et al.* 2014; Smith *et al.* 2008; Newton 2004; IASC 2002). Different accounts put the number of *Aloe* species between 450 and 600. The demands for *Aloes* in the medicinal and cosmetic industries is increasing at an alarming rate, but large-scale production schemes to meet the demand are limited (Dwivedi *et al.* 2014; Haque and Ghosh 2013). There is a lack of production of *Aloe* leaf to meet the industry demand and so it is necessary to undertake large scale cultivation of *Aloe* (Aggarwal and Barna 2004).

Propagation of Aloe vera by conventional methods or by means of offshoots has many drawbacks. Poor natural propagation by means of axillary shoots and the presence of male sterility are the two major barriers in rapid propagation of Aloe vera (Natali 1990; Demissew and Nordal 2010). To overcome this problem, plant tissue culture based clonal propagation system can of great help (Murashige 1974; 1978). The technique is particularly useful for plants where the rate of multiplication is very slow. Hence, there have been many attempts to multiply the crop *in vitro* by many workers. Several studies have reported rapid *in vitro* propagation of Aloe vera (Meyer and Staden 1991; Aggarwal and Barna 2004; Ahmed 2007; Gantait 2010; Mukesh Kumar 2011; Baksha *et al.* 2005). Shoot tips have been used as the explant source in most of the *in vitro* micro propagation protocols (Baksha *et al.* 2005; Singh and Sood 2009; Hosseini and Parsa 2007; Hashemabadi and Kaviani 2008; Kalimuthu *et al.* 2010). Scientists had obtained different results applying formulation of plant growth regulators with MS media. This study was conducted to develop a reproducible protocol of *in vitro* micro propagation of *Aloe indca* using the most common plant growth regulators to produce quality planting materials for large production.

Materials and Methods

Explants collection, preparation and sterilization for axenic culture

Two weeks old Aloe vera seedlings were collected from outdoor source. The seedlings were maintained under greenhouse condition up to 6 weeks to obtain enough amount of planting materials to start the experiment. A total of ten shoot tip explants were collected from the offshoot-derived from the mother plant. The explants were thoroughly washed in running tap water about 30 minutes. After that it trimmed to 3-4 cm and treated with Tween 20 for 5-10 minutes. Finally washed thoroughly with sterile distilled water for 2 to 3 times. Prior to inoculation, the explants were subsequently surface sterilized with 70% ethanol for 2-3 minutes, 20% sodium hypochlorite solution for 10 minutes and washed 3 to 4 times with sterile distilled water under laminar air flow.

Preparation of culture media

Culture media were prepared as per the standard protocol of Murashige and Skoog (MS) (1962) media supplemented with various concentrations of different PGRs, namely, BAP, Kn IBA, and NAA. Full-strength (for initiation and shooting) and half-strength (for rooting) MS media were prepared by adding appropriate stock solutions of micronutrients, macronutrients, and additives and by enriching them with 30-40 gm sucrose and 2.8 gm/L gelrite as a solidifying agent. pH of the medium was adjusted to about 5.8 by adding drops of 1N HCl and 1N NaOH as appropriate. The media were autoclaved at 121°C and 15 psi (pounds per square inch) for 20 minutes and allowed to cool at room temperature to about 60°C.

Culture conditions

The cultures were incubated at 25±2°C under cool white and fluorescent light of 2000-2500 lux, relative humidity about 60-80% and 16/8 hours photo and dark period were maintained in growth chamber respectively. Data on seed germination, multiple shoot induction, elongation and rooting were taken and statistically analyzed. Observations were recorded periodically. These culture conditions were used in all the experiments mentioned below unless otherwise stated.

Culture initiation for multiple shoot production

The initial cultures were established in full-strength MS medium supplemented with 1.0 mg/L BAP adding 3% sucrose. Cultures were also started with devoid of growth regulators as control treatment. Single explant was inoculated into each culture bottle. They were incubated for 4 weeks in a growth room adjusted to 25±2°C under fluorescent tube light with 16 hours photoperiod and 2000–2500 lux light intensity.

Induction of multiple shoots and optimization

The shoot tips collected from axenic cultures were cultured on MS medium supplemented with 0.0, 0.5, 1.0, 2.0, 3.0 mg/L of BAP and Kn alone and/or in combination along with 3-4 % sucrose. Physiological conditions and number of shoots per explant were observed periodically. Rate of multiplication of shoots was recorded up to 3-4 weeks of post inoculation. *In vitro* elongation was also attained on the same medium.

In vitro rooting of the shoot

In vitro elongated shoots (6-7 cm) were taken out from the culture vessel and transferred to half strength MS medium with different concentration (0.0, 0.5, 1.0, 1.5, 2.0 mg/L) of IBA for root induction and one control without PGRs. All treatments were replicated three times.

