JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

In vitro Micropropagation of Tinospora cordifolia - An Important Medicinal Plant

Dr. Nutanvarsha Pradiprao Deshmukh
Assistant Professor
Department Of Botany
Shri Shivaji Arts, Commerce and Science College, Akot MS.

Abstract

A protocol was established for rapid clonal propagation medicinal plant, *Tinospora cordifolia* (TC), through in vitro culture using nodal explants. Best shoot induction was observed on MS medium with 4.36 µM KIN produces 2.32□0.1 cm length with 1.8□0.1 numbers of shoots with 70% response when compared with other Cytokinin's BA and 2iP. After a month of shoot induced culture, creates a phenolic exudation problem in shoot formation, media had discoloration and explants were browning and the buds were break so, further analysis protocols were found by treating with different substances from that, silver nitrate (20%) with KIN (4.36 µM) gives 100% response with 3.01□1.0 cm length of shoots with 2.01□0.1 numbers of shoots within 16 days. Nodal explants of TC were cultured on MS medium with various concentrations of BA alone and in combination with NAA or IAA for shoot proliferation. From that, BA (8.82 µM) alone showed better growth response and produced 4.81□0.2 numbers of shoots with an average length of 3.1□0.1 cm after 20 days. Small sheetlet were transferred to shoot elongation medium with 8.82 µM of BA alone. An average length of 4.82□0.4 cm with 4.61□0.2 numbers of shoots produced, 76% of response. The elongated shoot lets transferred to half strength MS medium and 6.43 µM of IBA with 3% sucrose produce 5.2□0.2 rootlets per plant with average root length of 3.2□0.1 cm after 27 days. Rooted plantlets were transplanted ex vitro. Approximately 80% of plantlets survived.

Keywords: Micropropagation, *Tinospora cordifolia*, Nodal explants, Shoot proliferation **Introduction**

Nature has been a source of medicinal agents for thousands of years, and an impressive number of modern drugs have been isolated from natural sources, mainly based on their use in traditional medicine. These plantbased traditional medicine systems continue to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care. Plant products also have an important role in the health care systems of the remaining 20%, who reside in developed countries. This usually involves the use of plant extracts. Many medicinal plant species are disappearing at an alarming rate, as a result of rapid agricultural and urban development, deforestation and indiscriminate collection. If this trend continues, mankind will, forever, loss some of the most important sources of drugs. The tissue culture technique proved very efficient in rapid mass propagation and conservation of these important multipurpose medicinal plants. Tinospora cordifolia. It is a large, glabrous, succulent, perennial deciduous twiner with succulent stems and papery bark, climbing shrub belonging to the family menispermaceae, ascending to an altitude of 1200 m. Tinospora cordifolia (Guduchi) is Indian medicinal plant and has been used in Ayurvedic preparation for the treatment of various ailments throughout the centuries. Ancient Hindu physicians prescribed it for gonorrhoea. The plant is used in Ayurvedic, "Rasayanas" to improve the immune system and the resistance against infections. The whole plant is used medicinally; however, the stem is approved for use in medicine as listed by the Ayurvedic Pharmacopoeia of India. In folk and tribal medicine, the whole plant, powdered root and stem bark, decoction of root and stem, juice of the

root and paste or juice of leaves or stem of Tinospora cordifolia are used to treat various ailments such as fever, jaundice, general debility, cough, asthma, leucorrhoea, skin diseases, bits of poisonous insects and venomous snakes and eye disorders. Today the drug and a tincture prepared from Tinospora cordifolia are approved for use in the Indian pharmacopoeia. They are used for the treatment of general weakness, gonorrhoea, secondary syphilis, urinary diseases, impotency, gout and viral hepatitis. A variety of compounds have been isolated from aerial parts and roots of Tinospora cordifolia. They belong to different classes such as alkaloids, diterpenoids, lactones, glycosides, steroids, sesquiterpenoid, phenols, aliphatic compounds and polysaccharides. Tissue culture has greatly enhanced the scope and potential of mass propagation by exploiting the regenerative behaviour in a wide range of selected horticultural and agricultural plants including the medicinal ones. Many important medicinal herbs throughout the plants have been successfully propagated in vitro, either by organogenesis or by callus formation. The present study was therefore undertaken with a view to establishing an efficient protocol for in vitro rapid propagation of this important medicinal plant.

