Advanced Techniques in Biology & Medicine
ISSN: 2379-1764

Jani et al., Adv Tech Biol Med 2015, 3:2
DOI: 10.4172/2379-1764.1000125

Research Article Open Access

Phloroglucinol Plays Role in Shoot Bud Induction and *In Vitro* Tuberization in *Tinospora Cordifolia*- A Medicinal Plant with Multi-Therapeutic Application

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Abstract

This paper shows the potential of phloroglucinol for *In Vitro* regeneration and tuberization of microshoots of *Tinospora cordifolia*. Within a week of nodal explants culture, axillary bud in nodal segments sprouted in all the media investigated including basal MS media without any growth regulators. Maximum response (52.2 per cent) evinced by MS media supplemented with 6.98 µM Kin. The same media produced maximum response for multiple shoot formation out of total shoot bud sprouted fostering average 3 shoots with 3.9 cm shoot length and 4.2 number of leaves per shoot. Effect of PG also investigated for mass multiplication and *In Vitro* rooting. Basal MS + 6.98 µM Kin + 79.4 µM PG increased shoot bud induction from 52.2 per cent to 84.8 per cent and multiple shoot production per cent from 12.9 to as high as 60.3 percent and also supported maximum number of shoot per explants (7.5). The same treatment promoted highest axillary shoot proliferation in terms of shoot length (3.9 cm) and number of leaves per shoot (4.3). Phloglucinol induced *In Vitro* tuberization in microshoots. Maximum rooting response observed in ½ MS medium supplemented with 7.4 µM IBA and 793.7 µM PG with 81.1 culture responding taking just 8.7 days to sprout and producing maximum number of roots (3.2) with an average length (3.1 cm). Rooted explants successfully hardened in Soil: Vermicompost: Vermiculite (1:1:1) with 100% success.

Keywords: *Tinospora cordifolia*; Medicinal plant; Phloroglucinol; Nodal culture; *In Vitro* Tuberization

Abbreviation: BAP-Benzyl Amino Purine; IBA- Indole Butyric Acid; Kin- kinetin; µM-Micromolar; PG-Phloroglucinol

Introduction

Tinospora cordifolia (Willd.) Miers, commonly known as Guduchi, is a well known medicinal plant belonging to the family Menispermaceae. It possesses a reservoir of pharmacological properties for therapeutic applications [1,2]. *T. cordifolia* is used for the treatment of kasa (cough) and swasa (asthma), which is described in Ayurveda texts [3]. In the classical texts of Ayurveda like Charak, Sushruta, Ashtang Sangraha and other treatises, *T. cordifolia* is mentioned as useful in treating various diseases like fever, asthma, diabetes, chronic diarrhoea, anorexia, jaundice, gout and skin infections [4]. Its juice is also used for the treatment of chronic cough [5]. It's use for promoting longevity, enhancing immunomodulatory responses, treating arthritis, diabetes, improving liver function and as a chemopreventive agent has been well documented [6,7]. Recently its efficacy in allergic rhinitis, anti-diabetic potential and prevention of diabetic retinopathy has been assessed using molecular biology tools. The main chemical constituents of the plant include berberine, tinosporin, tinosporal, tinosporaside, tinosporic acid, tinocordiofolioside, columbin, etc.; all of which add to its medicinal property [8-11].

Various reports on its multiple medicinal use attracted attention for commercial exploitation of the plant to meet the requirements of the growing pharmaceutical industry. *T. cordifolia* natural stands are now fast disappearing and are threatened due to indiscriminate collection and over-exploitation [12]. *Tinospora cordifolia* suffers from poor seed set and poor germination in its natural habitat. Stem cuttings, though useful for propagation, are dependent upon weather conditions for proper growth so the conventional vegetative propagation of this plant has limited potential for large scale cultivation. Micropropagation may help in propagation and conservation of this plant. Culture of shoot meristems, especially through enhanced axillary branching, permits rapid propagation of certain plants and a high degree of genetic uniformity of the progeny [13,14].

In plant tissue culture research, there is a constant need to search for novel substances that could result in better or more efficient growth *In Vitro*. Phloroglucinol increases direct shoot formation and shoot multiplication and when added to rooting media together with auxin, phloroglucinol further stimulates rooting and reduces tissue browning [15-17].

