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Asian Journal of Plant Science and Research, 2015, 5(1):49-53

ISSN : 2249-7412
CODEN (USA): AJPSKY

In-vitro direct regeneration from nodal explants of *Toddalia asiatica* (L.) Lam.

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ABSTRACT

Micropropagation is an important biotechnological tool for select, multiply and conserve the critical genotypes of medicinal plants. It also offer an integrated approach for the production of standarized quality phytopharmaceutical through mass-production of consistent plant material for physiological characterization and analysis of active ingredients. The present study was developed an efficient micropropagation protocol by using nodal explants of T. asiatica. Explants are surface sterilized and inoculated into culture medium with different concentrations of plant growth regulators. Initially, explants were cultured on Murashige and Skoog's (MS) medium supplemented with different concentrations of BAP (0.5. 1.0, 1.5, 2.0, 2.5 & 3.0 mg/l) and in combinations with IAA. Highest number of shoots was obtained from nodal explants cultured on MS medium containing 2.5 mg/l BAP in combination with IAA (0.5 mg/l).The shoots developed through in-vitro regeneration were rooted on half strength MS media containing IBA and NAA and the highest number of roots was observed on MS medium with IBA (1.5 mg/l). The highest shoot sprouting efficiencies (95 %) and 38.1 shoots per explants, the highest shoot length were obtained about7.2 cm.

Keywords: Micropropagation, Nodal Explants, Phytohormones, *Toddalia asiatica*, Rutaceae

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INTRODUCTION

Since time immemorial, human poulation has been dependent on plants for food, flavours, medicinal and many other uses. The widespread use of herbal remedies and healthcare preparations, as those described in ancient texts such as the Vedas and the Bible, and obtained from commonly used traditional herbs and medicinal plants, has been traced to the occurrence of natural products with medicinal properties. About 80% of living population in developing countries depend on traditional medicines derived from plants for their primary health care. In several industrialized societies, plant-derived prescription drugs constitute an element in the maintenance of health. Medicinal plants are an integral component of research developments in the pharmaceutical industry [1]. About 40% of compounds used in pharmaceutical industry are directly or indirectly derived from plants because the chemical synthesis of such compounds is either not possible and/or economically not viable [2]. Therefore, rapid industrilization and urbanization has led to over exploitation and loss of valuable natural resources, including medicinally important herbaceous plants. Many species are subject to extensive, unregulated collection and are endangered or threatened with extinction.

Tissue Culture technology is used to produce high quality seedlings instead of the traditionally used cuttings. It has a high fecundity, producing thousands of propagules unlike conventional techniques. Micropropagation is an effective means for rapid multiplication of endangered species in which conventional methods present limitations. Established aseptic cultures and development of an efficient protocol for regenerated and multiplication of plants are required for developing *in-vitro* strategies for conservation. Micropropagation not only ensures the supply of quality planting material on regular basis but storage of germplasm in the form of *in vitro* cultures has been an additional advantage [3].

Toddalia asiatica (L.) Lam. (Rutaceae) (Fig. 1) is a climbing shrub or liana, commonly known as wild orange tree and it is a native of Asia from India and SriLanka to Malaysia; but also found in South Africa and Madagascar, Sumatra, Java, China. It is found almost throughout India up to an altitude of 2500m. It is common in the Nilgiris

and Palani Hills and in the shrub forests of Orissa and Andhra Pradesh [4]. *Toddalia asiatica* is an important plant used for the treatment of a range of diseases like cough, malaria, indigestion, influenza lung diseases and rheumatism, fever, stomach ailments, cholera and diarrhoea [5]. Coumarine derivatives with antiplasmodial [6], and antimicrobial [7], activity have been isolated from its leaves. The root bark is bitter, astringent and acrid and has been reported in *Siddha* classical literature as expectorant, anti-bacterial, diaphoretic, anti-pyretic, analgesic and anti-inflammatory [8]. A number of phytochemicals are also identified from this plant, which includes alakloids, coumarins, flavonoids and essential oils [9]. Therefore, the present study was conducted with an objective to establish an efficient direct regeneration protocol for *Toddalia asiatica*.

MATERIALS AND METHODS

Plant Materials

Young nodal explants were collected from Boda Hills, Namakkal District, Tamil Nadu india. The nodal explants were excised into 1 cm in length and were washed well in running tap water to remove the soil or sand particles adhering and also to reduce the microbial load in the surface of explants. After washing the plant materials, the explants were treated with tween 20 detergent solution for 5 minutes and rinsed in double distilled water for three times. Then the explants were treated with 70% ethanol for 30 seconds and washed with sterile distilled water for five times. Further the explants were immersed in 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 5 minutes. Finally the explants were repeatedly washed in distilled water for five times to remove the traces before it is inoculated on the culture medium.