Materials and methods

The healthy plants of Tinospora cordifolia were collected from wadner gangai village dist Amaravati MS. The explants were prepared from the nodal portion of the stems, internodes and shoot tip, which were first washed thoroughly in running tap water for about half an hour to remove soil and other superficial contamination, and then allowed to stay for 3–4 hrs in double distilled water to facilitate the phenolics and a characteristic gummy substance of polysaccharide leaching out of explants. The disinfected explants were surface sterilized under aseptic conditions in a laminar flow chamber. Later the explants (1cm each) with upper portion were washed with 1% sodium hypochlorite solution (v/v) for 5 min followed by thorough washing under running tap water for 15 min. Then single bud explants (1 cm each) with upper portion were washed with 1% Bavistin (w/v) for 5 min followed by 3 times washing with sterile distilled water. The explants were surface sterilized with 0.1% (w/v) mercuric chloride for 6 min. and later rinsed 4 or 5 times with sterile distilled water. Later the edges of the explants were trimmed with a sterile blade to eliminate possible residue of sterilant and the explants were then used for culturing. For, in vitro shoot initiation from various explants of Tinospora cordifolia, the surface sterilized explants such as nodes, internodes and shoot tips were cultured on full strength MS medium supplemented with various concentrations of Cytokinins BA (N6 – benzyladenine) (0.44–13.31 µM), KIN (Kinetin) (0.46–13.94 µM) and 2ip (N6 -2- isopentenyl adenine) (0.49–14.76 µM) were tested individually for shoot induction. The cultures were incubated under a 16 hours photoperiod (50 µm.m2 /sec provided by cool-white daylight Sylvania fluorescent lamps, USA) at 25 °C and 55% relative humidity in sterile environment condition. Different experiments were conducted to find out an optimum culture conditions for the maximum shoot initiation (budding) from the cultured explants. And use the different type of Phenolic exudation controlling substances such as polyvinyl pyrrolic one, activated charcoal, silver nitrate and ascorbic acid were used at the time of initiation. Different experiments were conducted to find out an optimum culture conditions for the maximum shoot proliferation and elongation from the cultured explants. Shoot proliferation and elongations were cultured on MS medium supplemented with various concentrations of Cytokinin BA (4.22–12.64 µM), KIN (6.23–12.44 µM) and 2ip (2.11–13.32 µM) were tested individually and combine with Auxins like NAA (α- naphthyl acetic acid) (4.22–12.88 µM) and IAA (Indole -3- acetic acid) (1.32–11.86 µM). After 20 days the culture was used to rooting, 1 to 2 cm long shoots were transferred to half strength of MS medium amended with 3% sucrose with Auxins IAA (1.76–17.13 µM), IBA (Indole-3-butyric acid) (1.32–14.70 µM) and NAA (1.34–16.11 µM) tested individually. All the experiments were done in six cultures with ten replicated experiments. The in vitro rooted shoots were carefully removed from the culture vessel and they were gently washed with sterile distilled water to remove every trace of media. Thereafter plantlets were dipped in 0.05% Bavistin (systemic fungicide) for 10 seconds to minimize the microbial infection. Again, a second wash was given with sterile distilled water. The treated plantlets were then transferred aseptically to small earthen pots containing mixture of vermiculite, sterilized red soil and farmyard manure at 1:1:1 ratio in growth chamber with controlled temperature, light and humidity to acclimatize with the outside environment. Half strength liquid medium was added periodically. The plantlets were covered with polythene bags to ensure a relative humidity of 70 to 80%. The acclimatized complete plantlets were then transferred to the field. For all the above studies, MS medium was used as sole basal medium. Four individual stock solutions viz., Macro, Micro, Fe, EDTA and Vitamins were prepared and stored in refrigerator; whereas meso-inositol and Plant Growth Regulator (PGR) stock solutions were freshly prepared for use. 3% of sucrose (30 g/l, w/v), meso-inositol (100 mg/l, w/v) and required amount of PGR were added to the MS medium and the pH was adjusted to 5.6 with 1N HCl or 1N NaOH. The gelling agent, agar at 0.8% (w/v) was added to the prepared media and mixed well before dispensing into glassware. The contents were labelled and sterilized in an autoclave at 15 lb pressure for 15 minutes at 121 oC. The cultures were incubated under a 16 hours photoperiod (50 µm.m2 /sec provided by cool-white day-light Sylvania fluorescent lamps, USA) at 25 °C

with 55% relative humidity in sterile environment condition. All the experiments were done in six cultures with ten replicated experiments. The data were subjected to ANOVA and means were performed by using SPSS (SPSS ver.16.0).

