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In vitro propagation and plant regeneration of *Torenia crustacea* (L.) Charm. & Schltdl; an important ethnic medicinal plant

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Abstract

Torenia crustacea (L.) Cham. & Schltdl is an important medicinal herb used in India, Indonesia and Malaysia. This herb is used by Bodo tribes in the Bodoland Territorial Region, Assam to treat various diseases like diabetes. The present experiment developed an efficient protocol for *in vitro* mass propagation technique for *Torenia crustacea* using its nodal explants. For tissue culture, the rapidly growing nodal explants of *Torenia crustacea* were used. Effective explant surface sterilisation was found at 15 min. of treatment with 2% sodium hypochlorite resulting in a maximum explant survival rate and a lower rate of explant contamination after 21 days of explant culture. Media containing full strength MS (Murashige and Skoog's) + BAP (6- Benzyl amino purine) (1 mg/l) were found most effective for the establishment of the explant. The highest shoot proliferation and multiplication were observed in the media containing full strength MS + BAP (1 mg/l) + NAA (Naphthalene acetic acid) (0.2 mg/l); in this combination, an average of 16 shoots formed per nodal explant. The rooting of explants was observed highest in MS media along with 0.4 mg/l concentration of NAA and 1 mg/l BA. The *in vitro* multiplied shoots were grown in plastic pots containing vermicompost fertiliser and soil mixture and successfully grown in the open field condition.

Keywords

Explant culture, MS media, shoot multiplication, surface sterilisation, tissue culture

Introduction

Numerous traditional medicinal plants exist in nature in different regions of India. The North-Eastern states of India are one of the richest areas of medicinal and aromatic plant resources in the world (1) and most of the medicinal plants are unexplored. Due to the high demands of crude drugs, the medicinal plants are being overexploited, resulting in the threat for many rare species and many important plants are disappearing from nature (2). Because of various anthropogenic activities, many species are on the way to extinction without being properly explored or identified which could have immense medicinal property. Hence, identifying and preserving these plants are very important as they are widely used by people. Plant tissue culture is one of the fundamental tools for plant science and is continuously employed in mass propagation, plant improvement and conservation of plant species (3). Plant tissue culture or micropropagation is one of those promising techniques for the rapid growth of the plant tissue and plant organ under sterile conditions in a culture medium. The propagation is uni-

form and synchronous, and disease-free plantlets are very important, especially for endangered plants. This technique is indispensable for those plants that are without proper seeds. *Torenia crustacea* (L.) Cham. & Schltdl is one such plant (Fig. 1) used as a medicinal plant by the people

Fig. 1. *Torenia crustacea* herb at 1X magnification.

of Assam. This herb is found in very less amounts and found seasonally, tissue culture of this herb could be an ultimate solution for this problem. It is a species of flowering plant known by the common name Malaysian false pimpernel belonging to the family Linderniaceae. It is used as an anti-diabetic by traditional village peoples of Assam. This herb also bears a significant role in anti-inflammatory, analgesic and antipyretic activities and is also a source of flavonoid, which has a potent antioxidant activity (4) also *T. crustacea,* traditionally used against human herpes virus infection and its active isolates could be effective against EBV (Epstein Barr Virus) (5).

The use of Plant Preservative Mixture (PPM) in the media (0.1 ml/l - 1 ml/l) can be effective against airborne contamination in the media. PPM showed a positive effect on shoot regeneration and multiplication in the chrysanthemum leaves, but shoot regeneration was inhibited at 2 ml/l PPM. At 0.1 ml/l concentration, the average number of the shoot was reduced by at least 35% (6). Plant Cell Technology recommends 0.5-1.0 ml/l PPM to minimise or reduce airborne contamination in the media (7). In the tissue culture process, the DNA transposons, retrotransposons and transpositions can be activated due to stresses; thus, these elements can be used as markers for genomic variations (8). Somaclonal variation is a predominant phenomenon in plant tissue cultured organisms, and it plays a significant role in research like plant improvement by enabling the breeders to obtain plants tolerant to stresses viz. drought, high/low soil pH, high salinity and disease (9).

