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# "MICROPROPAGATION OF *TAVERNIERA CUNEIFOLIA* ( ROTH) ARN. ; A SUBSTITUTE FOR COMMERCIAL LIQUORICE "

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## ABSTRACT

A protocol for *in vitro* micropropagation was developed for *Taverniera cuneifolia* (Roth) Arn; a substitute for commercial liqorice- *Glycyrrhiza glabra*, using nodal explants derived from *in vitro* raised 30 days old seedlings. The excised axillary tips were cultured on Murashige and Skoog (MS) medium containing BA, Kinetin alone or in combination with $AgNo_3$ for multiple shoot induction. The best condition for shoot growth was with 2 $mgl^{-1}$ BA and 5.07 $mgl^{-1}$ $AgNo_3$ in MS medium. Averages of 5.36 shoots were obtained per explant after 30 days of culture. The rooting of *in vitro* developed shoots (4~6 cm length) was best in MS medium containing 3 % sucrose and 2 $mgl^{-1}$ NAA. Micropropagated plants were successfully acclimatized (60 %) within 6 weeks after rooting. The standardized protocol reported in this study may help in the conservation of this plant which is currently exploited from nature.

**KEYWORDS: Axillary bud multiplication, *Glycyrrhiza glabra*, Medicinal plant, *Taverniera cuneifolia***

**Abbreviations : BA : N $^6$-benzyladenine, Kn : Kinetin , $AgNo_3$ : Silver nitrate , NAA : Naphthalene acetic acid , IAA : Indole-3-acetic acid, EMS: Ethyl methane sulfonate.**

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# INTRODUCTION

The genus *Taverniera* belonging to the family of Fabaceae, includes twelve species and is endemic to the Northeast African and Southwestern Asian countries ( Stadler *et al*. 1994). *Taverniera cuneifolia* (Roth) Arn. popularly known as Indian licorice is an herb and occurs along the banks of small streams. The roots of this plant taste sweet and are used by the tribes as a substitute for the commercial licorice- *Glycyrrhiza glabra* (Naik 1998). The roots of *G. glabra* are widely used in traditional systems of medicines all over the world (Grieve 1992) and are rich in bioactivities like antique , anti-inflammatory , antibacterial , antimalarial , antithrombic, antidiuretic , antitherrosclerotic, antifungal , estrogenic , antiallergic , antidiabetic and antimutagenic activities (Baltina 2003 ; Fukai *et al.* 2002 ; Kharazami *et al.* 1997 ; Mendes-Silva *et al.* 2003 ; Rastogi and Mehrotra 1993 ; Sebestain and Thampan 2003 ; Shibata 2000 ; Takil *et al.* 2001 ; Zani *et al.* 1993 ) *G. glabra* extract , glycyrrhizin and its derivatives are reported to inhibit growth of viruses like HIV, SARS , Hepatitis B & C , Influenza through the potentiation of the immune system , inhibition of reverse transcriptase and induction of interferon production (Cinatl *et al*. 2003 ; Sasaki *et al*. 2003 and Takahara *et al*. 1994).

Chromatography and spectral analysis of root extracts of *T. cuneifolia* and *G. glabra* has exhibited similarity in chemoprofile. The similar chromatophores included the sweetening principle, glycyrrhizin. Ethanol and chloroform soluble fractions of both the plant species possessed considerable *in vivo* anti-inflammatory and protective activity from EMS induced toxicity in *Salmonella typhumurium*. *T. cuneifolia* extracts showed inhibition of *Agrobacterium* induced tumors ( Zore *et al.* 2008). *T. cuneifolia* root extract have shown considerable antifungal activity ( Zore *et al.* 2003 ; 2004 ).

The commercial licorice has a huge demand in Indian system of medicine Ayurveda and is required to the tune of 5000 tons per year by the Indian pharmaceutical companies. This is totally imported from Pakistan and Afghanistan spending crores of Rupees (Anonymous 2000). Clonal propagation or micropropagation is an invaluable aid in rapid clonal multiplication of superior genotypes having desirable traits using tissue culture technology .Thus, selection and rapid multiplication of superior genotypes would help in the development of medicinal products. In the present communication , we report a protocol for regeneration of plants from nodal explants. This is the first report of direct regeneration of *T cuneifolia* plantlets through tissue culture. This protocol may be useful in conservation of this plant species, which is currently exploited from the nature.