Hence, the present study was aimed to develop an efficient *In Vitro* method for conservation and mass propagation of *Tinospora cordifolia* through *In Vitro* regeneration pathways, followed by successful restoration of the *In Vitro* grown plants into field conditions. The role of PG in micro-propagation of *T. cordifolia* also has been investigated.

Material and Methods

Explant preparation and culture condition

Nodal segment from fresh non lignified vines of *T. cordifolia*, collected from Medicinal Plant Nursery at ASPEE College of Horticulture and Forestry, NAU, Navsari were used as explants. Nodal segments were washed thoroughly under running tap water for 15 minutes to remove adhering dirt and then kept in 0.05 per cent Bavistin® and 0.01 per cent Streptocycline solution for approximately

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Received March 30, 2015; **Accepted** May 29, 2015; **Published** June 08, 2015

Citation: Jani JN, Jha SK, Nagar DS, Chauhan RS, Hegde H (2015) Phloroglucinol Plays Role in Shoot Bud Induction and *In Vitro* Tuberization in Tinospora Cordifolia- A Medicinal Plant with Multi-Therapeutic Application. Adv Tech Biol Med 3: 125. doi: 10.4172/2379-1764.1000125

two hour and then kept in 10 per cent solution of detergent (Tween 20) for 10 minutes. Traces of detergents were removed by repeated washing in double glass distilled water. To diminish browning of the tissue, explants were washed with antioxidant solution of citric acid (100 mg/l) for half hours. These explants were then surface sterilized with HgCl2 (0.1%; w/v) ascetically under laminar flow cabinet. Treated explants were washed thrice with double distilled water. Nodal segments were then inoculated on MS medium supplemented with different concentrations of growth regulator as per experiment. The pH of all media was adjusted to 5.8 ± 0.1 prior to gelling with 0.8% agar-agar (Hi-media), dispensed in culture tube (10 ml) and sterilized by autoclaving (121°C for 20 min). All the cultures were incubated in a culture room at a temperature of 25 ± 2 °C with relative humidity at 55 ± 5 per cent and exposed to 16 h light and 8 h dark photoperiod provided by 40 W cool white fluorescent tubes kept 50 cm above bench surface.

Culture establishment

Aseptically manipulated nodal segment of *T. cordifolia* were cultured on full strength solid MS media supplemented without any cytokinin or various concentration of BAP (2.2 µM, 4.4 µM, 6.7 µM and 22.2 µM) and Kin (2.3 µM, 4.7 µM, 7.0 µM and 23.3 µM). Observations on number of explants responding for shoot initiation, per cent of responding explants showing multiple shoot, average number of shoot, shoot length (cm) and number of leaves per shoot recorded after four week of culture.

Shoot bud proliferation

Nodal explants were cultured on solid MS media supplemented with 4.65 and 6.98 µM Kin in combination with various concentrations (39.7, 79.4, 198.5 and 397.0 µM) of PG. Observations taken on responding explants, explants producing multiple shoot, average number of shoot, shoot length (cm) and number of leaves per shoot recorded after four week of culture.

Rooting and acclimatization of plantlets

Shoots 1.5–2.0 cm in length were excised and transferred to half-strength solid MS medium supplemented with various concentrations of IBA (4.9 µM, 7.4 µM and 9.8 µM) alone or 4.9 and 7.4 µM IBA in combination with 396.8 µM and 793.7 µM PG. The rooted plants were removed from the culture tubes, washed free of agar with sterile distilled water and transferred to plastic pots with sterile media for hardening. The plantlets were maintained at 70% relative humidity by initially covering with transparent polyrthene. The plants were kept in 28 °C under a 12-h photoperiod for acclimatization. The plants were fertilized with 1/8th MS macro nutrients twice during the course of acclimatization at an interval of 4–5 wk. Established plants were placed in 20 cm diameter pots with sand:vermicompost: vermiculite mixture (1:1:1). Hardened plants were field-transferred and the survival rate was recorded.

Statistical analysis

All the data were analysed in complete randomized design for analysis of variance. Means were compared with DMRT. All contaminated cultures were removed from the initiation experiments, thus limiting the scope of thorough statistical analysis. Each treatments consisted 30 replication and repeated thrice.