In medicinal plants, somaclonal variation may have a very high impact on the production of secondary metabolites. There may be different types of somaclones may exist i.e.- phentypical. cytological, epigenetic or genetic (10), mostly somaclonal variation doesn't cause a change in the phenotype of the species, and hence detection of the genetic arrangement of the plant species is needed and detection should be based on appearance of somaclones (11). Molecular markers have proven to be a successful tool for detecting somaclonal variation (12). Also, there are chances for change in the phenotype of the species without somaclonal variation. In the study, it was reported that due to the long term subculture of explants and due to several *in vitro* conditions, the genetic variations in the explants were observed in banana tissue culture (13). Strong chemical mutagen-nitrosomethylurea was exposed to leaf explants of Begonia to study somaclonal variation, which resulted in no variation in RAPD profiles (14). Somaclonal variations have a negative impact (for clonal propagation) and a positive impact (it may influence the breeding). It has impacted positively the tissue culture of Phalaenopsis (15), Pelargonium (16), Petunia (17) and Saintpaulia (18).

However, there are no reports yet published on *in vitro* propagation and the effect of different growth regulators on *T. crustacea*. The objective of the present study is to develop an efficient *in vitro* propagation technique for mass propagation of the ethnic medicinal plant *T. crustacea* through callus formation from nodal explant for its high regenerative capacity and analyse the effects of different plant growth regulators on the species.

Materials and Methods

Media preparation

All the equipment, glassware and phyta jars required for the tissue culture process were washed and sterilised in an autoclave at 121 °C and 15 psi for 60 min. The components of MS media were added into sterile double distilled water, and the pH was adjusted to 5.6 with 1N HCl or 1N NaOH. Half strength and full-strength MS media containing 3%

(w/v) were prepared with different PGR ratios and 0-0.5 ml/l plant preservative mixtures, BAP (6- Benzyl amino purine 1 to 2 ml/l), NAA (Naphthalene acetic acid 0-0.4 ml/l) and 0-1 mg/l IBA. 0.8% agar media is used as a solidifying agent for the media. The media is sterilised at 121 °C and 15 psi for 15 min. The plant growth regulators were added to the media after sterilisation when the media temperature was 50–60 °C. The PGRs were filtered with the help of a 22 µm syringe filter and added to the media. The media were poured into the phyta jars, and it was kept under UV light overnight.

Explant collection

Rapidly growing, disease-free, healthy nodal explants of *Torenia crustacea* were collected from different regions of Baksa district (26°38'37.29"N and 91°34'35.67"E), Assam, India. Shoots containing nodes, roots and flowers were collected from the field and herbarium was prepared and the species was authenticated at the Department of Botany, Bodoland University bearing accession number BUBH0000576.

Explant Sterilisation

For sterilisation purposes, the explants were first washed under running water for 10-15 min. and then it was taken under laminar airflow and dipped in 500 ml sterile water containing 1-2 drops tween -20 for 30 min. Then the leaves from the explants were removed and the explants were treated in 0.5% bavistin for 35-40 min. followed by rinsing twice in the sterilised double-distilled water. The explants were now trimmed to 1-2 cm size containing nodes. Surface sterilisation is done using Mercuric Chloride (0.1%) for 0-5 mins followed by washing thrice in the sterilised double distilled water for 2, 3 and 5 min. respectively. The explants were inoculated into media containing different PGR ratios inside the LAF. In another experiment, for explant sterilization instead 0.1% mercuric chloride, 2% sodium hypochlorite treatment for 5 min. to 20 min. were done after the 0.5% bavistin treatment. The rest of the steps were kept similar to the above surface sterilisation methods.

Explant inoculation

For callus induction, the above explants were placed into the phyta jars containing full strength and half-strength MS media combination with different concentrations of plant growth regulators and the tissues are now incubated at 28 °C temperature and 12-16 hrs of photoperiod and 75±5% relative humidity for 4-6 weeks. After successfully establishing the explant in the media, the explants were subcultured in the fresh media to induce multiplication. Different concentrations of growth regulators are used to observe the different responses of the explant. The emerged shoots were taken out and transferred to different media, and the shoots were inoculated into different hormone concentration combinations.

Rooting, Hardening and acclimatisation

Both *in vitro* and *ex vitro* rooting were experimented on in the study. *In vitro* rooting was carried out in full strength MS or half-strength MS medium with different concentrations of PGRs. After successful callus formation from the explants, the explants were taken out, excised into parts and inoculated into media containing different PGR concentrations. After successfully forming shoots and roots *in vitro,* the plantlets were taken out and stacked agar media were removed. The plantlets were grown in small pots containing coco peat supplemented with vermicompost fertiliser and kept inside an agro net house (at 25 °C to 30 °C and 8-11 hrs of photoperiod). After about 10-14 days, the plantlets are taken out to the field and grown in the natural environment.