Results and Discussion

After surface sterilization the node, internodes and shoot tips explants were inoculated on MS supplemented with different concentration of BA, KIN and 2iP showed different response according to the hormonal concentration used. In the present study, the auxiliary buds on the nodal cuttings showed visible growth after five days in culture and most of them were grow into shoots within 20 days. Shoots formation was affected by the concentration of hormones used in the medium. Among the different Cytokinins (BA, KIN, and 2iP) the better result was produced only in nodal explants with KIN 4.36 µM, produces 2.23□0.1 cm length with 1.8□0.1 numbers of shoots with 70% response when compared with other Cytokinins (Table 1). In the initiation of shoot induction with nodal explants responded better than other explants such as internodes and shoot tips. After a month of shoot induced culture, creates a phenolic exudation problem in shoots formation, media had discoloration and explants were browning and the buds were break, so further analysis was needed to control the phenolic leaching out, the protocols were found by treating with different phenolic exudation controlling substances. The explants were allowed to stand for 3 to 4 hours in double distilled water to facilitate the phenolics and a characteristic gummy substance of polysaccharide leaching out of explants. Later single bud explants (1-2 cm each) with upper portion were washed with 1% Bavistin (w/v) for 5 min. followed by 3 times washed in sterile distilled water. The explants were surface sterilized with 0.1% (w/v) HgCl2 for 6 minutes, and later rinsed 4 or 5 times with sterile distilled water. Later the edges of the explants were trimmed and nodal explants (1 cm each) containing an axillary bud were used for the experiment.

Table 1: Influence of Cytokinins on shoots induction from nodal explants of Tinospora cordifolia in 20 days culture.

BA (µM)	KIN (µM)	2Ip (µM)	No. of Shoots	Shoot length (cm)	% of Response
00.44	0	0	callus	0	0
03.21	0	0	0	0	0
06.42	0	0	0	0	0
13.31			1.0 □ 0.0^{d}	0.34 □ 0.1^{e}	30^{d}
0	00.46	0	1.2□0.3 ^{bc}	1.34□1.1^{c}	40^{bc}
0	04.36	0	1.8□0.1a	2.23□0.1^{a}	70^{a}
0	08.46	0	1.4□0.1^{b}	2.-1□1.1^{b}	43^{b}
0	13.94	0	1.0□0.1^{d}	0.62□0.3c^{d}	32^{d}
0	0	00.49	Callus	0	0
0	0	04.26	1.2□0.0^{bc}	0.21□0.1^{f}	20^{e}
0	0	08.42	1.4□0.1^{b}	0.23□0.0^{f}	21^{e}
0	0	14.76	0	0	0

Explants were cultured on MS basal media supplemented with BA, KIN and 2iP. Data were recorded after 20 days of culture. Results represent mean □ SD of six cultures, ten replicated experiments. Values denoted by different letters in each column differ significantly at $p < 0.05$.

The different mediums used were polyvinyl pyrrolidone (0.01%), activated charcoal (1.5%), silver nitrate (20%), PVP (0.01%) + ascorbic acid (0.01%) and with 4.36 µM of KIN in MS medium.The results were given in Table 2. Among these combinations, silver nitrate (20%) with KIN (4.36 µM) has shown 100% response with 3.01□0.1 cm length of shoots with 2.01□0.1 numbers of shoots within 16 days of cultures. It indicates that Silver Nitrate at 20% along with KIN (4.36 µM) will control the Phenolic exudation in shoot induction. After, the determination of best treatments for shoots induction. The effect of different concentration of Cytokinin (BA, KIN, and 2iP) alone and combination of BA+ NAA and BA + IAA on shoot proliferation in nodal explants of Tinospora cordifolia in MS medium.

