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Micropropagation and antimicrobial activity of *Operculina turpethum* (syn. *Ipomoea turpethum*), an endangered medicinal plant

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Abstract

Operculina turpethum (syn. *Ipomoea turpethum*) - a plant in the morning glory family is an endangered medicinal plant of Bangladesh and its neighboring countries. An efficient micropropagation protocol was established for this plant through nodal segment culture as well as antimicrobial activity of the leaves was also evaluated. For micropropagation, nodal segments with axillary buds of field grown plants were surface sterilized in 0.1% $HgCl_2$ for 8 min. Rapid shoot bud proliferation (85.33%) along with a maximum of 14 shoots in each bud was observed when cultured in Murashige and Skoog medium supplemented with 1.0 mg l^{-1} BAP. Proper elongation (5-6 cm) of the primarily induced shoots was achieved by subculturing in 0.5 mg l^{-1} GA_3 plus 0.1 mg l^{-1} Kin. Indole-3-acetic acid (IAA) at a concentration of 1.0 mg l^{-1} was found most effective for root development. Well rooted plantlets were successfully acclimatized in the soil up to maturity. In terms of antibacterial properties petroleum ether and ethanol extracts of leaves showed potential antimicrobial properties against several human pathogenic bacteria with a minimum inhibitory concentration (MIC) ranged from 0.13-0.75 mg ml^{-1}.

Key words: Antimicrobial activity, endangered, *Ipomoea turpethum*, medicinal plant, micropropagation, *Operculina turpethum*

Abbreviations :BAP 6-Benzylaminopurine · GA_3: Gibberellic acid ·IAA: Indole – 3 acetic acid ·IBA: Indole–3 butyric acid · Kin: 6-Furfural amino purine · NAA: Napthalene acetic acid · PGR · plant growth regulator ·TDZ: Thidiazuron

Introduction

Opercliana turpethum (syn. *Ipomoea turpethum* L, Convulvulaceae) is large evergreen perennial climber blooms with fragrant showy flowers. The plant is native to Bangladesh and India although it can also be found in the Pacific Islands, China and Australia. In Bangladesh, it is found in the north-western districts and Chittagong home stead shrubberies. This plant has several medicinal properties. Root bark of the plant contains a resinous substance (turpethin), is a mild purgative and used in cases of debility, toxicity, etc (Ahmed, 1997). Paste of the root is used as an external application in hemorrhoids, chancres and ulcerations. Oil extracted from the root bark is used in skin diseases of a scaly nature. The fresh juice of leaves is dropped into the eyes for inducing lachrymation in ophthalmia. Unfortunately, this important medicinal plant has been enlisted as endangered species in Bangladesh and its neighboring countries (Ahmed, 1997). Rapid fragmentation of natural habitats is greatly narrowing the distribution of this plant and increasing the risk of loosing genetic diversity. Increase prevalence of multi-drug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting agents (Sieradski et al., 1999). Medicinal plants are the natural and safer source of phytochemicals to fight against new strains of microorganism. But due to rapid destruction of habitat they become extinct day by day. Among the different approaches, *in vitro* culture method provides new means of conserving and rapidly propagating valuable, rare and endangered medicinal plants (Nalawade et al., 2003; Thomas and Shankar, 2009; Rahman et al.,

Table 1. Effects of plant growth regulator on morphogenic responses of single-node explants of *Operculina turpethum* established *in vitro* (data were recorded after 6 weeks of culture in MS medium using 3% sucrose)

PGR mgl^{-1}	**Response frequency (%)**	**No. of Shoot/Culture** ($\bar{x}$±SE)	**Shoot Length** ($\bar{x}$±SE)	**Degree of Callus formation**
BAP				
0.5	62.42	7.47±0.50	2.85±0.22	+
1.0	85.33	11.65±0.80	2.10±0.08	++
1.5	56.18	5.33±1.12	2.36±0.15	+++
2.0	40.33	3.20±0.25	1.68±0.56	+++
Kin				
0.5	52.50	1.36±0.24	3.56±0.10	-
1.0	67.50	2.36±0.18	4.46±0.42	-
2.0	40.45	2.06±0.22	4.72±0.12	-
BAP+Kin				
1.0+0.2	66.33	5.56±0.32	3.06±0.15	+
1.0+0.5	70.33	7.60±0.45	3.25±0.24	+
1.0+1.0	59.50	5.14±1.33	4.10±0.36	-
2.0+0.5	62.47	3.36±0.87	3.50±0.25	+
BAP+NAA				
0.5+0.1	57.0	3.10±0.12	2.56±0.30	+
1.0+0.2	60.65	5.60±0.43	3.20±0.33	++
1.0+0.5	45.60	4.46±1.16	3.56±0.42	+++
BAP+GA_3				
1.0+0.1	58.33	8.33±0.27	3.56±1.02	+
1.0+0.2	65.36	6.20±0.46	4.63±0.44	+
1.0+0.5	50.25	3.38±0.42	4.90±0.18	-

