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Research Article

Micropropagation and Phytochemical Screening of *Tinospora cordifolia* (Willd.) Miers Ex.Hook. F. & Thoms.: A medicinal plant

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Abstract

Medicinal plants are an important source of compounds for the pharmaceutical industries and traditional medicine. Plants of economic interest in general and medicinal plants in particular are disappearing at an alarming rate due to various developmental activities. Since conventional method is insuffient to offset the destruction, therefore biotechnological methods could be employed for rapidly propagating, scaling up of secondary metabolites and conservation of valuable, rare and endangered medicinal plants. The present investigation was carried out with a view to standardize a protocol for rapid clonal propagation of the important medicinal climber, *Tinospora cordifolia* through micropropagation. Phytochemical screening revealed presence of alkaloids, tannins, saponins, terpenoids, phlobatannins and other bioactive compound suggesting it to be a potential medicinal plant. Shoots were initiated on Murashige and Skoog (MS) medium pplemented with 1.5mg/L kinetin. Among the cytokinins tested, N-6-benzyladenine is found more effective than kinetin for shoot proliferation with 90% response. Naphthalene acetic acid exhibited best results with 90 % rooting. Survival rate was found to be 80% when transferred to field conditions.

Key words: *Tinospora cordifolia,* Callus induction, Regeneration, Microprapagation, Phytochemical constituents, Conservation strategies.

1. INTRODUCTION

Tinospora cordifolia (Willd.) Miers Ex.Hook. F. & Thoms. is a genetically diverse shrub grown at high altitudes. It is known as a *rasayana* due to its potency of enhancing longevity and vitality. It is widely used in Ayurveda for a variety of purposes associated with inflammation, allergies, neurology and glucose metabolism[1]. The pharmaceutical significance of this plant is mainly because of the leaves, barks and roots, which contain various bioactive compounds such as alkaloids, glycosides, lactones, saponins, tannins, steroids, polysaccharides and aliphatic compounds having various medicinal importances. Some of the important applications are *viz*. immunomodulatory or immunostimulatory, anti-neoplastic, anti-oxidant, anti-hyperglycemia, anti-hyperlipidemia, anti-tuberculosis, hepatoprotection, anti-osteoporotic, anti-angiogenic, anti-malarial and anti-cancer[2].

This plant is disappearing rapidly from their natural habitats due to its wide applications in traditional medicines and also in modern medication. Plant is a good source for different kind of secondary metabolites but the concentrations of chemical agents are too little to be exploited commercially. Therefore, an alternative method needs to be formulated in order to enhance the concentration of secondary metabolites. In this perspective, biotechnological approach holds great promise; especially the establishment of tissue culture is a precondition for any advance study.

It is with this objective the present investigation has

been undertaken to enhance the biomass of the parent plant through micropropagation and also to establish a protocol for callus induction and regeneration.

2. MATERIALS AND METHODS

The plant material was obtained from the Medicinal Garden of the Department. The stem of the plant *Tinospora cordifolia* was washed thoroughly with running tap water and left over night in plain water to facilitate leaching out the phenolic compounds. Subsequently,washing was done in Tween 80 (3-5 drops in 250ml water) followed by series of treatments: 70% ethanol for 5 minutes; 0.1% $HgCl_2$ for 3 min and final washing was done with autoclaved double distilled water 4-5 times, under laminar airflow, to prepare the sterilized stem for inoculation.

The sterile stem containing nodal segment was inoculated in Murashige and Skoogs (MS) medium[3]with different concentrations of phytohormones; Naphthelene-1- acetic acid (NAA), Indole-acetic acid(IAA), Indole -3-butyric acid (IBA), Benzyl amino purine (BAP), and Kinetin (KN) either alone or in combinations. The pH of the medium was adjusted to 5.8 and the medium incorporated with phytohormone/s is dispensed 10-15 ml into culture tubes. The culture tubes were sterilized at 15 lbs psi for 20 min and allowed gradually to cool down.

The inoculation was done under laminar air flow cabinet and cultures were maintained in culture room under controlled conditions: temperature 25 ± 3°C; 16 hours light and 8 hours dark; light intensity 1500-3000 lux and relative humidity 60%.

