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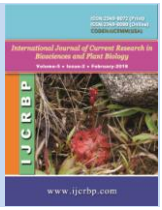

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An Efficient Micropropagation of *Achyranthes aspera* L. Using Shoot Tip Explant

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ABSTRACT

Traditional medicinal herb *Achyranthes aspera* L. holds an important place in Siddha and Ayurvedic medicines. Plant tissue culture studies have been carried out in this plant employing various explants. However, no report is available on micropropagation through shoot tip explant. Surface sterilization using Bavistin and $HgCl_2$, and *in vitro* germination procedure adapted for *A. aspera* provided very low level of contamination than other published reports, hitherto. Shoot tips cultured on Murashige and Skoog medium fortified with BAP at 2 mg/L produced maximum number of multiple shoots. Combinations of different concentrations of BAP with IAA did not facilitate the production of more shoots. Rooting of microshoots was successful in 2 mg/L of IBA. Vermiculite containing garden soil was suitable for hardening of somaclones.

Introduction

Thousands of plants have been utilized by the traditional medical practitioners to treat various diseases. Human population fulfils 80% of their medical needs through herbs (WHO, 1993). Foremost traditional medicines: Siddha and Ayurveda employ *Achyranthes aspera* L. for the treatment of various diseases. It is popularly known as "prickly-chaff flower" and it belongs to the family, Amaranthaceae. In Tamil it is referred as "*Nayuruvi*". This woody perennial herb is found in Puducherry, Tamil Nadu, other parts of Indian subcontinent and Bangladesh on waste lands and roadsides. Its habitat extends upto Cholistan desert and frequently used by the local inhabitants to cure chronic and acute diseases (Hameed et al., 2011).

A. aspera has found wide application in dropsy, piles, skin eruptions; used as a diuretic, astringent and purgative (Bhatnagar et al., 1973; Kapoor and

Kapoor 1980; Basak, 1997); in heart diseases, ascites, uterine–bleeding, as an antidote to snake bite (Selvanayagam et al., 1995); dysentery (Srivastava et al., 1980). Utilized also for the treatment of fractured bones (Singh and Ali, 1989); intestinal parasites, whooping cough, respiratory troubles, asthma (Reddy et al., 1988; 1989); abdominal disorders (Thakur et al., 1992), renal disorder, malarial fever, leucoderma and used as a laxative (Purohit et al., 1985), etc.

Production of true to type plants is been achieved by meristem cultures. Shoot tip of plants possesses apical mertistem (the embryonic tissue). Experiments had been carried out in *A. aspera* using leaf, node, internode and root as explants for *in vitro* callus development and/ or clonal propagation by scientific researchers. However, shoot tip has not been utilized as an explant. This study has been carried out to formulate a protocol for the micropropagation of *A. aspera* through shoot tip explants. Our results indicated that the procedure adapted for this study is suitable for an efficient *in vitro* germination of seeds and micropropagation of *A. aspera*.

Materials and methods

The experiments were conducted at Plant tissue culture laboratory of the department of Botany, Tagore Arts College, Puducherry. Shoot tips of *in vitro* germinated seedlings of *A. aspera* were used as explant. The mature seeds were collected from the college campus for this purpose. The plant identity was authenticated by Dr. K. Kadavul of Botany department.

Surface sterilization of seeds

Mature and healthy seeds were manually dehusked and surface sterilized using 1% fungicide- Bavistin (procured from PASIC, Puducherry) for 15 min with intermittent shaking. Fungicide solution was decanted and the seeds rinsed with sterile distilled water thrice. Later, they were subjected to 0.1% mercuric chloride ($HgCl_2$) solution treatment for 3 min and the solution was discarded. Subsequently, rinsed with sterile distilled water for 3 times. Entire culture experiments were divided into five stages:

Stage I: Culture medium and *in vitro* seed germination

The MS (Murashige and Skoog) medium was used with 3% sucrose (Himedia) (Murashige and Skoog, 1962). The *p*H of the media was set at 5.7 and gelled with 0.8% (w/v) agar. Three different PGRs were used: 6-benzyl aminopurine (BAP), Indole-3-acetic acid (IAA) and Indole-3-butyric acid (IBA). PGRs were added to the culture media just prior to autoclaving (20 lbs for 15 min). *In vitro* seed germination was done by inoculating the seeds on ½ strength MS medium with no PGR addition, i.e. ½ BM. Cultures were incubated at 25±1° C with a photoperiod of 12 h light:12 h dark in all stages.

