International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
www.chemijournal.com
IJCS 2020; 8(3): 1424-1429

Received: 24-03-2020
Accepted: 26-04-2020

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Vitro plantlet regeneration from *Tinospora cordifolia (Willd.)* Miers - a highly valuable medicinal plant and its chemo-profiling

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DOI: https://doi.org/10.22271/chemi.2020.v8.i3s.9397

Abstract
Tinospora cordifolia[(Willd.) Miers ex Hook. F. and Thom)] belongs to family Menispermaceae and is mostly found in tropical Indian subcontinent especially India, Sri Lanka and Bangladesh. It is an important medicinal plant species and considered as a rich source of alkaloids and terpenes which is used as a rejuvenating drug. The extract of the mature stem is useful in curing skin diseases and dry barks have anti-spasmodic, anti-pyretic, anti-allergic, anti-inflammatory and anti-leprotic properties. In the present investigation a viable *in vitro* regeneration protocol for mass multiplication of *Tinospora cordifolia* was standardized. Chemo-profiling of crude extract of *Tinospora cordifolia* was also investigated. The shoot buds were used for direct shoot induction and regeneration by using Murashige and Skoog (MS) medium supplemented with different concentrations of cytokinins BAP and Kinetin singly or in the combination. For auxiliary shoot proliferation and superior in shoot elongation, it was found that BAP was more effective than Kinetin among the cytokinins tested. An average multiplication rate of 6.3 shoots per explants was obtained with MS medium supplemented with 8.87 µM BAP alone. Small shootlets were transferred to MS medium supplemented with 8.87 µM BAP and 5.71 µM IAA for shoot elongation. The elongated shoots were rooted in half-strength MS medium supplemented with 2.22 µM BAP and 4.92 µM IBA. Rooted plantlets were successfully transferred to soil and established with 80% survival. The TLC profile was developed using hexane: ethyl acetate (25:5v/v) as a mobile phase and individual peak scanned at short wavelength 366nmto identify and separate the berberine in leaf, stem, and root of *T. cordifolia* In the antibacterial studies, it was found that *Tinospora cordifolia* has the most antibacterial properties against *Bacillus subtilis* as compare to other bacterial species. The methanolic extract of *Tinospora cordifolia* resulted a „zone of inhibition‟ of 18 mm at a concentration 80 mg/ml. Against *Styphylococcus aureus*, a 80 mg/ml methanolic extract worked best giving a „zone of inhibition‟ of 15 mm. The methanolic extracts of this species gave a „zone of inhibition‟ less than 1mm *Mycococcus*

Keywords: Antimicrobial activity; chemoprofiling; *in vitro* culture; micropropagation; *Tinospora cordifolia;* TLC.

Introduction
The genus *Tinospora* is one of the main genera of family Menispermaceae commonly known as ‘Guduchi' (Sanskrit), Gilroy' (Hindi) and 'Heart-leaved Moonseed plant'(English) (Mittal *et al*, 2014) [5] and there are about 70 genera and 450 species of this genus, commonly found in tropical low land regions. The members of this family are considered a rich source of alkaloids and terpenes. According to Kumar *et al*., (2019) among different species, five species of *Tinospora* (*T. bakis, T. cordifolia, T. crispa, T. malabarika and T. rumpii*) are well known for their medicinal value in treatments of different human ailments all over the world. The nature of the plant are generally climbing or twining, rarely shrubs, Leaves are altered and lobed; flowers are small cymose and seeds are usually hooked or reniform. In the Indian traditional system of medicine, this genus is of great importance and the most extensively second-hand as a raw drug in the manufacture of different Ayurvedic formulations. The stem, leaf, and root of [*Tinospora cordifolia* (willd.) Miers ex Hook. F. and Thoms] have been highly exploited for medicinal preparations because of their health-supporting properties. It is also known as a ‘rasayana’ due to its potency of enhancing longevity and vitality (Sinha and Sharma, 2015) [10]. *T. cordifolia* can cultivate in a wide range of soil from acid to alkaline and