- = no callus, + = little callus, ++ =moderate callus, +++ = huge callus

2009). A well established protocol including plant regeneration from tissue, organ etc. would help plant population from being extinct. Other advantages of the technique are the space exploitation, the improvement of the sanitary conditions of the plants and the facilitation of international germplasm exchange (Alderete et al., 2006).

Considerable progress have been done on *in vitro* propagation of related species *viz.* sweetpotato and *Ipomoea pes-caprae* (Kane et al., 1993; De Andrate and De Andrate, 1995; Verma et al., 2004; Alam et al., 2010a). A revision of the available literature showed no previous report on tissue culture of this important species. Consequently, the developed tissue culture technique will increase the scarce knowledge about the *in vitro* responses of this native germplasm with potential relevance as an endangered medicinal plant. Use of medicinal plants has been known for centuries in many parts of the world for the treatment of various human ailments. The use of antibiotics has revolutionized the treatment of various bacterial infections. However, their indiscriminate use has led to an alarming increase in antibiotic resistance among microorganisms, thus necessitating the need for development of novel antimicrobial compounds. Recent years have witnessed a renewed interest in exploring natural resources for developing such compounds. A number of plants have been documented for their biological and antimicrobial properties (Rios and Recio, 2005). Evidently, there is no sufficient scientific studies that confirm the antimicrobial properties of this plants. So, this study also looks into the *in vitro* antimicrobial activity of *Operculina turpethum* leaves against six pathogenic microorganisms. The present study reports a reliable protocol for the *in vitro* propagation of *Operculina turpethum*. In addition, the positive results on antimicrobial activity have been justified our work for saving this endangered medicinal plants using tissue culture.

Materials and Methods

Plant material

Micropropagation of *Ipomoea turpethum* has been carried out by culturing nodal segments having axillary bud of mature plants. Plant materials were collected from wild population of Rajshahi University Campus, Bangladesh. Initially, the collected nodes were washed with Savlon (commercial disinfectant, ACI Pharma, Bangladesh) for 5 min followed by treatment with 70% v/v in ethanol for 3 min in shaking. Finally the explants were sterilized 0.1% w/v mercuric chloride ($HgCl_2$) solution plus 3-4 drops of Tween-20 for 8 min with gentle shaking. Sterilized explants were washed 4-6 times with sterile distilled water to remove the effect of mercuric chloride. Then the explants were cultured in MS medium (Murashige and Skoog, 1962) containing different concentrations and combinations of BAP, Kin, NAA, and GA_3 for *in vitro* shoot proliferation (Fig A, B). The induced shoots were subcultured in medium containing different combination of GA_3 and Kin for

Table 2. Effect of different concentrations of IAA and IBA on rooting of *in vitro* elongated shoots of *Operculina turpethum* (data were recorded after 6 weeks of culture).

PGR (mgl^{-1})	Response frequency (%)	No. of Root/culture ($\bar{X}$±SE)
IAA		
0.5	62.50	5.29±0.24
1.0	85.33	13.41±0.18
2.0	67.50	7.16±0.22
IBA		
0.1	40.20	3.5±0.50
0.5	52.50	4.65±0.80
1.0	75.00	8.33±1.12
2.0	56.18	5.95±0.25

proper elongation. Roots were induced and proliferated in developed plantlets using different concentrations of IAA and IBA.

Physical culture condition

In all the cases, pH of the media was adjusted to 5.7±0.02 prior to gel with 0.8% w/v agar (BDH, England). Finally, media were sterilized at 121°C for 20 min under 1.06 $kgcm^{-2}$ pressures. The cultures were incubated in a growth chamber at 25±1°C under 16 h photoperiod regimes and provided by cool white fluorescent lights with 40-45 $\mu molm^{-2}s^{-1}$ illumination.

Data recording

To study the growth, development and proliferation of shoots in each treatment, percentage of explants induced multiple shoots, mean number of shoots per culture and mean length of shoots were evaluated. Frequency of roots formation and number of root per culture were estimated for rooting efficiency. Each treatment had at least 15 replications and all experiments were repeated twice. Mean and standard error were calculated for the treatments.