Stage II: Shoot induction on BAP media

After two weeks of seed inoculation, shoot tip (1 cm long with first node) of seedlings was excised and inoculated on culture tubes containing MS medium added with five different concentrations of BAP (1, 2, 3, 4 and 5 mg/L).

Stage III: Shoot induction on BAP+IAA media

Another set of shoot tip explants from *in vitro* raised seedlings were inoculated on MS medium containing BAP at the concentrations of 1, 2, 3, 4 mg/L and IAA (0.25 mg/L).

Stage IV: *In vitro* rhizogenesis

Microshoots formed from shoot tips on BAP and BAP+IAA media were microsurgically removed and utilized for rhizogenesis on IBA (1 to 4 mg/L) augmented media. Once the roots were formed they were counted and results tabulated. The microshoots with roots are termed as somaclones. The mean and standard deviation were calculated using the open source statistical software PSPP (GNU PSPP) version 0.7.8.

Stage V: Hardening of microshoots

Finally, rooted plantlets were transferred to plastic cups containing an equal amount of garden soil and vermicompost. They were kept at 25±1°C for two weeks. Once the somaclones established a good root system they were shifted to garden for further growth.

Results and discussion

Clonal propagation of plants through multiple shoot formation requires sequential steps. The findings of our study in each stage are presented below with the discussion.

In vitro seed germination

Fig. 1 shows the frequency of seed germination during first week of culture. Three fourth of the seeds germinated within 24 hrs. It was found that maximum number of seeds (85.6%) germinated on 5th day after inoculation. Germinated seedlings had cotyledonary and foliar leaves (Fig. 2A). Success of plant tissue cultures needs effective prevention of contamination. It was found to be very low (7.37%), however, with only fungal contamination. No increase in seed germination frequency evinced after 5th day. But the length of the seedlings increased further.

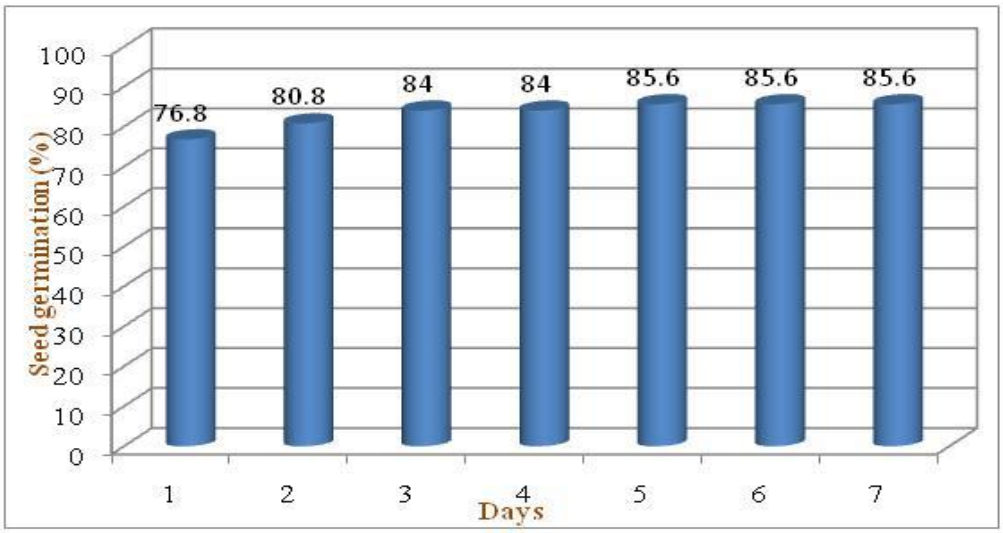

Fig. 1: *In vitro* seed germination of *A. aspera* in ½BM.