prefers modest soil moisture. It is found throughout tropical India ascending to an altitude of 300 m (Saran *et al*, 2019) [9] and also found in South Asia, Indonesia, Philippines, Thailand, Myanmar, China, and Srilanka. The stem of *Tinospora* is bitter, stomachic, diuretic; stimulates bile secretion; allays thirst, burning sensation, and vomiting; enriches the blood and cures jaundice. According to the Puratchimani and Jha, (2007) finding they suggest that the extract of the mature stem is useful in curing skin diseases and dry barks have anti-spasmodic, anti-pyretic, anti-allergic, anti-inflammatory and anti-leprotic properties. The root and stem of *T. cordifolia* are prescribed as an antidote to snakebite and scorpion sting in combination with other drugs (Kirtikar and Basu, 1975, Nadkarni *et al*., 1976) [2, 8].

Further, plants with medicinal importance are becoming endangered day by day at an increasing rate owing to urbanization, industrialization, deforestation and using complete plants for the medicinal preparation by pharmaceutical companies (Mohammed and Kumar, 2012). Similarly, the national medicinal plant board (NMPB) Govt. of India, has listed *T. cordifolia* in a prioritized medicinal plant list (Kala and Sajwan, 2007) [1]. This has led to its acute scarcity to meet the present-day demand. Previously, there are fewer reports on the micropropagation of *T. cordifolia* through nodal explants but the produced regeneration protocols are unreliable due to less number of *in vitro* shoot propagation (Sivakumar *et al*. 2014; Mittal and Sharma, 2017) [11, 4]. The present investigation illustrates an easy, reliable, efficient and reproducible protocol for the *in vitro* shoot multiplication of *T. cordifolia* using mature nodal explants.

Thin Layer Chromatography (TLC) technique is widely used techniques to separate new promising pharmaceutical therapeutics which could be used in pharmaceutical industries (Maripandi *et al.,* 2010). Chemo-profiling and TLC analysis are performed in the present research to identify and separate the berberine in leaf, stem, and root of *T. cordifolia*.

Materials and methods

Plant material

T. cordifolia plants were collected from All India Coordinated Research Project on Medicinal and Aromatic Plants, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha and established in the greenhouse of the Department of Agricultural Biotechnology OUAT, Bhubaneswar for consistent procurement of explants for micropropagation and chemo-profiling studies.

The explants were excised as single nodal segments, removing shoot tip and leaves except a small portion of the petiole. The standard surface sterilization method was used for surface sterilization of all the explants. The nodal segments (1-2 cm) were rinsed under running tap water for 30 min. These were disinfected with 0.1-0.2percent bavistin solution along with (2 percent, m/v) Tween 20 for 5 min and washed thrice with sterilized water. The explants were surface sterilized with 70 percent alcohol for 1 min followed by five rinses with sterile double distilled water. Further, the sterilization was done with 0.1 percent (m/v) aqueous mercuric chloride ($HgCl_2$) solution for 5 - 6 min and rinsed with sterile double distilled water for three times.

Culture media and growth conditions

Basal Murashige and Skoog (1962) [7] medium (MS) was used for the experiment. Stock solutions of macronutrients, micronutrients, iron source and organic supplements (except myoinositol) were prepared separately. Beside MS salts, sucrose 30 g/l as a carbon source and myoinositol 100 mg/l were added freshly. The pH of the medium was balanced to 5.6 to 5.8 using 0.1N HCL or 0.1N NaOH. Agar- agar 8 g/l as a gelling agent was added and dissolved by warming the medium. Subsequently, the nutrient medium was sterilized in autoclave at 121 ºC and 15 psi pressure for 20 min and kept for solidification.

The culture vials (25 X 150 mm) with molten media were incubated at 25 ± 2 ºC temperature under 16 h photoperiod with cool, white fluorescent lights (1000 lux) at light intensity of 40-50 µmol m-2 s-1and 8 h dark cycle. All experiments were set with eight replicates and repeated at least thrice. Cultures were observed daily for the assessment of any morphological changes, if occurred in the cultures.