Test of antimicrobial activities

Dried and powdered leaves were soaked separately in petroleum ether and ethanol (1:10 w/v) for 4-5 days at room temperature with occasional shaking. After filtration of the extracted solutions they were concentrated in a rotary evaporator at 40°C to dryness. For aqueous extraction, leaves were pasted using a mortar and pastle and soaked into sterile distilled water (1:10 w/v) in a conical flask and kept for 24 h with gentle agitation and then filtered off using sterile filter paper. This extract was subjected to water bath for evaporation and resulted crude extract. Collected solid residues (crude extract) were stored at 4 °C and were dissolved freshly in respective solvents prior to screening for antimicrobial activity. Two Gram positive such as *Bacillus subtilis* (NCIM 2063), *Streptococcus haemolytica* (clinical isolate), and three Gram negative bacteria including *Pseudomonas aeruginosa* (NCIM 5029), *Shigella sonnei* and *Shigella dysenteriae* (clinical isolates) were used in order to evaluate the antimicrobial activities. The referred strains and clinical isolates were obtained from the department of microbiology, Rajshahi Medical College, Bangladesh. The bacteria were cultured on nutrient agar medium at 37°C and maintained by subculturing periodically on agar slant at 4°C

Antibacterial activity was tested by disc-diffusion method (Bendahou et al. 2008). Extracts were prepared by reconstituting the extracts in respective solvents. A 100 µl of 24 h old broth culture of each tested bacteria (McFarland standard 0.5) was spread on the nutrient agar plate. Autoclave sterilized filter paper discs (6 mm diameter) were soaked with 3.0 mg ml^{-1} dissolved ethanol or 1.0 mg ml^{-1} petroleum ether extract and allowed them to dry for a few minutes and then placed on top of the seeded plate. The standard antibiotic discs (Hi-Media, Mumbai, India) of ampicillin and neomycine (10 µg) was served as positive control and ethanol, petroleum ether used as negative control. The assay plates were incubated at 37 °C for 24 h. The diameter of the zones of inhibition around each of the discs (disc included) was considered as measure of the antimicrobial activity. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded. The extracts, which showed antimicrobial activity in the disc, were subjected to the MIC assay. Serial two-fold dilutions of the extracts ranging from 0.004-4 mg ml^{-1} were prepared in 2 ml of nutrient broth. Then 40 µl of the test organism (0.5 McFarland turbidity standards) was added and incubated for 24 hours. The MIC values were interpreted as the lowest concentration of the extracts, which showed clear fluid with no development of turbidity. Blanks and positive controls were also included.

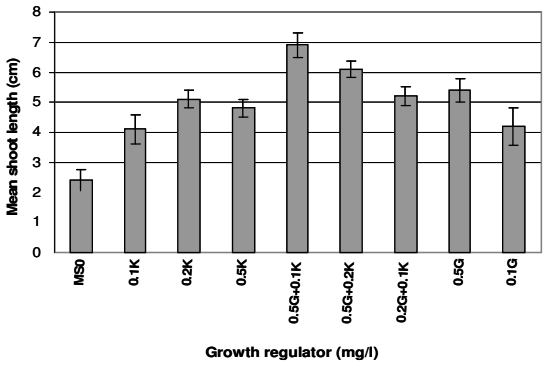

Fig 1. Effect of different plant growth regulators on shoot elongation of primarily induced microshoots in 1.0 mgl^{-1} BAP. Data were recorded after 21 days of subculture (vertical bars indicate standard error) G=GA_3, K=Kin).

Table 3. Antibacterial activity of *O. turpethum* leaf extracts against five pathogenic bacteria species

Extract / Bacteria	Zone of inhibition (mm)			
	Ethanol[1]	Petroleum ether[1]	Ampicillin[2]	Neomycine[2]
Streptococcus haemolytica	9.00	8.33	20.00	19.33
Bacillus subtilis	15.00	12.33	23.33	20.00
Pseudomonas aeruginosa	12.00	10.00	20.66	17.00
Shigella sonnei	13.66	11.33	20.00	19.33
Shigella dysenteriae	14.33	12.00	21.33	19.00

[1] 5.0 mg ml^{-1} (250µg/disc), [2]10 µg/disc

*No zone of inhibition was observed upon treatment with ethanol or petroleum ether (negative control)

Results and discussion

Explants inoculated in BAP containing medium showed better response than any other hormones or combinations of hormones used, and gave highest frequency of multiple shoot averaging 85.33% (Table 1). Among the various concentrations of BAP, 1.0 mg l^{-1} showed the best results producing highest 14 shoots with an average of 11.65±0.80 shoots per explants within 5-6 weeks of culture (Fig C). Increased concentration of BAP (>1.0 mg l^{-1}) produced high amount of undesirable basal callus resulting reduced shoot growth and number. Although use of Kin resulted no basal callus but number of shoot per culture was not encouraging, most of the cases it was solitary. However, it helps for increasing the length of shoot. When Kin was used with BAP, the number of shoot per culture was increased, which supported the earlier comment of using BAP for increasing the shoot number. When BAP was used with NAA or GA_3, the combination of BAP and GA_3 was moderately good for both number and length of shoots. No fruitful result was obtained when hormone-free (MS_0) medium was used, justified the necessity of growth regulator for both shoot induction and elongation.