Ex vitro rooting of the shoots

The *in vitro* grown elongated shoots were rooted in sand made propagation bed inside the mist house. The culture bottles with shoots were brought out from the growth room and kept under green house for 2-3 days losing the cap. Then the shoots were removed from the culture bottle and washed under tap running tap water until the medium is cleaned up. The cleaned shoots were then inoculated in the sand made propagation bed for rooting.

Hardening and acclimatization of plantlets

Cleaned rooted plantlets were transferred to sterile soil mix prepared by mixing garden soil, compost and sand in a 1:1:1 ratio and subjected to photoautotrophic culture system for 20 days prior to the greenhouse transfer. After transferring to the temperature and humidity controlled greenhouse, plants were hardened for a month time. Subsequently, well established plants were shifted to nursery beds.

Statistical analysis

All experiments were performed as Completely Randomized Design (CRD). Data were analyzed using statistical analysis system (SAS v9.3) and means were statistically compared using LSD test. The significance level was set up at $p < 0.05$. Three replications were considered for each treatment and repeated thrice.

Results

Culture initiation for multiple shoot production

Sometimes it seems to be difficult for establishing contamination free culture of Aloe vera. So, axenic culture of Aloe vera was established on MS medium from axillary shoot tip explants derived from greenhouse grown seedlings. Culture initiated from the axillary shoot tip of young seedling in MS medium supplemented with different concentrations of BAP (0.0, 0.5, 1.0, 1.5 mg/L). Under given conditions and over a culture period of 30 days explants from all the treatments produced multiple shoots simultaneously. The best media combination for culture establishment and multiple shoot initiation was found MS + 1.0 mg/L BAP + 4% sucrose. In this media combination, cultures were started to respond for new shoot initiation within 7 days and 100% of the cultures were established by the next 30 days.

Shoot induction and multiplication

The shoot multiplication rate was significantly different according to the various concentration of cytokinins supplemented in the media. To establish shoot regenerative potential and multiple shoot production, the single shoot was cultured on MS medium supplemented with different concentrations of cytokinins alone or in combination. Cytokinin level produced a significant response upon the number of shoots and leaves produced per plant. Treatment with BAP 1.0 mg/L induced comparable number of shoots (Fig.1). MS basal medium devoid of plant growth regulators (PGR) did not support the induction of multiple shoots. Among the different cytokinins, BAP at its 1.0 mg/L concentration evoked best response than the other concentrations. Shoots after their initial proliferation on medium containing 1.0 mg/L BAP were sub-cultured on same fresh medium after every 15 days. After excision of the multiple shoots, when the mother explants was cultured on the fresh shoot multiplication medium (MS+ 1.0 mg/L BAP) then the shoot numbers were increased significantly for the next four repeated transfers and reduced thereafter. Incorporation of Kn into MS medium supported shoot multiplication.

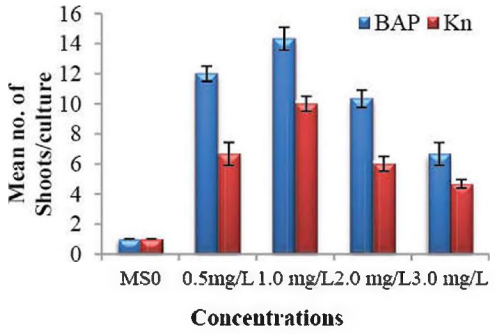

Figure 1. Effect of different concentrations of cytokinin on shoot multiplication of Aloe vera after 30 days of culture. Each value is the mean of three replications. Vertical bars indicated standard errors.

However, BAP proved to be a better choice than Kn because the maximum number of shoots per culture was obtained 14.33 on 1.0 mg/L BAP (Fig.1, Fig. 2B & 2C) and 10.0 for 1.0 mg/L Kn (Fig.1, Fig. 2D, 2E & 2F) after 30 days of culture respectively.

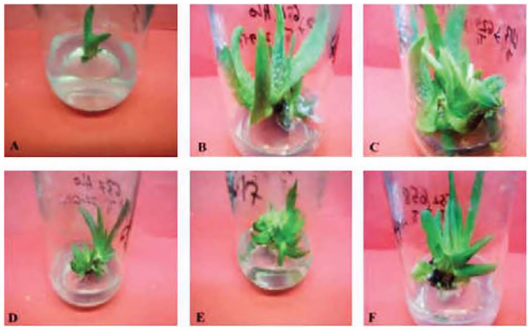

Figure 2. Effect of different concentrations of BAP and Kn in MS medium on multiple shoot production of Aloe vera. A. Control. B. MS + 1.0 mg/L BAP + 4% sugar after 15 days & C. after 30 days of culture. D & E. MS + 1.0 mg/L Kn + 4% sugar after 15 days and F. after 30 days.