Micropropagation

Direct shoot regeneration

Mature nodal segments (1.0-2.0 cm long) were inoculated on MS medium supplemented withvarious concentration of cytokinins viz., BAP (2.22, 4.44, 8.87 µM) and Kn (2.32, 4.65, 9.29 µM), singly or in combination to proliferate axillary buds to micro shoots. The culture tubes wereincubated at 25 ±2°C temperature, with 16 hours photoperiod and irradiance of 40 µmol $m^{-2}s^{-1}$ providedby cool white fluorescent tubes. Then leaves of elongated shoots were excised and remnant shoot bases in 4 - 5 mm long were aseptically transferred into MS media supplemented with BAP (2.22, 4.44, 8.87µM) along with IAA (0.57, 2.85 and 5.71µM) for further multiple shoot proliferation at 30 days interval.

Indirect shoot regeneration via callus

3 weeks old *in vitro* raised shoots were removed fromculture tubes and were cut into 4 -5 mm long explants, and aseptically transferred into MS medium supplemented with NAA (0.54, 2.68 and 5.37 µM), and BAP (2.22 and 4.44 µM) and Kn (2.32 and 4.65µM) for callus induction. Sub-culturing was made at every four weeks interval. The number of explants forming calli was scored to calculate callus induction frequency after two months of culture.

$$\textbf{Callus induction frequency} = \frac{\textbf{No.of explant froming callus}}{\textbf{Total no.of explants}} \times \mathbf{100}$$

Healthy calli were taken in a sterile petri dish and chopped into small pieces, then inoculated on shoot regeneration medium containing different concentration of BAP (2.22 and4.44 µM) and NAA (0.54, 2.68 and 5.37 µM). Green shoot primordia developed on the surface of calli within 4-5 weeks. The leaves and shoots elongated subsequently, so the number of shoots formed per callus were investigated after 4 weeks of culturing. Randomly selected individual regenerated shoots (2-3 cm long) with intact apices and three to four leaves were cultured for rooting in half-strength MS basal medium, having 3% (w/v) sucrose, supplemented with indole butyric acid (IBA) (1.23, 2.46 and 4.92 µM) with BAP for shoot multiplication and subsequent induction of best roots.

$$\textbf{Regeneration frequency} = \frac{\textbf{No.of shoot regenerated from calli}}{\textbf{Total no.of callus inoculated}} \times \mathbf{100}$$

In vitro rooting and acclimatization

The excised shoots were separated from culture and transferred to rooting medium, having 3% (w/v) sucrose, supplemented with indole butyric acid (IBA) (1.23, 2.46 and 4.92 µM) with BAP for shoot multiplication and subsequent

induction of best roots. Then number of shoots per explant; shoots length, number of roots per shoot and root length were recorded. all the data were statistically analysis (Harter, 1960). The rooted plantlets were removed from the media and washed with double distilled water properly to get rid of the traces of agar sticking to the roots. The plantlets were transplanted into plastic pots containing autoclaved mixture of soil, sand and vermi-compost (1:1:1) and kept in the greenhouse for hardening. Watering of plants was done at regular intervals.

Chemo-profiling analysis

The collected leaf, stem and root of *Tinospora cordifolia* were washed with deionized water, dried in the sun/shade for 1 week and again dried at 50°C in hot air oven for half an hour's and grounded in powder form.

Preparation of crude extracts

Approximately 15 g of sample was taken into a thimble and placed in a Soxhlet apparatus. The solvent methanol (100 ml) was added and extracted according to the boiling point at 40-60°C for 7 h and the complete extraction was effected within 24–30 h. The solvent was removed from crude extract at a reduced pressure with the help of rotary vacuum evaporator. The residue was collected in a beaker and preserved in the desiccators for further use in TLC analysis. The definite quantity of residue was dissolved in methanol to get final concentration (100 mg/ml). After completion of extraction the dark brown extract was stored in refrigerated condition until further use.