Primarily proliferated shoot bud clusters were not well developed during initial culture or when they subculture in the same medium. So, treatments with single use of Kin and GA_3, and their combinations were tested for proper elongation of the primarily induced microshoots. According to Figure-1, it was observed that shoots were able to attain a height of around 7 cm in 0.5 mg l^{-1} GA_3 plus 0.1 mg l^{-1} Kin combination (Fig D).This result supports that further treatment with proper hormonal combination was required for elongation of the primarily induced microshoots. The elongated shoots were found sturdy.

Root formation was absent during initial microshoot proliferation and elongation. So, IAA and IBA were tested for proper root induction from the developed shoots (Table 2). Between the two tested auxins, IAA (1.0 mg l^{-1}) induced more than 80% roots in shoots averaging 13.41 roots per culture (Fig E). Generally, roots were initiated after 10-14 days of transfer to rooting medium. The acclimatized plantlets were successfully established in soil (Fig. F, G) and the success was more than 85% after 4 months of transferring in *ex vitro*. Apparently, no abnormal morphological aberrations were observed in regenerated plants when compared with the wild populations.

Table 4. Minimum inhibitory concentration (MIC) of petroleum ether and ethanol form solvent extracts against five pathogenic bacteria.

Extract / Bacteria	MIC (mg ml^{-1})	
	Ethanol	Petroleum ether
Streptococcus haemolytica	0.75	0.50
Bacillus subtilis	0.13	0.25
Pseudomonas aeruginosa	0.50	0.75
Shigella sonnei	0.25	0.50
Shigella dysenteriae	0.25	0.13

Rapid shoot proliferation has been achieved with a wide range of species with initial explants being taken from normal aerial shoots of field grown herbaceous medicinal plants (Rai, 2002; Hall and Camper, 2002). Plant generation through axillary bud culture is considered one of the most convenient ways for multiplying a selected plant species true to the type. This *in vitro* experiment involves initially the establishment of nodal explants in culture, which resulted in the formation and elongation of multiple shoots followed by root development. BAP had a significant effect on induction of multiple shoot bud in *Operculina turpethum* although callus formation was concomitant with shoot induction. Optimum shoot multiplication using BAP is reported in a number of plants (Hiregoudar et al., 2006; Sharma et al., 2006; Alderete et al., 2006; Alam et al., 2010b). Role of BAP for callus formation during shoot multiplication is well known (Ilahi et al., 2007; Chandra et al., 2006). Subulturing in BAP was resulted much amount of undesirable basal callus with no further development of the proliferated shoots. For this, BAP induced multiple shoot clusters were further subcultured in different medium for proper elongation and development, which helped for elongation of shoots but not for new shoot induction. This inverse relationship between shoot multiplication rate and shoot length in response to exogenous growth regulator was also observed by Castillon and Cornish (2000). According to them selection of hormone and its further modification especially during proper developmental stage may be necessary to obtain desired multiplication rate and shoot length. So, our result of using GA_3 + Kin for