$HgCl_2$ is one of the powerful surface sterilants used in plant tissue culture studies worldwide (Roy et al., 2007). Surface sterilization with Bavistin (1%) and $HgCl_2$ (0.1%) exerted very positive effect in this study. The $HgCl_2$ at 0.1% is the most suitable concentration for surface sterilization of explants (Roy et al., 2007; Sen et al., 2013a; Mahadev et al., 2014). The percentage of seed germination in our study closely resembles the results of Sen et al. (2013c). In place of bavistin they used 75% ethanol (45 s) and got 90% seed germination. However, the rate of contamination was high in their study, 64.38% as against 7.37 % in our study. Mercuric chloride (0.1%) combined with Flugal (3%) resulted better for surface sterilization of nodal explant (Sen et al., 2013b). However, the seed germination recorded was 65% with the contamination rate of 35% and produced 4 shoots. Use of bavistin (1% for 10 min) and $HgCl_2$ (0.1% for 2-3 min) by them resulted in very low level of explant survival (20%) with 3.67 shoots. Our study using shoot tip showed much reduced contamination rate. This reduction in contamination was due to the increased incubation period in bavistin and combined effect of $HgCl_2$. It has been proved that bavistin combined with $HgCl_2$ exert positive effect on surface sterilization (Mahadev et al., 2014). Our report is the first one on *in vitro* germination of *A. aspera* seeds with very minimum level of contamination.

Multiple shoot production

Observation of results indicated that MS medium without any PGR, the basal medium (BM)/ control, could not induce axillary shoots (multiple shoots) even after many weeks (Table 1; Fig. 2B). Rather, shoot elongation and rhizogenesis occurred. Therefore, the experiment was continued using various amounts of shoot inducing cytokinin, BAP.

Table 1. Multiple shoot induction from shoot tip explant of *A. aspera* on MS medium augmented with BAP with or without IAA (data recorded in 11 weeks old cultures).

S. No.	Plant growth regulators (mg/L)		Shoot formation (%)	Culture responses Multiple shoots		No. of leaves
	BAP	IAA		Number	Length (cm)	
1	BM	0	0	0	0	0
2	1	0	37.5	3.00 (±1.5)*	1.61 (±0.5)	05.43 (±2.1)
3	2	0	87.5	7.63 (±3.0)	3.67 (±1.2)	12.29 (±7.0)
4	3	0	75.0	4.63 (±1.9)	3.56 (±1.1)	09.00 (±1.0)
5	4	0	75.0	4.88 (±2.3)	3.13 (±1.2)	10.43 (±4.0)
6	5	0	62.5	5.13 (±2.6)	2.71 (±1.2)	09.29 (±5.6)
7	1	0.25	62.5	4.00 (±2.3)	4.87 (±1.9)	09.71 (±2.8)
8	2	0.25	50.0	3.25 (±1.6)	3.91 (±1.7)	10.00 (±2.6)
9	3	0.25	62.5	3.75 (±2.2)	1.86 (±0.8)	06.86 (±2.0)
10	4	0.25	50.0	4.43 (±3.3)	2.70 (±0.9)	08.00 (±1.9)

Legend: * Data are mean ± SD of 8 replicates. The experiment was repeated once.