Chromatography

Chromatographic chemo-profile was performed on aluminium plate pre-coated with silica gel (60 F254 10 x 5 cm) TLC plates (Merck Bioscience, India), used as stationary phase. Extracts and reference standards berberine, were being applied onto the TLC plate with the help of CAMAG linomat V sample applicator equipped with 10µl syringe. Sample loaded plate was developed using the solvent system, used to identify berberine *viz.* hexane: ethyl acetate (40:10 v/v) under laboratory conditions (25-30°C and 40-50% relative humidity). The loaded plates were then placed vertically in the chamber previously saturated with solvent system for 30 min. After the solvent front moved up to a distance of about 90% of length, the plate was taken out, solvent front was marked and the plate was dried at room temperature. Developed plates were air dried and then immersed in a freshly prepared mixture of methanol and concentrated sulphuric acid (95:5 v/v) for derivatizing. After drying, the plates were heated at 110°C for 5 min to develop the color of the spots. For quantitative determination, spots corresponding to standards were scanned using a CAMAG TLC Scanner 3 at 366 nm (wavelength chosen to be appropriate for standards after staining) with a slit size of 6 × 0.4 mm. The Rf values were calculated using formula:

Rf = Distance travelled by solute / Distance travelled by solvent

Results

The present investigation was carried out for anti-bacterial characterization of *Tinospora cordifolia* and chemo-profiling of its active constituents. In addition to that standardization of viable *in vitro* regeneration protocol was carried out for *T. cordifolia* for large scale multiplication and secondary metabolite production for conservation of elite genotypes. The results of present investigations from different experiments are presented in this chapter.

Direct regeneration

The mature *in vivo* nodal explant (1.0-2.0 cm long) of *T. cordifolia* was being used through direct method of tissue culture to achieve in *vitro* regeneration successfully (Fig. 2). The effect of different concentration of cytokinins (BAP and Kinetin) on direct shoot multiplication was presented inTable-1.

Table 1: Effect of plant growth regulator on direct shoot induction of *Tinospora cordifolia*

S. No.	Phyto-hormones		Length of shoots (cm) after 2 weeks	No. of leaves per explants after 3 weeks
	BAP(µM)	Kinetin(µM)		
1	0.00 BAP	0.00	-	-
2	2.22 BAP	0.00	1.7	1.2
3	4.44 BAP	0.00	1.9	1.4
4	8.87 BAP	0.00	2.3	1.6
5	0.00	0.00 Kn	-	-
6	0.00	2.32 Kn	1.5	1.1
7	0.00	4.65 Kn	1.7	1.3
8	0.00	9.29 Kn	2.0	1.7
Mean			1.85	1.38
C. D. (p=0.5)			0.002	0.002

During the investigation, maximum shoot length after 2 weeks (2.3 cm) followed by (2.0 cm) were obtained on MS medium fortified with BAP (8.87µM) and MS medium fortified with Kn (9.29µM) respectively. The shoot length decreased as the concentration of BAP and Kn decreased beyond the concentrations mentioned above (Table-1). The explants grown on simple MS medium did not response to shoot growth and multiple shooting. The highest number of leaves per explants (5-6) after 3 weeks of culture were observed in MS medium fortified with Kn (9.29µM) (Table-1, Fig. 2).

The effect of plant growth regulators on multiple shoot induction was revealed, as the maximum number of shoots per explant (6.3 & 3.4 shoots/explant) were observed on MS media supplemented with optimized concentration of BAP (8.87 µM) + 5.71 µM IAA and 4.65 µM Kn + 2.85 µM IAA respectively (Table-2).

Table 2: Effect of BAP, Kinetin and IAA on shoot multiplication of *Tinosporacordifolia*

S. No.	Phyto-hormones			No. of shoots per explant
	BAP(µM)	IAA(µM)	Kinetin(µM)	
1	0.00	0.00	0.00	0.0
2	2.22	0.57	0.00	1.5
3	4.44	2.85	0.00	2.6
4	8.87	5.71	0.00	6.3
6	0.00	0.57	2.32	2.3
7	0.00	2.85	4.65	3.4
8	0.00	5.71	9.29	2.1
Mean				3.03
C. D. (p=0.05)				0.01

Very less numbers of shoots were observed from explant in the MS media supplemented with 2.22 µM BAP and 0.57 µM IAA (1.5 shoots/explant), whereas half MS media supplemented with 2.22 µM BAP + 4.92 µM IBA and 2.32 µM Kn + 4.92 µM IBA spontaneously gave good rooting (85%), (Table-3).