Media Preparation

MS basal media prepared with different plant growth regulators combinations and the pH was adjusted to 5.7. Media were steam sterilized at 121°C for 15 minutes. Murashige & Skoog [10] medium was tested and stock solutions of micro salts, vitamins (w/v) and plant growth regulators (PGRs) were prepared in sterile distilled water. All stock solutions were stored in a refrigerator at 4°C. During media preparation, macro salts were weighed separately and dissolved in distilled water one by one under continuous stirring. Stock solutions of micro salts, vitamins and PGRs were taken out from the refrigerator and allowed to attain room temperature. The required volumes of stock solutions of micro salts were taken using measuring cylinders. Vitamins and plant growth regulators like BAP, IAA, NAA and IBA were pipette out; sucrose and other additives if any, were weighed , dissolved and the media is made up to the desired volume. The pH of the medium was adjusted to the suitable range (5.6±0.2) with 1N NaOH or 1N HCL before adding 0.8% agar and dissolving it by heating at 80°C in a water bath. Interaction between the *in vitro* raised plantlets with the gelling agent in culture medium is a dynamic process and the changes in gel consistency affect the regeneration of plants or tissues. Traditionally, 0.8% agar is added to the culture medium to increase its viscosity. The required media were dispensed in culture vessels (15 – 20 ml medium in 25×150 mm culture tubes and 50-60 ml medium in 250 ml culture flasks) and closed tightly with non-absorbent cotton plugs. The medium was sterilized by autoclaving at 1.06 kg/cm pressure at 121°C for 15 min. Cultures were incubated at 24±2°C under cool white florescent light (with quantum flux density of 40 µ mol/m/s) with 16-8h regime photoperiod.

Shoot Induction

The surface sterilized explants were sized to 1 cm length contain a single node. The explants were inoculated vertically on the culture medium. Cultures were grown at 25±1 °C under 16 h photoperiod with a light intensity of 2000 – 3000 lux. *In vitro* shoot induction from nodal explants of *Toddalia asiatica* on MS medium supplemented with various concentrations of BAP and in combination with different concentrations of IAA is shown in Table 1.

Root Induction

The well grown shoots were then inoculated on half strength of MS medium with different concentrations and combinations of IBA and NAA for root induction.

RESULTS AND DISCUSSION

Micropropagation is an important biotechnological tool which is used for the commercial production of pathogen free plants to conserve the germplasm of rare and endangered species. The regeneration of plants under aseptic and controlled environmental conditions is referred to as micropropagation because very small pieces of plant tissue organs are used as starting vegetative tissues. *Toddalia asiatica* plant was efficiently regenerated from nodal explants. When nodal explants from field-grown plants of *Toddalia asiatica* were cultured on MS medium supplemented with various concentrations of BAP or in combination with IAA, the emergence of the adventitious shoot buds was observed at 12 days after inoculation. Data on different growth parameters from different treatments were recorded 4 weeks after culture initiation following one transfer to new medium.

Toddalia asiatica plants were efficiently regenerated from nodal explants. When nodal explants from field-grown mature plants of *Toddalia asiatica* cultured on MS medium supplemented with various concentrations of BAP and IAA the emergence of the adventitious shoot buds was observed at 12–15 days after inoculation (Figure 1). Data on different growth parameters from different treatments were recorded 4 weeks after culture initiation following one transfer to new medium.

At BAP concentrations higher than 1.5 mg/l, the number of shoots as well as the percentage response were lower (Table 1). Multiple shoots were induced when nodal explants were cultured on medium supplemented with BAP in combination with IAA. The mean number of shoots per explant varied among treatments of BAP plus IAA (Table 1). A significantly higher numbers of multiple shoots per explants (38.1 ± 0.15) were obtained on medium supplemented with BAP (2.5 mg/l) in combination with IAA (0.5 mg/l) (Figure 2). Other combinations of BAP with IAA also resulted in multiple shoot formation from nodal explants cultures however their number remained low as compared to BAP (2.5mg/l) and IAA (0.5 mg/l) combination. Under these conditions, the mean length of the shoot per explant was in the range of 1.4 -7.2 cm that remained low as compared to combination of optimal levels of BAP (2.5 mg/l) plus IAA (0.5 mg/l) (Table 1).