# MATERIALS AND METHODS

Mature fruits of *Taverniera cuneifolia* were collected from Osmanabad District in the state of Maharashtra of India . The plant material was authenticated by Dr. R. M. Mulani, in the Botany division and voucher specimen (NO. SRTMU/SLS/2011-102) is deposited in the herbarium of the School of Life Sciences, SRTM University, Nanded (MS) India. The shade dried seeds were used for seed germination. The seeds were thoroughly washed under running tap water in the tissue culture bottle for about 30 minutes. Seeds disinfected in 70 % v/v ethanol for 4 min. were surface sterilized in 1 % $HgCl_2$ for 8 min. Thereafter , the seeds were washed thrice with sterile distilled water to remove the traces of mercuric chloride prior to placing onto half strength of Murashige and Skoog ( MS ) ( 1962 ) medium (pH 5.8) containing 3 % sucrose and solidified with agar ( 0.9 % ) ( w/v) (HiMedia, Bangalore, India). Since seeds show seed coat dormancy, seeds were mechanically scarified with a blade on the opposite side of the embryo to facilitate the

intake of water and nutrients from the surrounding medium. In each bottle, five seeds were kept (350 ml Kasablanka tissue culture bottles with 50 ml medium in each). These *in vitro* raised one month old seedlings were used as a source of explants. Nodal explants were prepared by cutting the parts in aseptic conditions.

Nodal explants ( 1 cm ) were inoculated on Murashige and Skoog medium containing 0.9 % (w/v) Agar-agar and 3 % ( w/v) sucrose plus growth regulators with or without $AgNo_3$ : 1 $mgl^{-1}$ BA , 2 $mgl^{-1}$ BA and 4 $mgl^{-1}$ BA and 1 $mgl^{-1}$ Kn ; 2 $mgl^{-1}$ Kn and 4 $mgl^{-1}$ Kn (HiMedia, Bangalore, India) separately or these media were supplemented with different concentrations of $AgNo_3$ ( 1.69 $mgl^{-1}$ , 3.38 $mgl^{-1}$ and 5.07 $mgl^{-1}$ ) . The pH of the medium was adjusted to 5.8 with 1 N HCl and 1 N NaoH before addition of agar. The molten agar (Approximate 10 ml / test tube) dispersed into culture tubes (15 x 140 mm) was autoclaved at a pressure of 1.5 kPa at $121^0$C for 20 min. All cultures were maintained in culture room at 25 ± 2 $^0$C temperature under a 16 hour photoperiod at an intensity of 30 µmol $m^{-2}$ $s^{-1}$ provided by cool daylight fluorescent lamps (Philips, India). Humidity maintained in culture room was 70-90 %. Explants were subcultured after every 2 weeks. For each treatment 25 replicates were used. Nodal explants were cultured to standardize the regeneration protocol of this species.

Shoots (5-6 cm; 4-5 week old) were cultured on MS medium supplemented with different concentrations of NAA or IAA alone ( Himedia, Bangalore , India ). Cultures were maintained at 25 ± 2 $^0$C under 16 hour photoperiod at an intensity of 30 µM $m^{-2}$ $s^{-1}$ provided by cool daylight fluorescent tube lights. All media were supplemented with 3 % ( w/v ) sucrose and 0.9 % ( w/v) Agar-agar .

For acclimatization , 7-8 week old rooted shoots were removed from culture tubes . After washing away the adherent medium with distilled water, plantlets were transferred to plastic pots containing soil : sand ( 1 : 1 ) and sterilized natural sandy soil. The humidity was maintained initially by covering the pots with polyethylene bags. The polyethylene bags were progressively removed to reduce the humidity. After acclimatization for 15 days , plantlets were transferred to a shade house for one month with an average temperature of 25 $^0$C .

The culture responses were expressed in terms of percentage responding explants , number of regenerants ( shoots or roots ) per explant and average length of shoots or roots . A completely randomized block design with 3 replications was used. All data were subjected to one way analysis of variance ( ANOVA) and comparisons of means were made with least significant difference ( LSD) at the 5 % level . For each treatment of a replicate experiment , 25 explants were used.

## RESULTS AND DISCUSSION

Seeds show seed coat dormancy. Scarified seeds germinated in half strength of MS medium within 7 days while non-scarified seeds did not germinate for one month. The germinating seeds of *T cuneifolia* followed the synchronous pattern as most of the seeds germinated within 6 days of culture. Moreover , 100 % of the seeds had produced normal seedlings. Similar high value for *in vitro* seed germination (97 %) is reported for *Salvia africana-lutea* (Makunga and Staden 2008).