Table 3: Effect of BAP and IBA on root multiplication of *Tinospora cordifolia*

S. No.	MS medium	BAP(µM)	IBA(µM)	Kinetin (µM)	No. of shoots per explants
1	Full MS	0.00	0.00	0.00	0.0
2	½ MS	2.22	1.23	0.00	32
3	½ MS	2.22	2.46	0.00	65
4	½ MS	2.22	4.92	0.00	80
5	Full MS	0.00	0.00	0.00	0.0
6	½ MS	0.00	1.23	2.32	35
7	½ MS	0.00	2.46	2.32	45
8	½ MS	0.00	4.92	2.32	75
Mean					55.33
C. D. (p=0.05)					0.17

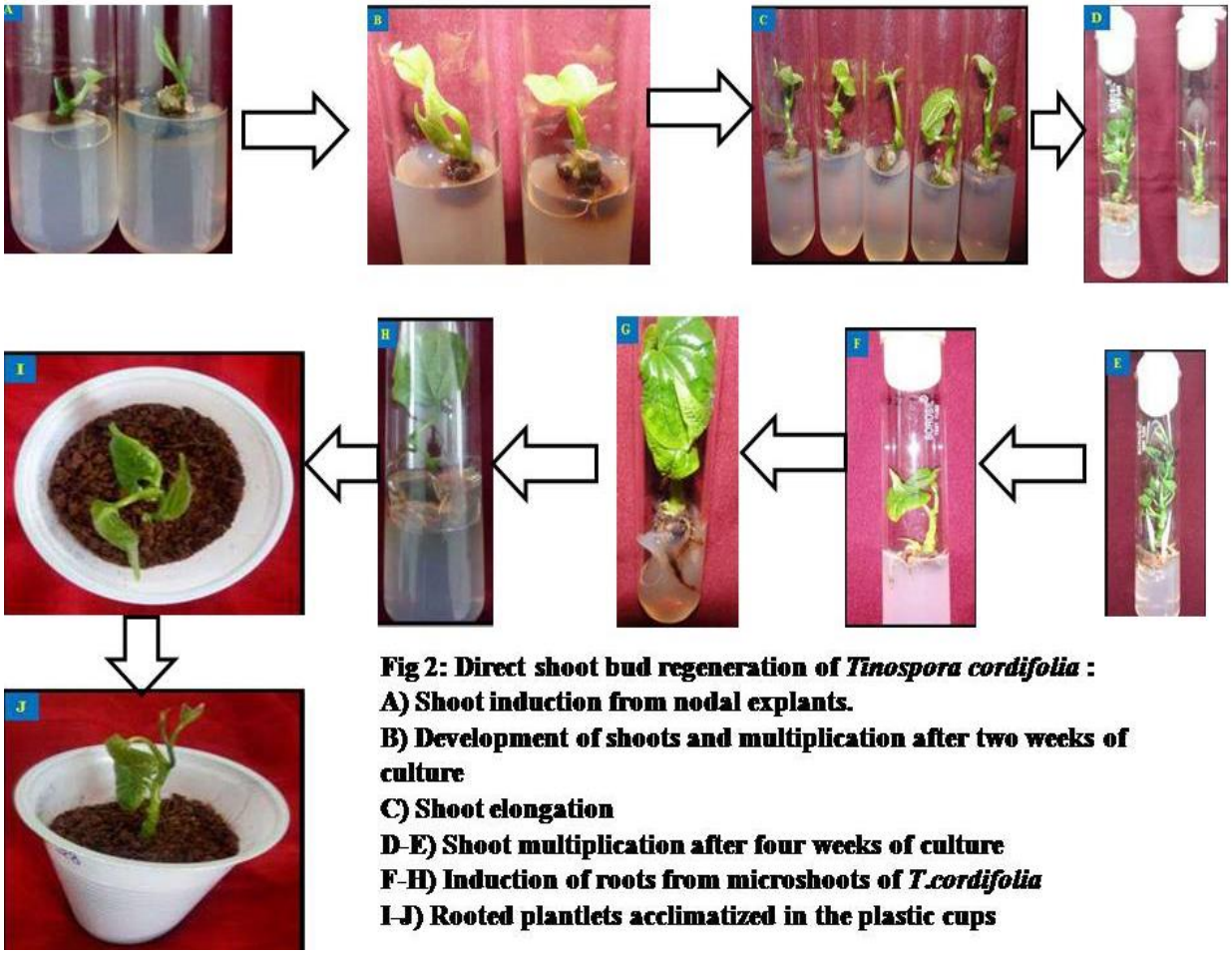

Fig 2: Direct shoot bud regeneration of *Tinospora cordifolia* :
A) Shoot induction from nodal explants.
B) Development of shoots and multiplication after two weeks of culture
C) Shoot elongation
D-E) Shoot multiplication after four weeks of culture
F-H) Induction of roots from microshoots of *T.cordifolia*
I-J) Rooted plantlets acclimatized in the plastic cups

Indirect regeneration

Callus initiation was observed when the nodal segments were inoculated on MS basal medium supplemented with various concentrations of NAA and BAP, Kn. The explants when cultured horizontally on to MS medium supplemented with 2.22 µM BAP + 5.37 µM NAA and 2.32 Kn + 5.37 µM NAA,gave optimum results and started swelling followed by callus formation within 3-4 weeks of inoculation (Fig. 3a-c). The same culture medium was resulted the highest callus induction (50.6 mg) along with 53% callus induction frequency. There was very low response to callus induction on MS medium supplemented NAA (0.54- 2.68µM) along with BAP and Kn (2.22µM & 2.32µM). The influence of different plant growth hormones on callus induction were presented in Table 4 & Fig.3.Positive response revealed for indirect shoot induction from callus using all the combination of hormones tested.