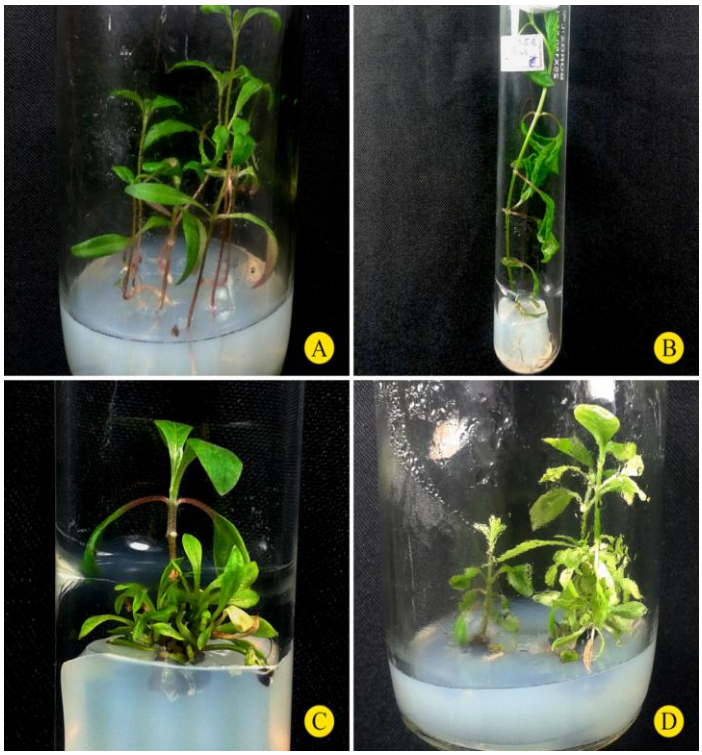

Fig. 2: *Achyranthes aspera* seedlings (2 weeks old) grown *in vitro* on ½ BM (A), and micropropagation through shoot tip explant in BM (B), 2 mg/L BAP (C), and 4 mg/L BAP + 0.25 mg/L IAA (D). Photos taken when the age of the cultures (B) to (D) were 8 weeks.

(A) Effect of BAP on multiple shoot formation

Initiation of multiple shoots/ microshoots occurred first on the 3rd day after inoculation. Maximum number of microshoots was formed after a week time. Of the five different concentrations of BAP tested, 2 mg/L proved better in shoot induction with 7.63 (±3.0) shoots per explant (Table 1; Fig. 2C). Increase in BAP amount could not enhance the shoot formation anymore. Second best response was found at 5 mg/L. Concentration at 2 mg/L was effective both in multiple shoots formation and growth of shoots. Besides these, the shoot length as well as the number of leaves produced were higher, 3.67(±1.2) cm and 12.29(±7.0), respectively. From the previous studies, it is learned that, BAP is the most preferred growth regulator for direct shoot induction in tissue culture experiments using nodal explant of *A. aspera* (Gnanaraj et al., 2012; Sen et al., 2013a), and the indirect shoot formation from *A. aspera* internode, leaf and root derived callus (Sen et al., 2014), and potato cv. Granula (Laboney et al., 2013).

Micropropagation of *A. aspera* by Gnanaraj et al. used nodal explant in MS medium (Gnanaraj et al.,

2012) noticed maximum number of shoot induction, 10.6 on 5 mg/L BAP medium and the frequency of regeneration was 79.4%. Same concentration resulted in the formation of only 5.13 shoots with 62.5% response in our study. Gnanaraj and coworkers had highest shoot forming efficiency, 93.6% at 3 mg/L BAP only (Gnanaraj et al., 2012). In the present study highest number of shoot production occurred was 7.63(±3) with the frequency of 87.5%, not with the above said concentrations but with 2 mg/L. Change in shoot inducing efficiency in the current study might be due to the difference in explant type (shoot tip) and explant source, i.e. explant derived from *in vitro* grown seedlings.

In another study carried out by Sen et al. (2013a) also had a good number of shoot formation from nodal explant in ½ MS medium fortified with 3 mg/L BAP. Lower concentration responded better in the present study. Same PGR concentration produced appreciable number of shoots in sugarcane cultivar, CoC-671 (Biradar et al., 2009). This has proved that BAP at 2 mg/L is suitable for shoot induction in *A. aspera* shoot tip explant.

Shoot tip is a suitable explant for the micropropagation of other plants, to name a few: *Dendrobium primulinum* (Pant and Thapa, 2012), *Phyllanthus amarus* (Bhattacharya and Bhattacharya, 2001; Sen et al., 2009), potato cv. Granula (Laboney et al., 2013), sugarcane cultivars HSF-240; CP-77-400 and CPF-237 (Khan et al., 2008).