Results

There was major problem of phenolics exudation from the cut surface of explant in all the media even after pre-treatment with citric acid. The phenolic exudation from cut end led to arrest of growth of sprouted shoot and most of the explants engulfed by dark brown callus mass at basal end within four week of culture. Albeit the effort was made to control this problem by sub-culturing of explants on fresh media, the problem of browning remained uncontrolled.

Within a week of culture, axillary bud in nodal segments sprouted in all the media investigated including basal MS media without any growth regulators. But, the frequency of bud sprout varied significantly between treatments. Maximum response (52.2 per cent) evinced by MS media supplemented with 6.98 µM Kin (Table 1). The same treatment took minimum number of day for bud sprouting (Personal observation).

Unlike some other crops where multiple buds sprout from nodal explants, this species showed tendency of producing single shoot per axillary bud (Figure 1a) which is a challenge in economically viable mass multiplication of species *In Vitro*. Very less number of culture showed multiple shoot bud. Maximum response for multiple shoot formation out of total shoot bud sprouted was produced by 6.98 µM Kin (12.9 per cent), it fostered average 3 shoots with 3.9 cm shoot length and 4.2 number of leaves per shoot (Table 1).

To overcome problem of phenolics and challenge of less multiplication rate, Phloroglucinol (PG) either alone or in various combination with Kin supplemented in MS medium. PG showed apparent reduction in browning and callusing of culture. Role of PG in control of exudes was evident even at lowest concentration tried.

PG alone could not show better multiplication response unless it applied in combination with Kin. Under the influence of PG, Kin showed better multiplication rate as compare to Kin alone. Treatment MS+6.98 µM Kin +79.4 µM PG increased shoot bud induction from

BAP (µM)	KIN (µM)	Percent of responsive culture	Percent of responsive culture with multiple shoot	Average number of shoot per explants	Average length of shoot (cm)	Average number of leaf per shoot
0.00		26.7 ± 1.9^{c}	0.0	1.0 ± 0.0^{c}	1.4 ± 0.2^{cd}	3.0 ± 0.1^{d}
2.2		42.2 ± 4.5^{ab}	0.0	1.0 ± 0.0^{c}	1.5 ± 0.1^{cd}	4.1 ± 0.2^{ab}
4.4		38.9 ± 2.9^{bc}	0.0	1.0 ± 0.0^{c}	1.6 ± 0.1^{c}	3.4 ± 0.1^{c}
6.7		25.5 ± 9.1^{c}	0.0	1.0 ± 0.0^{c}	1.1 ± 0.0^{cd}	3.3 ± 0.1^{cd}
22.2		26.7 ± 1.9^{c}	0.0	1.0 ± 0.0^{c}	1.0 ± 0.1^{d}	3.0 ± 0.0^{d}
	2.3	48.9 ± 1.1^{a}	9.2 ± 2.5ab	1.4 ± 0.1^{b}	1.5 ± 0.0^{cd}	3.1 ± 0.1^{cd}
	4.7	48.9 ± 2.2^{a}	11.3 ± 2.2ab	2.8 ± 0.1^{a}	2.9 ± 0.1^{b}	3.8 ± 0.1^{b}
	7.0	52.2 ± 2.9^{a}	12.9 ± 0.7a	3.0 ± 0.2^{a}	3.9 ± 0.4^{a}	4.2 ± 0.1^{a}
	23.3	34.4 ± 1.1^{b}	9.7 ± 0.3b	1.0 ± 0.0^{c}	1.1 ± 0.1^{cd}	3.1 ± 0.1^{cd}

Table 1: Effect of BAP and Kin on per cent of culture responding to shoot induction, number of culture showing multiple shoot, number of shoot per explants, average number of shoot and average number of leaves per shoot in the nodal culture of *Tinospora cordifolia* (after four weeks of culture).

Figure 1: *In Vitro* regeneration of *Tinospora cordifolia* nodal culture (a) tendency of tinospora nodal culture to sprout single shoot only (b) & (c) multiple shoot bud induction under influence of phloroglucinol (d) type of root produced by different media (i- MS+7.4 µM IBA+793.7 µM PG, ii- MS+7.4 µM IBA,iii- MS+4.9 µM IBA+396.8 µM ,iv- MS+4.9 µM IBA) (e) *In Vitro* tuberization in phloroglucinol supplemented media (f) hardening.