2. 1 Phytochemical Screening

The plant samples (leaves and stems) were air-dried and ground into uniform powder.The methanol extract of each sample was prepared by soaking 10 gm of dried powdered samples in 100 ml of methanol. The extracts were filtered after continuous soaking and shaking for 72 hours using Whatman filter paper No 1. The chemical tests adopting standard procedures were carried out either on the methanolic extract or extract powder so as to identify the secondary metabolites as constituents are described by many workers. [4,5,6.]

- **Test for tannins:** About 0.5 gm of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.
- **Test for phlobatannins:** Appearance of a red precipitate when a methanolic extract of each plant sample was boiled with 1% hydrochloric acid suggests the presence of phlobatannins.
- **Test for saponins:** 2 gm of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken thoroughly, then observed for the formation of emulsion thereafter in plant sample (filterate/powder).
- **Test for flavonoids:** 5 ml of dilute ammonia solution was added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2SO_4. A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing after few minutes.
- **Test for steroids:** To the test solution added 10 ml of chloroform then filtered. To the 2 ml filterate added 2 ml of acetic anhydride and con. H_2SO_4. Blue green ring indicate the presence of steroids in the sample.
- **Test for terpenoids:** To the test solution added 2 ml of chloroform and 1 ml H_2SO_4, reddish brown color at interface, indicate the presence of terpenoids.
- **Test for cardiac glycosides:** To an extract, added 4 ml of glacial acetic acid, few drops of ferric chloride and concentrated sulfuric acid (2 ml) was added. Brown ring obtained at interface, indicate the presence of cardiac glycosides.

2.2 Field Transfer

The *in vitro* raised plantlets developed from nodal cuttings were subjected to hardening process before field transfer. Shooted plantlets at an early stage (about 1-2 cm in length) were transferred from filter paper to polycups containing a mixture of sterile garden soil: sand (3:1), which was covered with polypropylene bags and irrigated with 10x diluted MS liquid medium. The plants were kept in a culture room for 15 days and sprayed with bavistin to check fungal infections. After 15 days, the polycups hardened plants were transferred to pots and kept in green house.

3.RESULTS

MS medium supplemented with BAP or KN alone or in different concentrations, showed shoot formation from nodal segment. The leaf was given of from the nodal point in the fourth week. Initially, single leaf was seen but after seven days more leaves were seen arising from the same point. MS + KN (1.5 mg/L),

shoot regeneration percent was found to be the minimum (10%). Increased concentrations of KN led to high frequency of regeneration, 40% and 60% at 3mg/L and 4mg/L of KN, respectively (Table 1).

On the other hand, per cent of cultures showing micro-shoots recorded to be 90% in MS+ BAP (4 or 5 mg/L). However, 30% shoot formation was observed in 2mg/L BAP, whereas per cent regeneration of shoot declined in 5 mg/L to 60%. Combination of BAP (3mg/L) and KN (6mg/L) showed good response (78%) with 3-4 shoots bearing healthy leaves (Fig.1; Table 2).

For roots induction, MS medium was supplemented with auxins (NAA, IBA). The frequency of cultures showing roots was 90% in 6 mg/L NAA (Fig.2; Table3) and 72% in 3mg/L IBA (Fig.3; Table5); however, the number of roots per explant was maximum up to 10-11(Table 3). MS + NAA proved to be futile with nodal and internodal segment for rooting at high concentration; however response was not good with decreasing concentration of NAA. In combination of NAA (2.5mg/L) +BAP (1mg/L) response of rooting was 70 % (Table 3). However, scanty callusing was also observed in this combination.

Phytochemical screening of leaf and stem of *Tinospora cordifolia* showed that it contains saponins, tannins, alkaloids, steroids, terpenoids, glycosides, phlobatannins, flavonoids, cardioglycosides and alkaloids (Table 6). However phlobatannins was found to be absent in the leaves of *Tinospora cordifolia.* Results show presence or absence of secondary metabolites. Dark colour or precipitation is indication of high concentrations of the compounds in question.

The success rate of field transfer was recorded to be 75- 80% in the present investigation.The *in vitro* rhizogenesis was not a precondition with this plant as the stem cutting itself readily regenerate large numbers of roots, thus facilitates the establishment of plantlets in field condition.