During caulogenesis some amount of callus was formed in lower cut end of shoot tip explants in some cultures containing BAP. On the contrary no callus formation was noticed on BM. In BAP media, callus at cut end was 50% in 1 mg/L, 87.5% in 2 and 4 mg/L and 75% in both 3 and 5 mg/L. The callus size was pronounced in the cultures grown on 5 mg/L BAP.

(B) Synergistic effect of BAP and IAA

Further study was continued using different concentrations of BAP in combination with low amount of IAA (0.25 mg/L). Synergistic effect of both the PGRs did not result in better response when compared to BAP alone containing cultures (Table 1). Multiple shoots produced in BAP (4 mg/L) + IAA was high but only 4.43±3.3 (Fig. 2D). In general, shoot length and number of leaves produced in shoots had mixed response. The BAP+IAA could not induce many shoots when compared to respective concentrations of BAP alone supplemented media. Similar poor response was reported from the study on shoot tip culture of *Phyllanthus amarus* by Bhattacharya and Bhattacharya (2001). The possible reason could be due to the inhibitory role of IAA. Perhaps *A. aspera* is having high level of endogenous auxin which is evident from the formation of callus at the cut end of shoot tips in media having no auxin.

In vitro clonal propagation through shoot tip culture of *A. aspera* has not been studied so far. Shoot initiation from nodal explant of *A. aspera* with BAP (3 mg/L) + IAA (2 mg/L) resulted maximum (only 3) shoots per explant in one of the previous studies (Sen et al., 2013a). In the present study, 3.75 shoots per shoot tip explant has been produced in BAP (3 mg/L) + IAA (0.25 mg/L) medium. We utilized only 1/8 amount of IAA as against used by Sen et al. (2013a) in shoot tip culture. Again the increase in shoot forming capacity might be due to the type of explant and synchronized growth of *in vitro* derived explant.

Similar to BAP media, the callus at cut end of explants was found in BAP+IAA media. Callus size at cut end of explants was pronounced in BAP media having 1-3 mg/L. Multiple shoots produced had more shoot length at BAP (1)+ IAA (0.25) and more number of leaves at the medium having BAP (2)+ IAA (0.25). Though, more shoots were formed in sugarcane CoC-671 by BAP (2 mg/L), shoot length and strength were found high with the cultures having 1 mg/L BAP as in our study (Biradar et al., 2009). From this study we come to a conclusion that IAA supplementation to BAP media is not needed for efficient shoot formation from *A. aspera* shoot tips.

Table 2. Root induction of *A. aspera* L. microshoots on MS medium containing IBA media.

Sl. No.	PGR (mg/L)			Response (%)	No. of roots formed
	Medium I (*in vitro* germination)	**Medium II (multiple shoot formation)**	**Medium III (root induction)**		
1	½ BM	BAP (2)	IBA (1)	57.14	03.43 (±5.4)
2			IBA (2)	80	07.60 (±5.7)
3			IBA (3)	100	05.00 (±5.2)
4			IBA (4)	100	05.80 (±4.9)
5		BAP (4)	IBA (1)	100	05.20 (±3.3)
6			IBA (2)	100	12.00 (±4.2)
7			IBA (3)	66.66	05.67 (±9.0)
8			IBA (4)	100	13.50 (±5.0)

Legend: Data are the mean ± SD of 3-7 replicates. Experiment was repeated once.