Results and Discussion

Explant selection and surface sterilisation

Explant selection and sterilisation is the most crucial step in the tissue culture process. Disease-free, rapidly growing nodal explants are the best choice for *in vitro* propagation. Most of the contaminations in media or culture arose after 7-20 days of inoculation. Sometimes it is seen after the callus formation also. For explant surface sterilisation, 0.5% bavistin treatment for 30-60 min and 0.1% mercuric chloride were preferred. The most effective surface sterilisation treatment process resulted in 0.1% mercuric chloride for 2 min. where most of the explants (about 60%) survived and less rate of contamination was seen (Table 1).

Table 1. Explant sterilisation with 0.1% mercuric chloride and 2% sodium hypochlorite

Sl. No.	Percentage of treatment	Time (in min)	Rate of survival of the explant after 21 days of inoculation	Rate of contamination
1	Control	0	0%	100%
2	Mercuric chloride (0.1%)	1	20%	80%
3	Mercuric chloride (0.1%)	2	60%	40%
4	Mercuric chloride (0.1%)	3	55%	30%
5	Mercuric chloride (0.1%)	4	21%	25%
6	Mercuric chloride (0.1%)	5	12%	0%
7	Sodium hypochlorite 2%	5	25%	75%
8	Sodium hypochlorite 2%	10	42%	58%
9	Sodium hypochlorite 2%	15	84%	16%
10	Sodium hypochlorite 2%	20	67%	0%

Explant inoculation and shoot multiplication and rooting

Though most of the explants of *T. crustacea* were successfully established in the media with any concentrations of plant growth regulators (Fig. 2A), the response of the explants varied in different PGR ratios. Most effective explants response forming callus showed at full strength MS with 1 mg/l BAP at 28 °C and 16 hrs of photoperiod (Fig. 2B). After successful formation of callus, these callus formed shoots most effectively in the medium containing MS with a PGR concentration of 1 mg/l BAP and 0.2 mg/l NAA (Fig. 2C). The maximum numbers of root formation were observed in the media containing MS with 1 mg/l BAP and 0.4mg/l NAA (Table 2).