4. DISCUSSION

Plant tissue culture is presently of great interest to molecular biologists, biotechnologists, plant breeders and industrialists because it is used for both basic and applied aspects. [7-9]

There are so many plants which are difficult to be propagated through conventional methods. Micropropagation technique is used for the mass multiplication of existing stocks of germplasm for biomass enhancement and also for the conserservation of important elite and rare plant species that are threatened or on the verge of extinction. [10-17]

In the present investigation also plants were regenerated through micropropagation employing different combination of phytohormones. Single cytokinin or more than one type of cytokinins facilitated shoot regeneration. In the initiation of shoot induction with nodal explants responded better than other explants such as internodes and shoot tips.The results are in agreement with the finding of Sivakumar *et al* [18.] Root regeneration from regenerated micro-shoots was not a problem in the present investigation as micro-shoots readily regenerated roots on transfer to sterile soil. However, there are large numbers of reports where second treatment is required for the induction of roots before acclimatization. [19,20,21] Some work on *T. cordifolia* has been done by other workers too. [2,22, 23,24] Chemistry and medicinal properties of *Tinospora cordifolia* were reported by many workers. [25,26,2] Different parts of the plant especially stem and leaves of *T. cordifolia* are very rich in different types of phytochemicals such as tannins, cardiac glycosides, saponins, phlobatannins, terpenoids etc. These phytochemicals have been attributed to the medicinal properties of the experimental plant. [2,24,26-30]

5. CONCLUSION

Callus induction, regeneration and micropropagation of medicinal plants of economic importances in general are found to be highly useful for commercial production of medicinally important compounds. The efficient techniques of plant tissue culture plays a crucial role in serving the life science and opening new horizons in preserving endangered species compiling our green world.

Plant tissue culture in the present investigation proved to be a very good on account of callus induction, regeneration and micropropagation. Presence of different phytochemicals in the experimental plant suggesst its profuge therapeutic efficacy.

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Table 1
Effect of Cytokinins on Shoot Induction.

Concentration of Kinetin (mg/L)	Percentage of Shoot induction	Number of Shoots/Explants.
KN(0.5mg/L)	-	-
KN(1 mg/L)	-	-
KN(1.5 mg/L)	10%	1
KN(2 mg/L)	10%	1
KN(3 mg/L)	40%	1-2
KN(4 mg/L)	60%	2-3
BAP(1 mg/L)	-	-
BAP(2 mg/L)	30%	1-2
BAP(3 mg/L)	90%	3-4
BAP(4 mg/L)	90%	4-5
BAP(5 mg/L)	60%	1-2

Table 2
Effect of Combinations of Cytokinins (BAP and KN) in Different Concentrations on Shoot Induction.

Concentration(mg/L)	Percentage of Shoot Induction	Number of Shoots/Explant.
BAP(0.5mg/L)+KN(1mg/L)	15%	1
BAP(1mg/L)+KN(2mg/L)	40%	1
BAP(2mg/L)+KN(4mg/L)	70%	2-3
BAP(3mg/L)+KN(6mg/L)	78%	3-4
BAP(4mg/L)+KN(8mg/L)	30%	1-2

Table 3
Effect of Auxins (NAA and BAP) alone or in combination on Root Induction.

Concentration(mg/L)	Percentage of Root Induction	Number of Roots/Explant
NAA(1 mg/L)	10%	1 root (Callus also observed)
NAA(2 mg/L)	10%	1 root (Callus also observed)
NAA(3 mg/L)	40%	1 root (Callus also observed)
NAA(4 mg/L)	58%	1-2 roots (Callus also observed)
NAA(5 mg/L)	78%	3-4 roots(Callus also observed)
NAA(6 mg/L)	90%	10-11 roots(Callus also observed)
NAA(2.5 mg/L)+BAP(1 mg/L)	70%	1 root (Callus also observed)

Table 4
Effect of Auxin (IAA) on Root Induction.

Concentration(mg/L)	Percentage of Root Induction	Number of Roots/Explant
IAA(1 mg/L)	Slight swelling	_
IAA(2 mg/L)	Slight swelling	_
IAA(3 mg/L)	42%	2 roots (Callus also observed)
IAA(4 mg/L)	67%	3-4 roots(Callus also observed)
IAA(5 mg/L)	87%	3-4 roots(Callus also observed)

Table 5
Effect of Auxin (IBA) on Root Induction.