PG (µM)	KIN (µM)	Percent of responsive culture	Percent of responsive culture with multiple shoot	Number of shoot per explant	Average shoot length (cm)	Number of leaves per shoot
		45.6 ± 1.1^{e}	7.3 ± 0.2^{e}	1.2 ± 0.0^{e}	1.1 ± 0.0^{e}	3.7 ± 0.0^{b}
79.4		47.8 ± 1.1^{e}	9.2 ± 2.1^{e}	1.3 ± 0.1^{e}	1.1 ± 0.1^{e}	3.7 ± 0.3^{b}
198.5		38.9 ± 2.2^{f}	14.2 ± 2.7^{e}	1.1 ± 0.0^{e}	1.1 ± 0.0^{e}	3.9 ± 0.0^{b}
397		34.4 ± 1.1^{g}	13.0 ± 3.5^{e}	1.4 ± 0.1^{e}	1.1 ± 0.0^{e}	3.7 ± 0.0^{b}
39.7	4.7	75.6 ± 1.1^{c}	50.0 ± 2.2^{b}	4.5 ± 0.3^{c}	2.6 ± 0.3^{bc}	3.9 ± 0.2^{b}
79.4	4.7	84.4 ± 1.1^{a}	56.6 ± 1.8^{a}	5.8 ± 0.4^{b}	2.5 ± 0.0^{bc}	4.3 ± 0.0^{a}
198.5	4.7	70.0 ± 1.9^{d}	54.0 ± 3.6^{a}	3.8 ± 0.2^{d}	2.9 ± 0.1^{b}	3.8 ± 0.0^{b}
397	4.7	78.9 ± 1.1^{b}	39.4 ± 1.2^{cd}	3.5 ± 0.1^{d}	2.3 ± 0.1^{c}	3.7 ± 0.0^{b}
39.7	7.0	81.1 ± 1.1^{a}	57.9 ± 1.2^{a}	6.0 ± 0.1^{b}	2.2 ± 0.1^{c}	4.2 ± 0.0^{ab}
79.4	7.0	84.4 ± 1.1^{a}	60.3 ± 3.3^{a}	7.5 ± 0.2^{a}	3.9 ± 0.4^{a}	4.3 ± 0.0^{a}
198.5	7.0	71.1 ± 1.1^{c}	45.4 ± 2.2^{bc}	3.5 ± 0.2^{d}	1.2 ± 0.1^{de}	3.7 ± 0.1^{b}
397	7.0	70.0 ± 1.9^{d}	33.6 ± 5.5^{d}	3.7 ± 0.3^{d}	1.5 ± 0.0^{d}	3.8 ± 0.1^{b}

Table 2: Effect of Phloroglucinol (PG) on on per cent of culture responding to shoot induction, number of culture showing multiple shoot, number of shoot per explants, average number of shoot and average number of leaves per shoot in the nodal culture of *Tinospora cordifolia* (after four weeks of culture).

52.2 per cent to 84.8 per cent and multiple shoot production per cent from 12.9 to as high as 60.3 percent as compared to MS+6.98 µM Kin (Table 1) (Table 2) also fostered maximum number of shoot per explants (7.5). The same treatment promoted highest shoot length (3.9 cm) and number of leaves per shoot (4.3) (Table 2) (Figure 1b, Figure 1c). Overall, PG supplemented in low concentration increased shoots bud multiplication. Higher PG in media showed direct rooting on explants cultured and reduced shoot multiplication rate.

The micro shoots more than 1.5 cm length were excised and set on the ½ MS media supplemented IBA alone or in combination with PG. Effect of various treatments on *In Vitro* rooting is shown in Table 3. Basal MS media supplemented with PG produces tuberous root on the cut surface of micro shoot within four weeks of culture. All the media supplemented with IBA alone also produced rooting however the frequency of root induction and quality was quite low as compared to those supplemented by PG (Table 3) (Figure 1d). Maximum rooting response observed in ½ MS medium supplemented with MS+7.4 µM IBA+793.7 µM PG which produced 81.1 percent responsive culture and took minimum days (8.7) to sprout. Maximum number of roots (3.2) and average length (3.1 cm) observed in the same media (Table 3). PG produced tuberous well developed root system (Figure 1e).