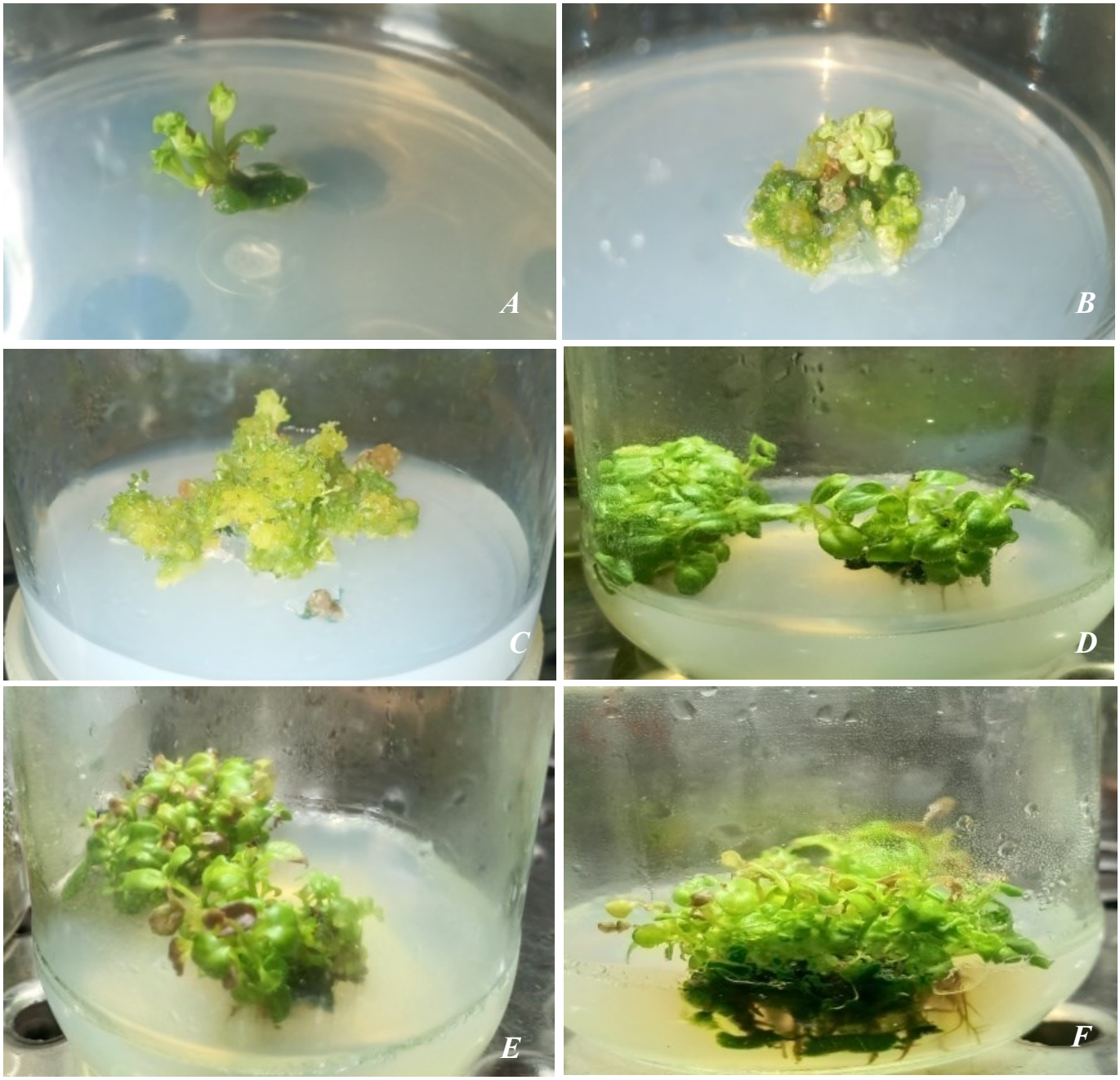

Fig. 2. (1X magnification). *In vitro* propagation of *L. crustacea*, **A**; initiation of explant (7 days), **B**; callus formation from explants of *L. crustacea* (14 days), **C**; callus formation and multiplication (21days), **D** and **E**; multiplication stage (42 days), **F**; *in vitro* rooting of *L. crustacea* (42 days).

Table 2. Showing effect of various growth regulators on explant multiplication and shoot proliferation and rooting, Mean ±SE of 5 replica

Sl. No.	Media with PGR concentrations	Explant responded after 42 days of explant culture	Mean Shoot number after 42 days of explant culture (>2cm length)	Mean Shoot length after 42 days of explant culture	Mean number of roots after 42 days of explant culture	Remarks
1	MS Control (BM1)	75%	3± 1.09	2.162± 0.54	6.4± 12.41	Moderate response of explants forming callus.
2	½ MS+ 1mg/l BAP (BM2)	58%	5.2± 1.16	2.26± 0.52	7.8± 15.41	Minimum number of inoculated explants responded that formed callus
3	MS+ 1mg/l BAP (BM3)	91%	7.2± 1.16	3.1± 0.18	8.8± 17.56	Maximum number of inoculated explants formed callus and moderate rate of shoot multiplication.
4	MS + 1mg/l BAP+ 0.2mg/l NAA (BM4)	91%	16.2± 1.16	4.16± 0.38	11.2± 22.72	Highest numbers of shoots proliferation and maximum number of inoculated explants formed callus.

Explant selection and collection need to be done carefully for the rapid growth of the explants and to minimise the rate of contamination. The selection of infected and damaged explants may lead to a higher risk of contamination in the culture medium. Nodal explants were preferred for *in vitro* culture; the cells in the axillary meristem in the nodal explant undergo rapid mitosis so that there is less possibility for genetic changes in the shoots (19). Surface sterilisation is the most important step for tissue culture and improper surface sterilisation of explants leads to contamination in the culture. For explant sterilisation, 0.5-1% Bavistin treatment for 30 min. to 1 hr and 0.1% mercuric chloride were used. 2 min. treatment with 0.1% mercuric chloride is found to be the most effective for *in vitro* propagation of *T. crustacea*. No mercuric chloride treatment and also at 1 min. treatment with mercuric chloride showed maximum tissue survival but got contaminated after 5-10 days. Again, 3-5 min. of treatment resulted in mostly damaged and burnt explant, causing in death of the explant (Table 1).

In another surface sterilisation experiment, 2% sodium hypochlorite treatment for 5 min. and 20 min. was used. In the experiment, most effective results was found at 15 min. of 2% sodium hypochlorite treatment, where 84% of explants survived after 21 days of culture. In the 2% sodium hypochlorite treatment for 5 min., only 25% of explants survived, 10 min. of treatment resulted 42% explant survival and 20 min. of treatment showed 67% explant survival after 21 days of explant culture.