Concentration(mg/L)	Percentage of Root Induction	Number of Roots/Explant
IBA(1 mg/L)	_	_
IBA(2 mg/L)	_	_
IBA(3 mg/L)	Slight swelling	_
IBA(4 mg/L)	46%	1-2 roots (Callus also observed)
IBA(5 mg/L)	62%	1-2 roots (Callus also observed)

Table 6
Phytochemical Screening of Secondary Metabolites in *Tinospora cordifolia.*

S.No	Plant part	Saponin	Tannins	Sterioids	Terpenoids	Glycosides	Phlobatannin	Flavanoid	Cardiac glycosides	alkaloids
1.	Stem	+	++	++	++	+	++	+	+	+++
2.	Leaf	+	+	+	+	+	--	++	+	+

(+++): appreciable amount; (++): moderate amount; (+): trace; (-): absent

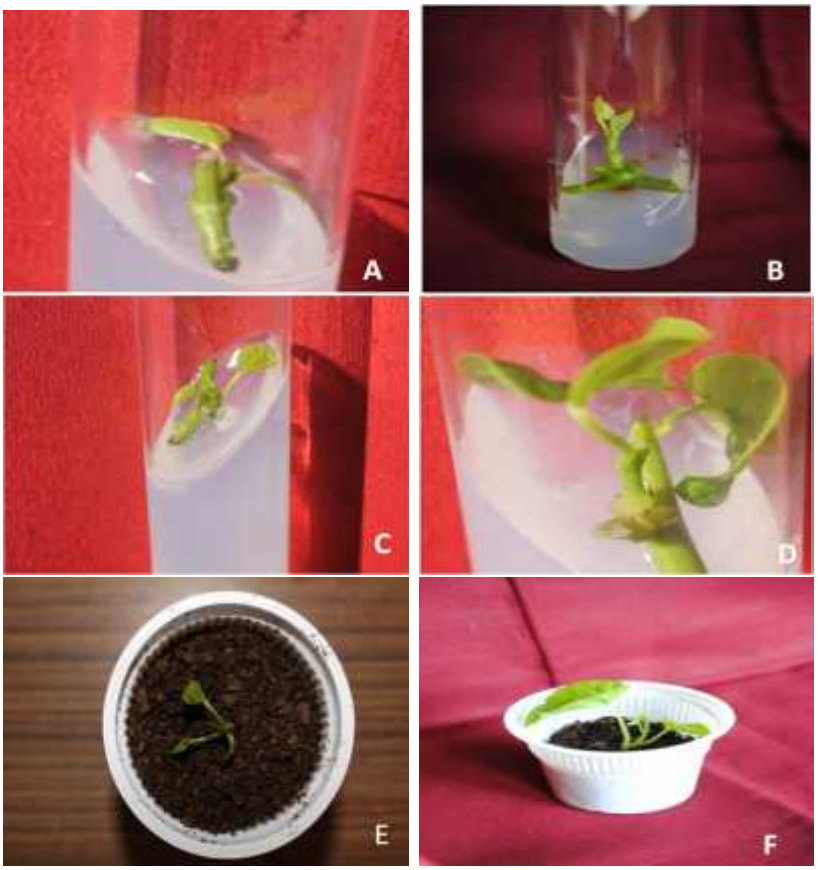

Fig 1
Shoot regeneration in MS medium incorporated with different concentrations of cytokinins in combinations or singly and transfer in pot : (A) BAP (1.5mg/L) +KN (3mg/L); (B) BAP (2 mg/L) +KN (4mg/L); (C) BAP (4mg/L); (D) BAP (3 mg/L) +KN (6mg/L); (E) 42- day-old; and (F)71- day- old Plantlets.