Table 2: Standardization of controlling in Phenolic exudation in shoot induction from nodal explants of Tinospora cordifolia in 20 days of culture

KIN(µM)	Gelling Agent	Control measures	No. of shoots	Shoot length (cm)	% of Response
4.36	Agar	Polyvinylpyrrolidone (PVP, 0.01%)	1.01 ±0.3^{c}	2.34±0.0^{b}	60^{d}
4.36	Agar	Activated Charcoal (AC, 1.5%)	1.22±0.1^{b}	2.23 ± 0.2^{c}	85^{b}
4.36	Agar	Silver Nitrate (AgNO3, 20%)	2.01±0.1^{a}	3.01 ± 0.1^{a}	100 ^{a}
4.36	Phytagel	PVP (0.01%) + Ascorbic Acid (0.01%)	1.12 ± 0.1^{b}	2.42 ±0.2^{b}	75 ^{b}

Explants were cultured on MS basal media supplemented with KIN and different gelling agents. Data were recorded after 20 days of culture. Results represent mean ± SD of six cultures, ten replicated experiments. Values denoted by different letters in each column differ significantly at $p < 0.05$.

Among the different experiment, BA (8.82 µM) alone showed better growth response and produced 4.81±0.2 numbers of shoots with an average length of 3.9±0.1 cm after 20 days of culture with 80% of response (Table 3). Similar observations have been reported for Bauhinia variegate and Hogarthian antidysentery (Fig. 1. E; F and G). Other experiments of KIN and 2iP different concentrations produced a less number of shoots, whereas 8.82 µM BA with different concentration of NAA produced very less number of shoots and high concentration of NAA produced dark green cultures. The same results of dark green were obtained in BA 8.86 µM with different concentration of IAA (data was not showed). Between the three Cytokinins tried, BA had more positive effective on KIN and 2iP for shoot multiplication.

Table 3: Effect of different concentration of Cytokinins on shoot proliferation in nodal explants of *Tinospora cordifolia* on MS medium

BA (µM)	KIN(µM)	2iP(µM)	No. of shoots	Shoot length (cm)	% of Response
04.22	0	0	2.42± 0.1^{c}	3.6± 0.3^{b}	60^{b}
08.82	0	0	4.81± 0.2^{a}	3.9±0.1^{a}	80^{a}
12.64	0	0	3.21±0.0^{b}	3.4± 0.1^{c}	52^{b}
0	06.23	0	2.11± 0.1^{c}	2.5± 0.4^{e}	55^{cd}
0	09.46	0	2.32± 0.2^{c}	2.2±1.0^{fg}	63^{b}
0	12.44	0	2.0± 0.1^{d}	2.1± 1.1^{g}	60^{b}
0	0	03.34	1.26±0.3e	2.5± 0.5e	55^{cd}
0	0	08.68	1.32± 0.5e	2.8± 0.3d	54^{cd}
0	0	13.32	2.0± 0.1d	2.1 ±0.2g	55^{c}

Explants were cultured on MS basal media supplemented with BA, KIN and 2iP. Data were recorded after 20 days of culture Results represent mean ± SD of six cultures, ten replicated experiments. Values denoted by different letters in each column differ significantly at $p < 0.05$

Fig 1: Micropropagation of *Tinospora cordifolia* in various Stages

A. Shoot induction from nodal explant of *Tinospora cordifolia* on shoot induction medium containing silver nitrate allowing with supplementary of 4.36 µM KIN. **B.** Shoot induction with 4.36 µM KIN in M.S. media after 20 days. **C**. Second sub culturing of the growing initiation culture along with leaves. **D**. Elongation culture with supplementary of KIN (9.46 µM) to form single large leaf. **E**. Initial stage of multiplication in shoot culture medium with BA (8.82 µM). **F**. Multiple shoot proliferation from nodal explants on shoot induction medium. G. multiplication of shoot in M.S. culture medium allowing with BA (8.82 µM) after 4 weeks. **H**. Shoot elongation on M.S. medium with BA (6.88 µM) and KIN (8.22 µM). **I & J**. Elongated shoot supplementary with BA (6.88 µM) and KIN (8.22 µM) after 12 and 28 days respectively. **K.** *In vitro* rooting on Half strength M.S. medium with IBA (6.43 µM). **L & M**. Acclimatized plantlets in pots. **N.** Five month old tissue cultured *Tinospora cordifolia* plants in pot.