Nodal explants are preferred explants for micropropagation due to the presence of preexisting meristems , which can be developed easily into shoots while maintaining clonal fidelity ( Ahuja , 1993). Multiplication of plants through the sequential subculture of nodal explants has been achieved for a large number of medicinal plants. Nodal explants were cultured on different concentrations of BA and Kn alone to optimize cytokinin concentration and elicit the regeneration

response. Induction of adventitious shoots took place in all concentrations of BA . Kn-supplemented medium also showed induction of adventitious shoots at 2 $mgl^{-1}$ concentration with 2 shoots per explant whereas media without plant growth regulator showed 1.0 shoot per explant. A maximum of 3.04 ± 0.14 shoots developed per nodal explant at 2 $mgl^{-1}$ BA with 60 % response. The promotive role of BA for shoot differentiation has been documented in *Cercis canadensis* var. *mexicana* ( Mackay *et al*. 1995) , *Schinopsis balansae* ( Sansberro *et al.* 2003), *Vernonia anthelmintica,(* Shahnaz and Veena 2011 ) *Holarrhena santidysentrica* ( Kumar *et al.* 2005 ), *Searsia dentata* ( Prakash and Staden 2008). However, addition of $AgNo_3$ to BA containing MS medium facilitated the growth of axillary and adventitious shoots. The best response of 5.36± 0.17 shoots was recorded at 5.07 $mgl^{-1}$ $AgNo_3$ and 2 $mgl^{-1}$ BA with 80 % response ( Fig. 1). Developed shoots elongated slowly. Supplementation of $AgNo_3$ to Kn-supplemented media did not improve the shoot number per explant (Table 1). Also , supplementation of IAA , IBA or NAA to BA supplemented media did not enhance the shoot number ( data not shown ).

**Table 1**

***Effect of BA, Kinetin & $AgNo_3$ at different concentrations on regeneration of Taverniera cuneifolia after 30 days of culture.***

| Growth regulators / $AgNo_3$ | Concentration ($mgl^{-1}$) | Multiplication (%) | Number of shoots per explant ( Mean ± SE) | Average shoot length in (cm) ( Mean ± SE) |
|---|---|---|---|---|
| Hormone Free Medium | | 60 | 1.00± 0.00[a] | 1.50± 0.12[a] |
| BA | 1.0 | 80 | 1.92± 0.12[a] | 2.88± 0.16[a] |
| | 2.0 | 60 | 3.04± 0.14[b] | 3.96± 0.17[b] |
| | 4.0 | 70 | 2.08± 0.11[a] | 3.88± 0.17[c] |
| Kn | 1.0 | 80 | 1.20± 0.15[c] | 3.08± 0.14[a] |
| | 2.0 | 40 | 2.00± 0.12[a] | 2.12± 0.12[d] |
| | 4.0 | 80 | 1.08± 0.05[d] | 3.08± 0.14[a] |
| BA +$AgNo_3$ | 2.0+1.69 | 60 | 3.12± 0.12[e] | 3.80± 0.18[e] |
| | 2.0+3.38 | 70 | 3.16± 0.14[f] | 5.20± 0.14[f] |
| | 2.0+5.07 | 80 | 5.36± 0.17[g] | 5.84± 0.13[g] |
| Kn +$AgNo_3$ | 1.0+1.69 | 60 | 1.20± 0.11[h] | 3.00± 0.15[a] |
| | 2.0+3.38 | 70 | 1.88± 0.18[a] | 2.64± 0.15[a] |
| | 4.0+5.07 | 80 | 1.08± 0.10[i] | 2.16± 0.07[h] |

***Values are the average mean ± standard error and treatments denoted by the different letter in a same column are significantly different ( $P \leq 0.05$ ).***

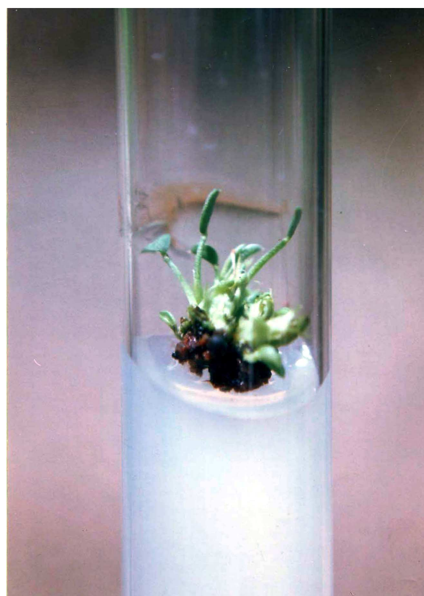

**Figure I**
***Induction of multiple shoots from nodal tip explant in MS + 2mgl$^{-1}$ BA and 5.01mgl$^{-1}$ AgNo$_3$.***