The rooted microshoots were carefully removed from each media separately, washed thoroughly to clear it from adherent agar and planted in polybags filled with soil:vermicomspost: Vermiculite (1:1:1).

IBA (µM)	PG (µM)	Percent of responsive culture	Days taken for root induction	Type of Root	Number of root	Average root length (cm)
-	-	-	-	-	-	-
4.9	-	27.8 ± 2.2^{e}	14.7 ± 0.3^{b}	Slender	1.5 ± 0.3^{d}	1.6 ± 0.3^{c}
7.4	-	53.3 ± 1.9^{c}	12.3 ± 0.3^{cd}	Slender	2.0 ± 0.1^{cd}	2.1 ± 0.1^{bc}
9.8	-	34.4 ± 1.1^{d}	13.3 ± 0.7^{c}	Slender, abnormal	1.6 ± 0.1^{d}	1.3 ± 0.2^{c}
4.9	396.8	62.2 ± 2.2^{b}	11.3 ± 0.3^{d}	Tuberous	2.4 ± 0.2^{bc}	2.4 ± 0.1^{ab}
7.4	396.8	58.9 ± 1.1^{c}	12.3 ± 0.7^{cd}	Tuberous	2.2 ± 0.1^{b}	1.6 ± 0.2^{c}
4.9	793.7	65.6 ± 2.9^{b}	9.3 ± 0.3^{e}	Tuberous	3.0 ± 0.2^{a}	2.8 ± 0.5^{ab}
7.4	793.7	81.1 ± 2.2^{a}	8.7 ± 0.3^{a}	Tuberous	3.2 ± 0.2^{a}	3.1 ± 0.2^{a}

Table 3: Effect of IBA and Phloroglucinol (PG) on percent response to root induction, days taken for root induction, type of root, number of root and average root length (cm) of *In Vitro* grown microshoots of *Tinospora cordifolia* (after four weeks of culture).

All the rooted microshoots successfully hardened (100%) after four weeks of culture.

Discussion

Initial establishment of culture is difficult in this species due to phenolics exudation. Early workers reports same difficulty for micropropagtion of this species [13,18]. PG effectively controlled tissue browning in *T. cordifolia* in present investigation. Prevention or reduction of tissue browning by PG in culture has also been reported by Kim et al. [17].

More number of shoot bud activated and resulted in more number of shoot in media supplemented with PG. Enhancement of *In Vitro* growth, axillary shoot proliferation, and adventitious root formation in woody plants by the addition of PG has been reported [19-21]. The effectiveness of certain phenolic substances in enhancing shoot formation is well established in plant tissue cultures [21,23]. Higher concentration of PG resulted in direct root formation in *Tinospora cordifolia*. The promotive effect of PG on rooting has been identified in several plant species [15,19,16]. PG has been reported in adventitious root formation in micropropagated shoots of adult wild cherry, *In Vitro* multiplication of Tebernaemontana fuchsiaefolia L. and direct shoot bud formation in Psoralea corylifolia [23,24,16]. The beneficial effect of PG is due to its ability to depress the peroxidase activity within the cultured explants, thereby protecting the endogenous auxin from peroxidation catalyzed oxidation [25,26]. PG had stimulatory effect on tuber induction. 81.1 per cent of explants showed tuber development. *In Vitro* tuberization have also been reported for potato under the influence of PG, PG is a trihydoxyphenol and it is known to protect the auxin by keeping the cell at a low redox potential [27]. All the plants rooted in PG supplemented media showing well developed tubers were suucessfully hardened. This may be due to better lignifications of plants *In Vitro* and better root development.

Conclusions

Phloroglucinol as media additive has immense potential for *In Vitro* regeneration of *T. cordifolia*. It enhances shoot bud in nodal culture as well as induces *In Vitro* tuberous root formation on microshoots. The present protocol will be beneficial for conservation and mass multiplication of this multi therapeutic medicinal plant. Application of phloroglucinol can also be beneficial for other crops where phenolic exudation and less rooting and multiplication rate is a problem.

Acknowledgements

Authors acknowledge all the member of Forest biotechnology laboratory at Foresty College, Navsari agricultural university, Navsari for their support.

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