The multiple shoots obtained from our experiments were short (below 2cm) and had condensed nodes. Therefore, transfer of these shoots to shoot elongation medium contains Cytokinins (BA, KIN and 2iP) alone or combinations. The best result was produced (Table 4) with the combination treatment of BA (6.88 µM) + KIN (8.22 µM) produces 86% of response with 4.8□1.4 numbers and 6.8□3.1 cm length of shoots produced within 20 days of cultured (Fig. 1. H, I & J). Higher concentration of KIN show increase in shoot length 4.8 cm and better growth with enlargement of single leaves.

Table 4: Effect of BA and KIN in different concentrations on elongation of shoot regenerates from nodal explants of Tinospora *cordifolia* on MS medium.

BA (µM)	**KIN(µM)**	**No. of shoots**	**Shoot length (cm)**	**% of Response**
2.44	4.66	3.4□1.1^{b}	5.2□0.0^{b}	80^{c}
6.88	8.22	4.8□0.4^{a}	6.8□0.1^{a}	86^{a}
8.82	12.44	3.6□1.3^{b}	4.87□1.1^{c}	72^{a}

Explants were cultured on MS basal media supplemented with BA and KIN. Data were recorded after 20 days of culture. Results represent mean □ SD of six cultures, ten replicated experiments. Values denoted by different letters in each column differ significantly at $p < 0.05$.

The regeneration of shoots was excised and cultured on half strength of MS supplemented with 3% sucrose and different concentration of Auxins in IAA (1.76–17/13 µM), IBA (1.32– 14.70 µM) and NAA (1.34–16.61 µM) were used alone. The results were provided in Table. 5. Rooting was noticed in all the concentrations of Auxins used, however, a maximum number of shoots rooted in 6.43 µM IBA at 85% of response (Fig. 1. K) followed by 10.21 µM which produces 80% of response when compared with other concentrations of Auxins at 27 days on medium. Several authors reported that IBA was an effective Auxin in the induction of roots in different ornamental, medicinal and After 30 days of growth, lateral roots were produced from the main root. The rooted plants were transplanted *ex vitro* and raised in pots (Fig. 1. L and Fig. 1. M) containing red soil, vermiculite and farmyard manure in 1:1:1 ratio, kept under greenhouse conditions for one month followed by their field transfer. Approximately 80% of plantlets survived (Fig. 1. N). Regeneration protocols have been standardized for many medicinal plants for their continuous supply and conservation. Available studies also recorded that the regenerated plant material exhibited higher activity of active compounds compared to that recorded for the field collected material.

Table 5: Effect of different Auxins on rooting from *in vitro* Elongated shoots of *Tinospora cordifolia* in half strength MS medium.

IAA (µM)	IBA (µM)	NAA (µM)	No. of roots	Root length (cm)	% of Response
01.76	0	0	4.3 □ 1.1^{d}	3.0□0.4^{bc}	79^{c}
04.32	0	0	4.2□1.1^{ef}	2.9□0.3^{c}	78^{c}
08.56	0	0	4.3□0.3^{d}	2.2□0.1^{ef}	74^{d}
17.13	0	0	4.5□0.4^{c}	3.2□0.2^{a}	79^{c}
0	01.32	0	4.3□1.1^{d}	2.1□1.3^{g}	74^{d}
0	06.43	0	5.2□0.2^{a}	3.2□0.1^{a}	85^{a}
0	10.21	0	5.0□1.1^{bc}	3.2□0.4^{a}	80^{bc}
0	14.70	0	4.7□1.0^{c}	2.3□0.2^{ef}	78^{c}
0	0	01.34	3.2□1.0^{g}	2.1□1.0^{g}	60^{r}
0	0	05.43	4.1□0.3^{f}	2.1□0.1^{g}	60^{r}
0	0	10.22	4.6□1.1^{c}	2.5□0.0^{d}	62^{e}
0	0	16.11	3.5□0.2^{g}	2.8□1.0^{c}	61^{e}

Explants were cultured on half strength MS basal media supplemented with IAA, IBA, NAA and 3% of Sucrose. Data were recorded after 27 days of culture. Results represent mean SD of six cultures, ten replicated experiments. Values denoted by different letters in each column differ significantly at $p < 0.05$.