In vitro rooting of regenerated shoots

The regenerated shoots were well rooted in ½ MS +0.5 IBA+ 2% sucrose medium. Roots were induced within one week of culture at the base of the shoots. The half strength of MS medium without any PGR was failed to induce rooting of regenerated shoots. However, shoots were capable to induce root when cultured on medium containing auxins. Auxins in different concentrations induced roots when incorporated in the medium containing ½ strength of MS salts. The best rooting response, about 99% of the cultures was observed on medium containing 0.5 mg/L IBA supplemented with 2% sugar and 2.8 g/L gelrite after 28 days. The maximum mean number of roots per culture was recorded 13 with a length of 10 cm after 28 days of culture (Fig. 3, Fig. 4A, 4B, 4C & 4D).

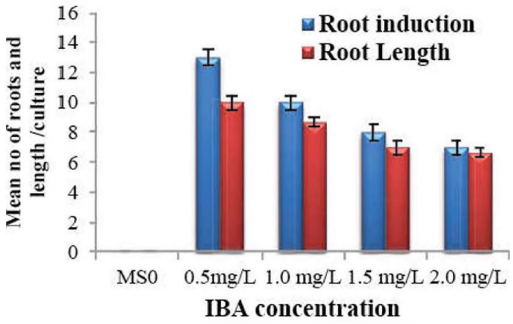

Figure 3. Effect of different concentrations of IBA on root induction of Aloe vera from *in vitro* regenerated shoots after 4 weeks of culture. Each value is the mean of three replications. Vertical bars indicated standard error.

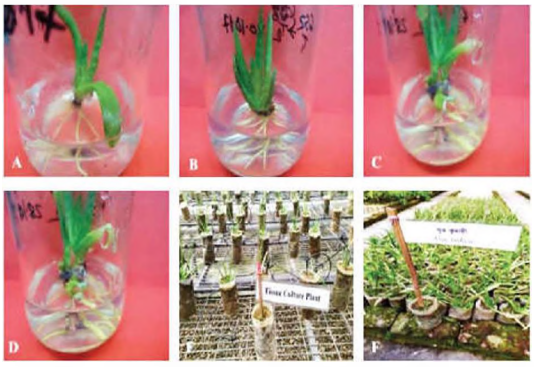

Figure 4. *In vitro* rooting and plant production of Aloe vera. A, B, C & D. shoots with roots in culture bottles. E. Plantlets transferred in poly bag for hardening under green house. F. Tissue cultured plants in the nursery bed.

Ex vitro rooting of regenerated shoots

The regenerated shoots were also rooted under *ex vitro* condition in the mist house. Four weeks old *in vitro* grown shoots were brought out from the growth room and kept inside the green house for 3 to 4 days losing the cap of culture bottle. Later the shoots were washed under running tap water and cleaned the base from medium. The cleaned shoots were inoculated in the sand made propagation bed for rooting. The results showed that 100% shoots were survived and started for rooting at the base within one month after transferred in propagation bed. The shoots also produced axillary shoots at a various level during rooting period. The highest mean number of root per plant was recorded as 14 with a length of 4.0 cm after 8 weeks (Table 1, Fig. 5).

Table 1. Root initiation and plant production of *in vitro* grown shoots of *Aloe vera* in propagation bed.

Days	Morphogenic response			
	% of shoot induced root	Mean no. of root /plant	Mean length of root /plant	Mean no. of new shoots/ plant
2 weeks	0	0 ± 0.0	0 ± 0.0	1 ± 0.0
4 weeks	50	8 ± 0.57	2 ± 0.28	2 ± 0.57
6 weeks	70	11 ± 0.76	3 ± 0.0	3 ± 0.50
8 weeks	100	14 ± 0.57	4 ± 0.50	3 ± 0.28

The rooted plants were transferred in polybag containing sterile soil: cow dung: sand (1:1:1) for hardening. The plantlets were hardened and grown well in polybag at nursery bed for further developing new shoots.

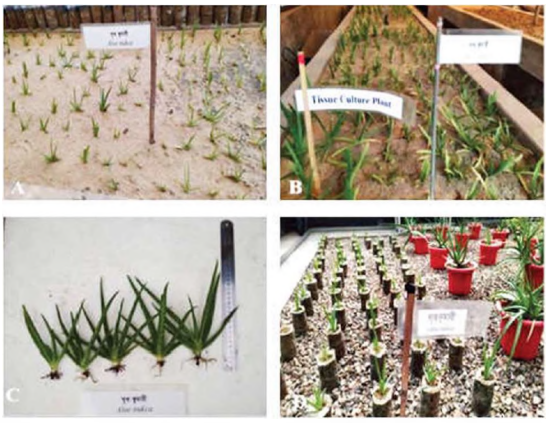

Figure 5. *Ex vitro* rooting of *in vitro* grown shoots of Aloe vera in propagation bed and plant production. A) Shoots are inserted in propagation bed from culture bottle. B) Shoots at the propagation bed after 8 weeks. C) Rooted shoots, & D) Plantlets in polybags for hardening.