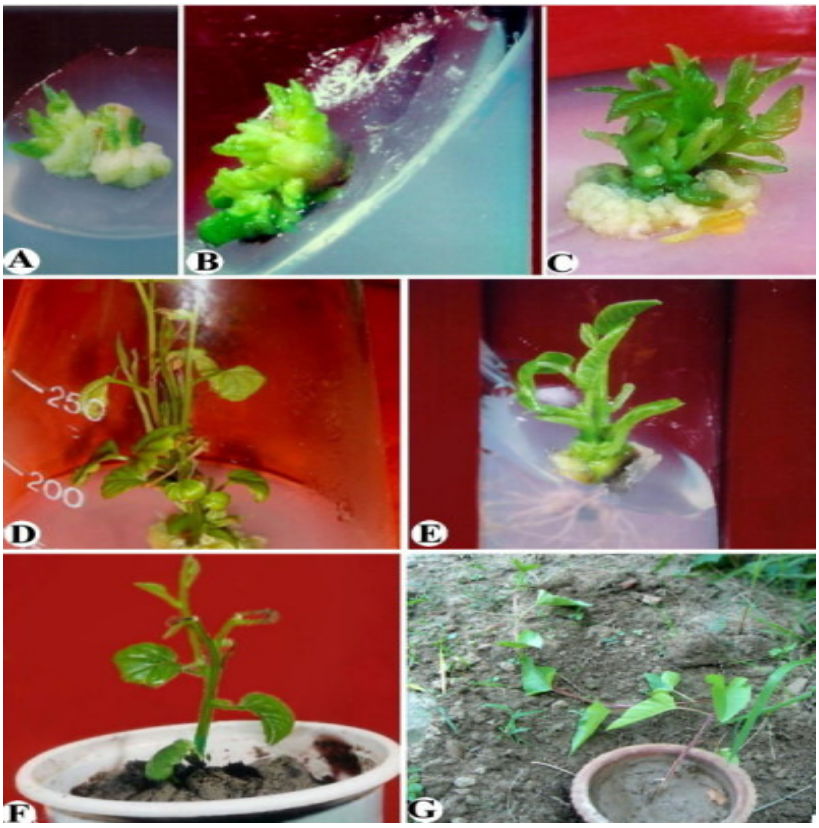

Fig 2. *In vitro* propagation following *ex vitro* establishment of *Operculina turpethum*
A, B: Induction of shoots from axilliary bud of nodal segment in MS+BAP (1.0mgl^{-1}).
C: Five-week-old culture showing multiple shoots proliferation with basal callus.
D: Elongation of shoots in MS+0.5 mgl^{-1}GA_3+0.1mgl^{-1}Kin.
E: Rooting in plantlets using 1.0 mgl^{-1}IAA.
F, G: Acclimatization of plantlets in soil.

shoot elongation justified the idea. Gibberellins have the unique ability among plant hormones to promote extensibility of stem of many species. In many cases BAP induced shoots were further elongated using GA_3 singly or combined with BAP (Ye et al., 2002). But in our case BAP-GA_3 combinations or single use of GA_3 was not so encouraging. Responses of plants to cytokinins and gibberellins and their different types may also change according to species, ecotype and even presumably location of plants in systematic. It is also reported that GA_3 has a role to block the action of 6-benzylamino-purine (BAP) and 1-naphthaleneacetic acid (NAA), (Heide and Ola, 1969). However, many reports supports GA_3 or combination of Kin and GA_3 is helpful for elongation of shoot prior to rooting (Baskaran and Jayabalan, 2005; Mohamed et al., 2006) Besides the discussed growth regulators (Table 1), different concentrations of thidiazuron (TDZ) were also tested, but trace effect was observed for shoot induction rather producing huge amount of undifferentiated green and compact callus (data not shown). This is supported by Lima et al (2008) where it is mentioned that thidizuron, a non-purine compound showed high cytokinin activity in promoting growth of cytokinin-dependent callus culture. Besides adventitious or axillary shoot production, TDZ is also capable to induce somatic embryogenesis in many dicot plant species (Lu, 1993; Murthy et al., 1998).

In our studied species although induction of root was absent during shoot multiplication and subsequent elongation stage but in other *Ipomoea* species especially *Ipomoea batatas* shows spontaneous rooting during shoot induction (Lu, 1993; Gonzalez et al., 1999). However, Verma et al. (2004) reported that IAA is the most effective for rooting in *Ipomoea batata*s, which supported our result for *Operculina turpethum.*
The antimicrobial activities of two solvent extracts compared with those of ampicillin, neoycine as positive control and solvent ethanol, petroleum ether used as negative control are summarized in Table 3. Assay of the extracts revealed that ethanol extracts showed a varied degree of inhibition against patho- genic bacteria while aqueous extract did not show significant zone of inhibition. In general, ethanol extracts showed higher activity than petroleum ether extracts and produced inhibition zones ranging from 9 to 14 mm in diameter at a concentration of 5.0 mg ml^{-1}. There are many repots available that ethanol extract of plant showed antimicrobial activity against human pathogenic bacteria (Trakulsomboon et al., 2006; Abdelsalam et al., 2007). The Minimum inhibitory concentration (MIC) values of active extracts were

determined by the broth dilution method and presented in Table 4. The MIC value of extracts ranged from 0.13 to 0.75 mg ml^{-1}. Ethanol extracts had lower MIC values comparable with petroleum ether extract against the tested strains.

In conclusion, we described here a simple and reproducible micropropagation method for *Operculina turpethum* that would be helpful for mass propagation of this endangered species. Evidence of strong antimicrobial components in the leaves would be useful in developing antimicrobial substances in medicinal industries.

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