Conclusion

As a result of the over-exploitation of plant material from natural stands for traditional medicinal purposes, the standardization of the regeneration protocols for *Tinospora cordifolia* medicinal plant was becoming important. The protocols will facilitate conservation of the species and could also serve as an alternative source of materials for use.

Acknowledgement

The regeneration protocol described herein would benefit the conservation of *Tinospora cordifolia*, which was extensively used in traditional medicine.

References

1. Dubey NK, Kumar R, Tripathi P. Global promotion of herbal medicine: India's opportunity. Current science 2004; 86:37-41.

2. Fay MF. Conservation of rare and endangered plants using *in vitro* methods. *In vitro* cellular and Developmental Biology 1992; 28:1-4.
3. Sinha k, Mishra NP, Singh J, Khanuja SPS. *Tinospora cordifolia* (Guduchi), a reservoir plant for therapeutic application: a Review. Indian journal of traditional knowledge 2004; 13:257–270.
4. Singh SS, Pandey SC, Srivastava S, Gupta KS, Patro B, GhoshAC. Chemistry and medicinal properties of *Tinospora cordifolia*. Indian J Pharmacol 2003; 35:83-91.
5. Sivakumar V, Dhana RMS. Antioxidant activity of *Tinospora cordifolia* experimental diabetes. IJPSR 2011; 2:93 95.
6. Hassan AKMS, Roy SK. Micropropagation of *Smilex zeylanica* L., A Perennial climbing medicinal shrub, through axillary shoots proliferation. Bangladesh J Life Sci 2004 16:33-39.
7. Joshi M, Dhar U. *In vitro* propagation of *Saussurea obvallata* (DC.) Edgew. An endangered ethno religious medicinal herb of Himalaya. Plant Cell Rep 2003; 21:933- 939
8. Raha S, Roy SC. *In vitro* plant regeneration in *Holarrhena antidysenterica* Wall. through high frequency axillary shoot proliferation. *In Vitro* Cell. Dev Biol Plant 2001; 37:232–236.
9. Mathur J, Mukuntha K. Micropropagation of *Bauhinia variegata* and *Parkinsonia aculeata* from nodal explants of mature trees. Plant Cell Tiss. Organ Cult 1992; 28:119–121.
10. Kumar R, Sharma K, Agrawal V. *In vitro* clonal propagation of *Holarrhena antidysenterica* (L.) Wall. through nodal explants from mature trees. *In Vitro* Cell Dev Biol Plant 2005; 41:137– 144.
11. Martin G, Geetha S. Raghu PAV, Balachandran I, Ravindran PN. Micropropagation of *Holarrhena pubescens*. J Trop Med Plants 2005; 6:111–117.
12. Hoque MI, Jahan MT, Sarker. RH. *In vitro* shoot regeneration and *ex- vitro* rooting in *Chrysanthemum morifolium ramat.* Plant Tissue Cult 1998; 8:157-164.
13. Shibli AR, Ajlouni MM, Shatnawi AM, Abdullah AB. An effective method for *in vitro* production of disease – free carnation (Dianthus caryophyllus CV. Balady*).* Plant Tissue Cult 1999; 9:159-166.
14. Sarker RH, Islam MR Hoque MI. *In vitro* propagation of neem (*Azadirachta indica* A. Juss.) plant from seedling explants. Plant tissue Cult 1997; 7:125-133.
15. Caboni E, Tonelli MG. Effect of 1,2- benzisoxazole -3-acetic acid on adventitious shoot regeneration and *in vitro* rooting in apple. Plant Cell Rep 1999; 18:985-988.
16. Vanisree M, Lee CY, Lo SF, Nalawade SM, Lin CY, Tsay HS. Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. Bot Bull Acad Sin 2004; 45:1–22.
17. Rout GR. Micropropagation of *Clitoria ternatea.* Linn. (Fabaceae)—An important medicinal plant. *In Vitro* Cell Dev Biol Plant 2005; 41:516–519.
18. Massot B, Milesi S, Gontier E, Bourgaud F, Guckert A. Optimised culture conditions for the production of furanocoumarins by micropropagated shoots of *Ruta graveolens*. Plant Cell Tissue Organ Cult 2000; 62:11–19.
19. Collin HA. Secondary product formation in plant tissue cultures. Plant Growth Regul 2001; 34:119