Acclimatization of plantlets

The culture bottles with rooted shoots were brought out from the growth room and kept under green house for 2-3 days losing the cap. Then the shoots were removed from the culture bottle and washed under tap water until the medium is cleaned up from the roots. Cleaned rooted plantlets were transferred to sterile soil mix prepared by mixing sand and cow dung in a 1:1:1 ratio. After transferring to the temperature and humidity controlled greenhouse, plants were hardened for a month. Subsequently, well-established plants were shifted to nursery beds. Similarly, the well rooted shoots in the propagation bed under mist house were transferred to soil in polybag and hardened for further growth and development (Fig. 4E & 4F, Fig. 5D).

Discussion

In vitro optimization of shoot production for mass propagation of Aloe vera was established in MS medium supplemented with different cytokinins. It was observed that shoot proliferation was faster with the addition of

cytokinins in the culture media than the media devoid of plant growth regulators. The results revealed that the supplementation of plant growth regulators were positively influenced the shoot proliferation of Aloe vera. Rahman *et al.* (2018) stated that the apical shoot tip of *Phylanthus emblica* was able to produce multiple shoots in MS medium supplemented with different concentrations of cytokinins BAP and Kn. The shoot tip culture of *Gymura procumbens* proliferated faster with the addition of cytokinins than the medium devoid of plant growth regulators (Rahman *et al.* 2019). However, growth regulators, mainly cytokinins are the most important factors affecting the shoot proliferation. Different concentrations of cytokinin i.e. BAP, kinetin and 2-ip have been used in micropropagation research work (Bhojwani and Razdan 1992). BAP is the most reliable and useful cytokinin which is demonstrated by a wider survey of existing literature. In the present study, shoot proliferation also occurred in presence of cytokinin. Signs of shoot proliferation were showed after 7 days of culturing. Multiplication of shoot was best on MS medium with 1.0 mg/L BAP. The percentage of shoot proliferation and number of shoots were 90 and 15, respectively. BAP variations affecting shoot proliferation were also reported by Bhandari *et al.* (2010), Gantait *et al.* (2010) and Mangal *et al.* (2009). Abrie and Staden (2001) and Chaudhuri and Mukundan (2001) had also reported the use of BAP in shoot proliferation of *Aloe polyphylla* and *Aloe vera,* respectively. It was also reported that the highest shoot proliferation in *Aloe vera* was found in MS medium containing BAP and IBA (Aggarwal and Barna 2004; Mukesh *et al.* 2011 and Meyer and Staden 1991). This is in contrast to earlier reports in *Aloe vera* by Natali *et al.* (1990) where better proliferation occurred on medium containing kinetin instead of BAP. Baksha *et al.* (2005) also reported that the enhancement of shoots was observed by using BA and NAA. Rooting response of micro shoots was also reported with the use of growth regulators such as NAA and IBA in medium (Bhojwani and Razdan 1992). In the present study, healthy rooting was observed in IBA (0.5 mg/L) medium. Healthy roots were obtained in medium with IBA 0.5 mg/L in 30 days of time. The highest root response in *Aloe vera* was reported in hormone free medium (Bhandari *et al.* 2010; Aggarwal and Barna 2004). The highest shoot proliferation was also reported in MS medium containing BAP 1.0 mg/L and IBA 0.2 mg/L (Mukesh *et al.* 2011) while highest percentage of root induction (80%) was observed in MS medium supplemented with IBA 0.5 mg/L. Similar result was reported by Abrie and Staden (2001) in *Aloe vera*. Based on hardening, explants were transferred to plastic cups containing sterilized sand and every day moistened with 10 times diluted MS broth. After two weeks they were transferred to earthen pots containing soil and sand under greenhouse conditions for 3-4 weeks for acclimatization. The survival rate was 99% and the plants established well in 4-6 weeks of growth at nursery bed.

Conclusion

Like many species of *Aloe vera*, *Aloe indica* can be a good source of phytochemicals with medicinal, nutritional, and pharmaceutical potential. Exploring into the *in vitro* micropropagation of the plant is one effort in a large-scale project aiming at elucidating its regeneration, physicochemical, and agronomic characteristics. As the present study shows, the plant can easily and successfully be propagated *in vitro* with the help of the commonly used PGRs, namely, BAP, Kn and IBA. The results of the study will serve as important foundation for future research using many different media formulations and combinations to develop an optimized protocol of large-scale *in vitro* micropropagation. The developed protocols

enable to produce large number of healthy tissue culture plants of aloe vera within a short period of time for future demand.

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