Table 1: Effect of BAP and IAA on shoot proliferation from nodal explants of *Toddalia asiatica* (L.) Lam

S.No.	Phytohormones (mg/l)		Shooting response (%)	Mean no of shoots	Mean length shoots
	BAP	IAA			
1	0.5	-	24.8 ± 0.15	8.2 ± 0.2	1.9 ± 0.03
2	1.0	-	32.1 ± 0.41	8.9 ± 0.26	1.4 ± 0.01
3	1.5	-	34.7 ± 0.37	6.9 ± 0.15	2.4 ± 0.20
4	2.0	-	35.8 ± 0.56	6.3 ± 0.26	2.5 ±0.15
5	2.5	-	44.9 ± 0.20	13.1 ± 0.15	2.7 ± 0.1
6	3.0	-	24 ± 0.2	5.9 ± 0.1	2.1 ± 0.17
7	0.5	0.5	61 ± 0.25	20.7 ± 0.25	1.8 ± 0.01
8	1.0	0.5	72.1 ± 0.20	25.3 ± 0.47	2.9 ± 0.02
9	1.5	0.5	80.9 ± 0.11	23.1 ± 0.20	3.4 ± 0.05
10	2.0	0.5	82.2 ± 0.20	26.3 ± 0.36	5.8 ± 0.01
11	2.5	0.5	95 ± 0.11	38.1 ± 0.15	7.2 ± 0.17
12	3.0	0.5	65 ± 0.15	20.8 ± 0.20	1.4 ± 0.05

Table 2: Effect IBA and NAA on root induction from *in vitro* induced shoot of *Toddalia asiatica* (L.) Lam

S.No.	Phytohormones (mg/l)		Rooting response (%)
	IBA	NAA	
1	0.5	-	61
2	1.0	-	66
3	1.5	-	87
4	2.0	-	58
5	-	0.5	34
6	-	1.0	31
7	-	1.5	45
8	-	2.0	51

Figure 1. Natural Habitat

Figure. 2. Multiple shoots production from nodal explants on MS medium containing BAP (2.5 mg/l) and IAA (0.5 mg/l)

Figure. 3. Root formation from elongated shoot lets on MS medium containing IBA (1.5 mg/l)

It is well known that cytokinins stimulate plant cell division and participate in the release of lateral bud dormancy, induction of adventitious bud formation, growth of lateral buds, and in the cell cycle control. Whereas, auxins exert a strong influence in initiation of cell division, meristem organization giving rise to un-organized tissue (callus) or defined organs (shoots), cell expansion, cell wall acidification, apical dominance, promotion of vascular differentiation, and root formation [11]. The role of BAP and IAA in shoot formation has also been recorded in other medicinal plants [12,13,14]. After 4 weeks, well developed shoots with were transferred to half strength MS media supplemented with various levels of IBA (0.5, 1.0, 1.5, 2.0 mg/l) singly and in combination with NAA (Table 2). Root initiation occurred after 10 days of culture at all concentrations of IBA (Figure. 3).

In different concentration of IBA tested, 1.5 mg/l IBA in half strength was found to be most suitable for root induction. Other hormones not produced well roots compare to the IBA. The highest shoot sprouting efficiencies (95 %) and 38.1 shoots per explants, the highest shoot length were obtained about7.2 cm. The supplementation of auxin either singly or in combination was also reported in many plant species [15, 16, 17]. However, the addition of IBA also favored rooting in other medicinal plants like *Ocimum kilimandscharicum* [18], *Sida cordifolia* [19], *Withania somnifera* [20] and *Ocimum basilicum* [21].

The rationale behind the favorable effect of reduced macronutrient concentration is that the concentration of nitrogen ions needed for root formation is much lower than for shoot formation and growth [22]. Of the two types of auxins, IBA was more effective for producing roots than NAA at the different concentrations tested. The stimulatory effect of IBA of root formation has been reported in many other medicinal plant species, including *Ocimum basilicum* [23], *Centella asiatica* [24], *Murraya koenigii* [25] and *Tylophora indica* [26].

CONCLUSION

In this work we developed the efficient and reliable micropropagation protocol for *in-vitro* regeneration of *Toddalia asiatica* from nodal explants. It can be used for large scale propagation and should become a valuable part of strategies for *ex-situ* conservation of this important medicinal plant.

Acknowledgments
The authors are profoundly thankful to the University Grants Commission (UGC) New Delhi for the financial support to this research work under the Major Research Project Scheme (UGC F.No. 41-440/2012(SR).

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