PPM is mostly used to inhibit or reduce the rate of contamination in the culture media. The reports showed that shoot multiplication and regeneration decreased when PPM was added to the medium. PPM helps to fight against airborne contamination by reducing the rate of contamination. PPM penetrates the cell wall of microbes and inhibits important enzymes in the TCA cycle and Electron Transport system (7), but it cannot penetrate the plant cell wall due to its complexity and the plant cell wall can prevent the entry of PPM molecules inside the cell hence plant cell does not get harmed by PPM (7). In the present study of *in vitro* propagation of *T. crustacea* there was no remarkable effect of PPM on the shoot proliferation, and a reduction in the rate of contamination was observed.

The influence in the shoot induction directly depends on the plant growth regulators; the concentrations of PGRs for shoot induction vary from species to species (20). Some reports showed BAP alone was found effective over other cytokines for shoot regeneration of *B. monnieri* (19).

In the present study, BAP (6 benzyl amino purine), IBA (indole-3-butyric acid) and NAA (naphthalene acetic acid) were used as growth regulators. Although explants in the control medium without growth regulators also showed response but, the rate of response was low (approximately 56%). Explants were found to be successfully initiated and formed callus in the full strength MS media with 1 mg/l BAP in which the highest (approximately 91%) explants formed callus and full-strength MS with 1 mg/l BAP and 0.2 mg/l NAA showed 91% of the tissue response and formation of maximum shoot number from callus (Fig. 2D & E). Although all the basal medium resulted in rooting of the plantlets (Table 2), basal media with PGR ratios containing 1 mg/l BAP and 0.4 mg/l NAA resulted in the highest number of rooting *in vitro* (Fig. 2F).

Previously work reported on *in vitro* propagation of the family Linderniaceae named *Lindernia antipoda* L. (Alston), an aquatic herb. In the study (21), a protocol for shoot multiplication and *in vitro* flowering from the axillary bud of *Lindernia antipoda* had been developed. The study obtained maximum callus formation in half-strength MS in combination with 1 mg/l BAP, shoot induction and best rooting were observed in the combination of 1 mg/l BAP with 0.4 mg/l NAA.

ANOVA test

One way ANOVA was performed for the cultures using different media and response of tissues (shoot numbers, shoot length and root numbers) after 42 days of culture. The test was performed for 5 cultures of *Lindernia crustacea*. From the ANOVA test of shoot numbers, the F value was $F(6,28) = 27.89$, which indicates significant differences in the shoot number formations among the different basal media. From the ANOVA test of shoot length the F value was $F(6,28)=12.05$, which showed significant differences in shoot length among the different basal media. From the ANOVA test of root formation number the F value was $F(6,28)=14.19$, which showed a significant difference in the root numbers among the different basal media. All the experiments were performed at 5% level.

Acclimatisation and Hardening

The above *in vitro* rooted plantlets were taken out from the MS media bottles and planted in small pots containing coco peat supplemented with vermicomposting fertilisers, and the plantlets were planted in a controlled environment for 5 to 7 days. Later, the pots containing plantlets were transferred to the agro net house and finally successfully grown in the field.

Conclusion

Medicinal plant tissue culture is often used technique for the production of active compounds for herbal and pharmaceutical industries, this technique also help in the conservation of threatened species. In the above study, an efficient *in vitro* propagation technique for multiplication and large scale production of *Torenia crustacea* has been successfully developed. For the experiment of surface sterilisation of *T. crustacea* it was found that 2% sodium hypochlorite treatment for 15 min. showed a maximum of 84% explant survival rate. The explants of *T. crustacea* responded most effectively in BM3 media (MS+ 1 mg/l BAP), Highest numbers of shoots proliferation were observed in BM4 media (MS+ 1 mg/l BAP + 0.2 mg/l NAA). As this herb is found in very smaller quantities and found seasonally, using this *in vitro* technique large scale propagation and also propagation of the herb can be done in any season. This

technique will help in the large scale *in vitro* propagation, supply of uniform, disease-free cloned plantlets also will help in the secondary metabolites production for pharmaceutical uses. Further studies on the somaclonal variation of the species due to several *in vitro* conditions and effect of somaclonal variation on the production of secondary metabolites could be done. In the previous work it was reported that secondary metabolite production capabilities can be increased by identifying and cloning of the high yielding cells (22), hence experiments on inducing of some target genes for more production of required secondary metabolite from the species.

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Authors contributions

TB carried out the field surveys, experiments and manuscript writing. SD participated in experimental design, coordination and supervision.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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