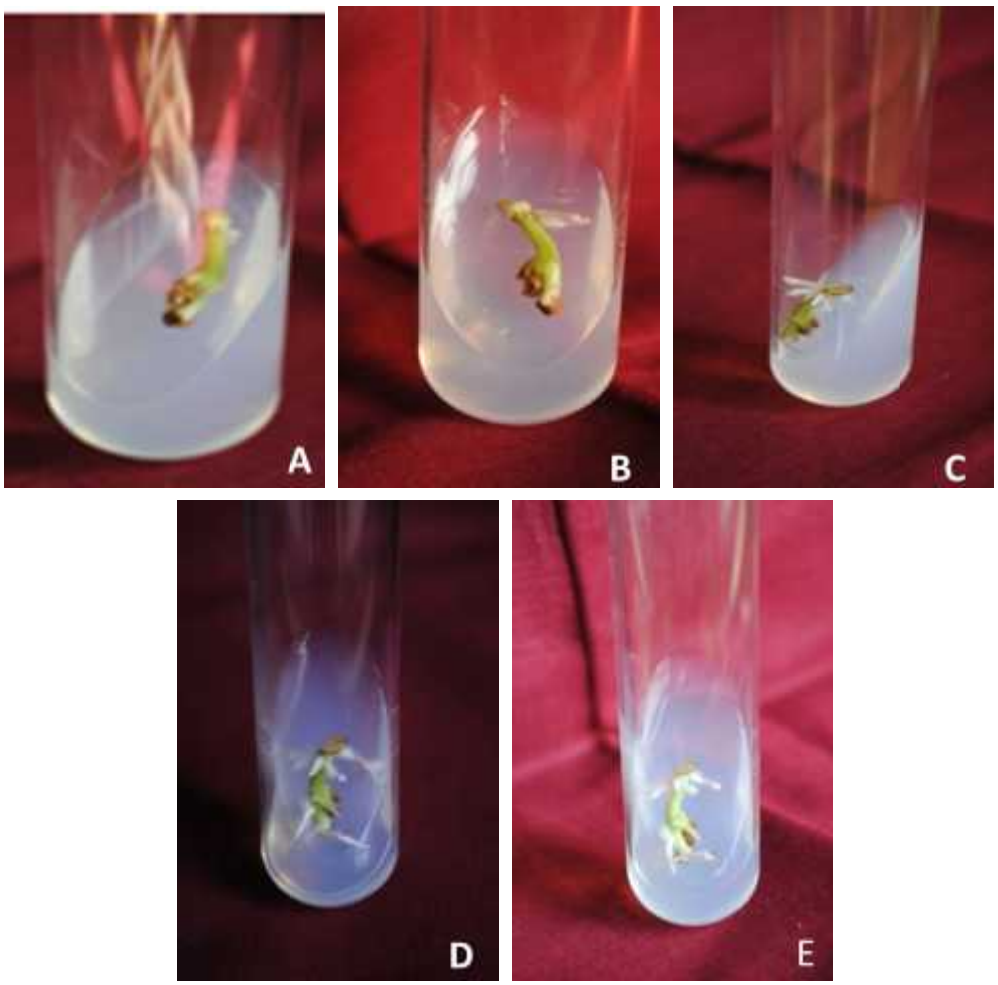

Fig 2

Nodal culture showing root induction in MS + NAA (6mg/L): (A) 17 days old; (B) 22days old**;** (C) 36 days old; D) 45 days old; E) 50 days old

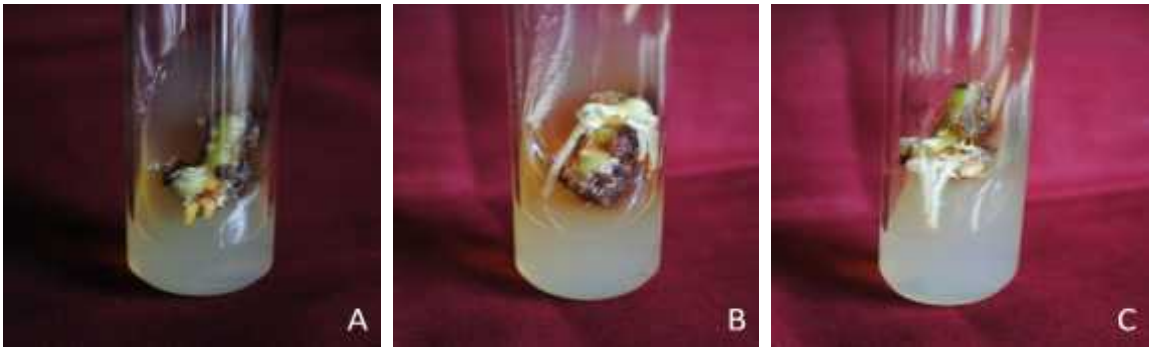

Fig 3

Rooting in different concentrations of Auxins.(A)IBA (5mg/L); (B)IAA(5mg/L); (C)NAA(6mg/L).

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