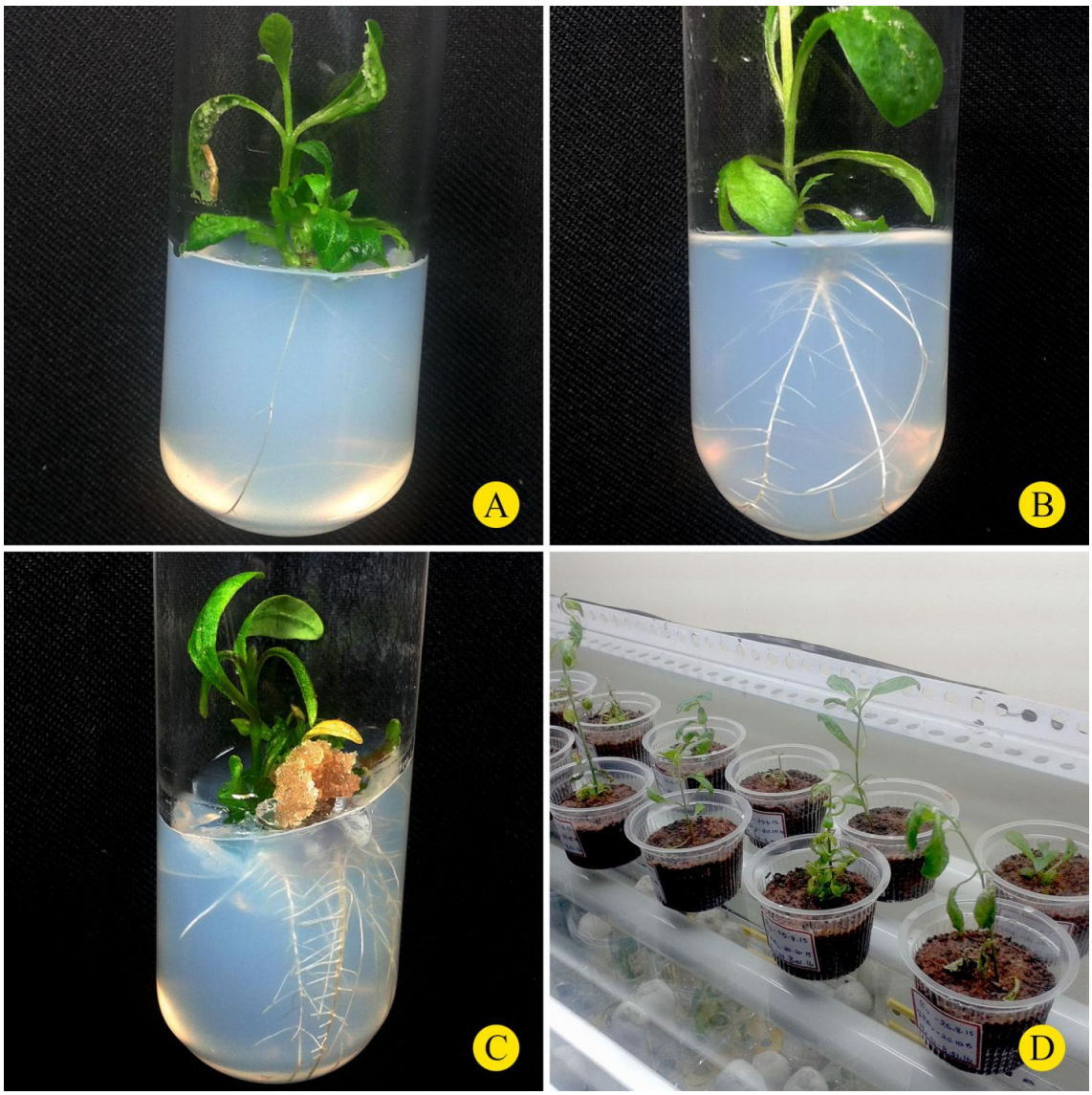

Fig. 3: Rhizogenesis of *A. aspera* somaclones in BM (A), 2 mg/L BAP derived shoot in 2 mg/L IBA (B), 4 mg/L BAP derived shoot in 2 mg/L IBA with callus (C), and hardening of plantlets in vermiculite and garden soil (1:1).

Incubation of cultures for 8 weeks evinced more growth with nodes and increase in the number of microshoots. During these periods the colour of the plantlets became dark green. Shoot initiation started first on the 3rd day after inoculation in almost all the explants grown on BAP+IAA media. Shoots were formed directly from the nodal region of shoot tip not from the callus.