Ethylene is recognized as a ubiquitous plant hormone which influences growth and development of plants. It inhibits shoot multiplication. AgNo$_3$ has been known to inhibit ethylene action. The exact mechanism of AgNo$_3$ action on plants is unclear. However, few existing evidences suggest its interference in ethylene perception mechanism (Kumar *et al.* 2009). Ethylene insensitive mutations ( Hall *et al.* 1999) and silver ions are thought to perturb the ethylene binding sites ( Rodriguez *et al.* 1999). Ethylene receptor ,ETR1, contains one ethylene-binding site per homodiamer and binding is mediated by a single copper ion ( Cu) present in the ethylene-binding site. The replacement of the copper co-factor by silver also serves to lock the receptor in to a confirmations such that it continuously represses ethylene responses ( Zhao *et al.* 2002).
Silver nitrate is known to promote and enhance multiple shoot formation in different economically important plants. The addition of BA with AgNo$_3$ enhanced the rate of sprouting of *Coffea* buds ( Ganesh and Sreenath 1996). Plus *et al.* (1993) effectively addressed this issue by incorporating AgNo$_3$ in culture medium to restore the regeneration potential. AgNo$_3$ enhances shoot regeneration frequency in *Albizia julibrissin*. Inhibition of ethylene action by AgNo$_3$ stimulates regeneration of shoots from cotyledon explants of *Helianthus annus* (Chraibi *et al.* 1991). Silver nitrate induced shoot multiplication in *Chichorium intybus* (Bias *et al.* 2000). Silver nitrate was found to be beneficial in the regeneration and clonal propogation of several economically important plants like *Arachis hypogea* (Ozudogru *et al.* 2005) , *Punica granatum* ( Naik and Chand 2003) *Gossypium* sps ( Divya *et al*. 2008) *Raphanus sativus* ( Pua et al. 1996) *Albizzia julibrissim* (Sankhla *et al.* 1995) and *Capsicum annum* (Joshi and Kothari 2007).
Roots developed from shoots (5-6 cm) placed on half strength MS basal medium supplemented with any of the three tested auxins (IAA, NAA and IBA) at 1 mgl$^{-1}$, 2 mgl$^{-1}$ and 3 mgl$^{-1}$ ; they appeared 4 weeks after transfer. Rooting was best on medium with 2 mgl$^{-1}$ NAA (Fig II) with 7.16 roots per shoot, whereas media without plant growth regulator and with IAA, IBA showed lower rooting frequency (Table II). NAA is widely used for

induction of roots on regenerated shoots in medicinal plants like *Chlorophytum borivilianum* (Purohit *et al.* 1994) and *Curculago orchoides* (Bhavisha and Joshi 2003).
The plantlets were transferred in plastic cups. The humidity was maintained covering cups with the polythene bags. After the development of new leaves , the plants were moved to a shade house. The survival was 60 % in both methods. The survived plantlets grew in without any obvious morphological aberrations.

**Table 2**
***Rhizogenic response of in vitro regenerated shoots of Taverniera cuneifolia on MS medium supplemented with different concentrations of NAA and IAA, IBA after 30 days of culture .***

| Growth hormones ($mgl^{-1}$) | | | Rooting (%) | Number of roots per shoot ( Mean ± SE) | Average root length in (cm) ( Mean ± SE) |
|---|---|---|---|---|---|
| NAA | IAA | IBA | | | |
| Hormone free | | | 60 | 1.3±0.11[a] | 1.00±.012[a] |
| 1 | | - | 60 | 4.92±0.15[a] | 2.20±0.11[a] |
| 2 | | - | 80 | 7.16±0.18[b] | 3.52±0.23[b] |
| 3 | | - | 60 | 4.04±0.25[c] | 2.48±0.11[a] |
| - | 1 | - | 60 | 3.32±0.23[d] | 2.68±0.13[c] |
| - | 2 | - | 50 | 4.52±0.26[a] | 2.60±0.12[a] |
| - | 3 | - | 50 | 3.32±0.19[e] | 2.16±0.18[a] |
| - | | 1 | 40 | 2.68±0.00[e] | 2.16±0.11 [a] |
| - | | 2 | 30 | 2.20±0.00[e] | 2.16±0.23 [a] |
| - | | 3 | 30 | 2.20±0.00[e] | 2.20±0.18 [a] |

***Values are the average mean ± standard error and treatments denoted by the different letter in a same column are significantly different ( P ≤0.05 ).***

Regeneration protocols have been standardized for the continuous supply of many medicinal plants. As a result of the overexploitation of plant material from the natural stands for traditional medicinal purpose, the standardization of the regenaratation protocols for such plants is becoming important. Our protocol may facilitate the conservation of the species and could severe as an alternative source of materials for use. The regeneration protocol described herein would benefit the conservation of *T. cuneifolia* , which is extensively used in traditional medicines by the local people.

**Figure II**
***Roots developed from shoots transferred to MS medium supplemented with 2 $mgl^{-1}$ NAA***

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