Table 4: Callus induction from leaf extract of *Tinospora cordifolia* on MS basal medium supplemented with a combination of NAA, BAP and Kinetin

S. No.	Phyto-hormones			Fresh weight (mg) of callus after 30 days of culture	Callus induction frequency (%)
	NAA(µM)	BAP(µM)	Kinetin(µM)		
1	0.00	0.00	0.00	0.0	0.0
2	0.54	2.22	0.00	0.0	0.0
3	2.68	2.22	0.00	30.0	35
4	5.37	2.22	0.00	50.6	53
5	0.00	0.00	0.00	0.0	0.0

6	0.54	0.00	2.32	0.0	0.0
7	2.68	0.00	2.32	35.0	26.0
8	5.37	0.00	2.32	40.7	43.0
Mean				38.75	39.25
C. D. (p=0.05)				0.11	0.14

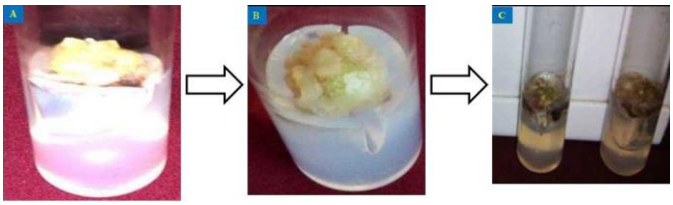

Fig 3: Callus induction for indirect regeneration of *Tinosporacordifolia*

A) Callus formation at the cut end surface of the explants after 2 weeks ofculture
B) Mass of calli developed from bud base after 3-4 weeks of culture
C) Calli developed after subsequent sub culture in similar medium

Chemo-profiling of *Tinospora cordifolia*

Extraction of secondary metabolites from stems and its purification

The healthy stems were collected, washed with distilled water and shade dried for about one week. The stems were grinded into powder using electric grinder. Extraction was done in methanol as solvent using soxlet apparatus and dry recovery was measured in milligrams. The quantity of dry extract and recovery percentage were also estimated and represented in Table 5. These extracts were dissolved in pure methanol (100mg/5ml) and stored at 4°C for HPTLC and biochemical analysis.