Rhizogenesis of microshoots

Root formation in shootlets is an important step in micropropagation. Multiple shoots formed from shoot tip explants on BAP (2 and 4 mg/L) were separated individually and cultured on four different concentrations of the auxin, IBA (Table 2). There was 100% root initiation response on IBA at 3 and 4 mg/L on microshoots derived from 2 mg/L BAP medium. Shootlets formed in BAP at 4 mg/L showed 100% rhizogenesis in all IBA media except at the concentration of 3 mg/L.

As far as the number of roots formed was concerned, it varied between the concentrations of IBA. Maximum number was formed in 2 and 4 mg/L of IBA (Table 2; Fig. 3). The synthetic auxin, IBA has been reported to be a most suitable PGR for rhizogenesis of many species (Ramakrishnan and Kulandaivelu, 2007; Gnanaraj et al., 2012). Maximum number of roots per shootlet was recorded in ½ strength MS medium (½ BM) supplemented with 1.0 mg/L of IBA in the study conducted by Gnanaraj et al. (2012). Considering the better shoot formation and rhizogenesis, BAP and IBA both at 2 mg/L, respectively were most suitable for the cultures of *A. aspera*.

Hardening of plantlets

Proper hardening of plantlets is needed for the successful survival of the plantlets in the field. Plantlets produced from the above procedures were transferred to plastic cups containing garden soil and vermicompost in the ratio 1:1 (Fig. 3D). They were watered with distilled with distilled water every alternate days. Plantlets were maintained under 12 hrs photoperiod at 25° C, for 2 weeks. During this period, growth of shoots occurred with thick and large leaves.

After this period these plantlets were shifted to the garden and kept under shade for a week and rooted in the soil. For successful hardening of plantlets an equal amount of vermiculite and garden soil was used. This has been reported in the micropropagation of *Curculigo orchioides* Gaertn. and *Corbichonia decumbens* (Forssk.). Exell (Wala and Jasrai, 2003), and *Manihot esculenta* Crantz (Yandia et al., 2018). Vermicompost is a source of rich organic nutrients and supports the growth of various plants *in vivo*. Hence, the choice of using this for hardening (acclimatization) of microshoots proved useful.

Even though tissue culture experiments on *A. aspera* have been studied by various researchers concentrating on surface sterilization and *in vitro* seed germination (Sen et al., 2013a), nodal culture (Sen et al., 2013a), callus formation from leaf (Kayani et al., 2008; Naz and Khatoon, 2014), node, internode (Naz and Khatoon, 2014) root (Sen et al., 2014) and stem (Senthilmanickam et al., 2012), multiple shoot formation from node (Sen et al., 2013c; Gnanaraj et al., 2012). No research utilized shoot tip explant for micropropagation. Hence, our study is significant in the formulation of a procedure for an efficient *in vitro* germination with least contamination rate and successful micropropagation through shoot tip culture.

Conclusion

It is concluded that for the surface sterilization of *A. aspera* dehusked seeds, Bavistin (1%) and $HgCl_2$ (0.1%) are suitable which show very low level of contamination. For clonal propagation using shoot tip explant, 2 mg/L BAP augmented MS medium is needed. For rhizogenesis of microshoots, IBA at the concentration of 2 mg/L is ideal. Garden soil and vermicompost in equal proportions are suitable for hardening of the somaclones under laboratory conditions for 2 weeks.

Micropropagation of *A. aspera* L. through shoot tip

explant requires 2 weeks for *in vitro* seed germination in ½ MS basal medium (½ BM), 11 weeks for multiple shoot induction with appropriate size on BAP (2 mg/L) medium, 4 weeks for rhizogenesis on IBA (2 mg/L) medium and 2 weeks for hardening the somaclones under laboratory conditions in plastic cups which are filled with garden soil and vermicompost in equal amount. Totally 19 weeks (ca. 4.5 months) are needed for the micropropagation. From the above study it is learned that *A. aspera* could be propagated using the above procedure. Further research is needed to compare and validate the efficiency of shoot tip explants of *in vivo* and *in vitro* grown plants for micropropagation.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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