Table 5: Extraction of secondary metabolites from stem of *Tinospora cordifolia*

S. No.	Species Name	Stem powder wt. taken for extraction (gm)	Methanol for extraction (ml)	Dry extract wt. (gm)	Recovery %
1.	*Tinospora cordifolia*	15	100	5.14	34.26

Antimicrobial activity

The antimicrobial activity was tested as per the zone of inhibition formed by the plant extract against the bacteria (*Bacillus subtillis, Staphylococcus aureus, Mycococcus luteus, E.coli*). The methanolic extracts of *Tinospora cordifolia* were tested against the pathogenic bacteria namely *Bacillus subtilis, Staphylococcus aureus, Mycococcus luteus and E.coli at* different concentrations of extract *viz.* 20, 40, 60, 80 mg/ml. The methanolic extracts of *T. cordifolia* showed antibacterial activity against these four bacteria and the zone of inhibition was recorded in millimetre (mm) (Fig. 7). Methanolic stem extract showed maximum antibacterial activity against *Bacillus subtilis* (18 mm) at 80 mg/ml concentration. Less antibacterial activity showed against *Mycococcus luteus* (1mm) at 20 mg/ml

Discussion

During the investigation, maximum shoot length after 2 weeks (2.3 cm) followed by (2.0 cm) were obtained on MS medium fortified with BAP (8.87µM) and MS medium fortified with Kn (9.29µM) respectively. Raghu *et al.* 2006 has also reported the successful multiplication of shoots using BAP in *T. cordifolia*as well as in other medicinal plants such as *Portulaca oleracea*, *Asparagus racemosus* and *Cedrela fissilis* (Sharma *et al.* 2011; Thakur *et al*. 2015; Araga ˜o *et al.* 2016) [13, 14] which supports the present results. 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* (2014) [11].

The methanolic extracts of *T. cordifolia* were tested against the pathogenic bacteria namely *Bacillus subtilis, Staphylococcus aureus, Mycococcus luteus and E.coli* at different concentrations of extract viz. 20, 40, 60, 80 mg/ml. Methanolic stem extract showed the maximum antimicrobial activity against *Bacillus subtilis* (18 mm) at 80 mg/ml concentration. The highest zone of inhibitions against *staphylococcus aureus* were measured by methanolic extract (80 mg/ml) of (15 mm). Jeyachandran *et al.* (2003) had investigated the antibacterial activity of the aqueous, ethanol and chloroform extracts from the stems of *T. cordifolia* was studied using disc diffusion method against *Escherichia coil*, *Proteus vulgaris, Enterobacter faecalis, Salmonella typhi* (Gram-negative), Staphylococcus aureus and *Serratia marcesenses.* (Gram-positive). Results suggest that the ethanolic extract has significant antibacterial activity against tested bacteria.

Conclusion

From the investigation we concluded that best shoots were initiated on Murashige and Skoog (MS) medium supplemented with different concentrations of cytokinins BAP and Kinetin singly. The basal media found to be superior to MS medium for the induction of multiple shoots when tested in the combination. For auxiliary shoot proliferation and superior in shoot elongation, it was found that BAP was more effective than Kinetin among the cytokinins tested. An average multiplication rate of 6.3 shoots per explants was obtained with MS supplemented with 8.87 µM BAP alone. Small shootlets were transferred to MS supplemented with 8.87 µM BAP and 5.71 µM IAA for shoot elongation. The elongated shoots were rooted in half-strength MS medium supplemented with 2.22 µM BAP and 4.92 µM IBA. Rooted plantlets were successfully transferred to soil and established with 80% survival. TLC was performed using hexane: ethyl acetate (25:5v/v) as a mobile phase. The assay combined the separation and quantification of the analysts on silica gel 60 F254 plates was used as a stationary phase with visualization under UV and scanning at 366nm

Acknowledgement

The authors wish to acknowledge to the Department of Biotechnology, Government of India for providing student research grant under PG-HRD course program and All India Coordinated Research Project on Medicinal and Aromatic Plants, Odisha University of Agricultureand Technology, Bhubaneswar, Odisha for their support.

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