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Optimized conditions for callus induction, plant regeneration and alkaloids accumulation in stem and shoot tip explants of *Phyla nodiflora*

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Abstract

The present study describes callus induction and the subsequent plant regeneration with alkaloids accumulation in stem and shoot tip explants of *Phyla nodiflora*. Both explants were cultured on different media (MS, B5, SH and WPM) for callus induction. Stem explants showed better callus biomass (dry weight) than shoot tip explants with green compact callus when cultured on MS medium containing 1.5 mg L^{-1} α-naphthalene acetic acid. Shoots were regenerated from the callus on MS medium with 1.5 mg L^{-1} α-naphthalene acetic acid and 1.0 mg L^{-1} benzyl adenine. The rooting of all regenerated shoots was successfully performed on half-strength MS medium with 1.0 mg L^{-1} indole-3-butyric acid. The plantlets were acclimatized and established in soil (90%) and exhibited morphological characteristics similar to those of the mother plant. In addition, the alkaloids content was higher in regenerated callus than intact stem and shoot tip explants, which were analyzed by a gravimetric method, TLC (thin layer chromatography) and HPTLC (high performance thin layer chromatography). The proposed method could effectively be applied for the conservation and clonal propagation to meet the pharmaceutical demands of this medicinally important species.

Additional key words: callus biomass; gravimetric method; growth curve; HPTLC; rooting; TLC.

Resumen

Condiciones optimizadas para inducción de callos, regeneración de plantas y acumulación de alcaloides en explantes de tallos y brotes de *Phyla nodiflora*

El presente estudio describe la inducción de callos y la posterior regeneración de plantas con la acumulación de alcaloides en los explantes de tallos y brotes de *Phyla nodiflora*. Para la inducción de callos, ambos explantes fueron cultivados en diferentes medios (MS, B5, SH y WPM). Los explantes de tallos tuvieron mejor biomasa (peso seco) de callo que explantes de ápices de naturaleza verde compacta cuando se cultivaron en un medio MS con 1,5 mg L^{-1} de ácido α-naftalén acético. Los brotes fueron regenerados a partir de los callos en medio MS con 1,5 mg L^{-1} de ácido α-naftalén acético y 1,0 mg L^{-1} de benciladenina. El enraizamiento de todos los brotes regenerados se realizó con éxito en un medio ½ MS con 1,0 mg L^{-1} de ácido indol-3-butírico. Las plántulas fueron aclimatadas y establecidas en suelo (en un 90%) y mostraron características morfológicas similares a las de la planta madre. Además, el contenido de alcaloides fue mayor en los callos regenerados que en los explantes de tallo y brotes intactos, que fueron analizados por un método gravimétrico, TLC y HPTLC. El método propuesto se podría aplicar con eficacia para la conservación y propagación clonal, a fin de satisfacer la demanda farmacéutica de esta especie medicinalmente importante.

Palabras clave adicionales: biomasa de callo; curva de crecimiento; enraizamiento; HPTLC; método gravimétrico; TLC.

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Abbreviations used: BA (benzyl adenine); BFC (brown friable callus); DMRT (Duncan's multiple range test); GCC (green compact callus); GFC (green friable callus); HPTLC (high performance thin layer chromatography); IAA (indole acetic acid); IBA (indole-3-butyric acid); KN (kinetin); NAA (naphthalene acetic acid); PGR (plant growth regulator); WFC (white friable callus); WPM (woody plant medium); WWC (white watery callus); 2,4-D (2,4-dichlorophenoxy acetic acid).

Introduction

Phyla nodiflora (L.) Greene (= *Lippia nodiflora* (L.) Mihex) belongs to Verbenaceae family, which is widely distributed in South Africa and Central America (Terblanche and Kornelius, 1996). It is a runner plant with scanty roots possessing various ethanobotanical and medical applications in adenopathy, chronic indolent ulcers, etc. (Kirtikar and Basu, 1975). It has been used as a traditional and Unani system of medicine to treat diarrhea, pain in knee joints, lithiasis, cold, and fever, which also has been used for inducing bowel moments in the stomachic patients (Nadkarni and Nadkami, 1954). *P. nodiflora* contains flavonoids, sugars, sterols, essential oils, resins, tannins and other medicinally valuable constituents. In addition, the compounds halleridone and hallerone serve as anti-cancer, anti-tumor, anti-malarial, anti-fungal and other cytotoxic activities (Ravikanth *et al.,* 2000). The aerial parts of this plant are used as anodyne, antibacterial, diuretic, emmenagogue, parasiticide, refrigerant and febrifuge agents (Agarwal, 1997). Several researchers have reported various pharmacological properties including antispasmodic, hypotensive, anti-inflammatory, analgesic, antipyretic (Forestieri *et al.,* 1996), antibacterial, antinociceptive, antifungal, antioxidant and free radical scavenging activities (Shukla *et al.,* 2009). *P. nodiflora* extracts have been used to cure multiple skin diseases and hair afflictions (Abbasi *et al.,* 2010).

This plant is over-exploited due to its high medicinal value and hence, propagation of this plant by tissue culture may be mandatory, which offers a greater potential to deliver large quantities of disease-free, true-to-type healthy stock within a short span of time (Hussain *et al.,* 2001). While, the systematic manipulation of media, phytohormone concentrations and selection of suitable explants are among several key factors that control the process of shoot regeneration from callus; the callus-mediated regeneration protocol is a critical requirement as it allows exploitation of *in vitro* selection, somaclonal variation and genetic engineering techniques, which aims at the genetic improvement of plants (Georges *et al.,* 1993). Since the last decade, very few *in vitro* studies have been done in this genus; the latest studies have been performed on the propagation of *Lippia junelliana* and *Lippia alba* (Juliani *et al.,* 1999; Gupta *et al.,* 2001). Direct shoot propagation using axillary node explants has been implemented in our previous studies and somatic embryogenesis was observed to be very successful in this medicinal plant (Ahmed *et al.,* 2005 and 2011). The present study is an advancement over the earlier protocol, because it describes the hormonal regulation, callus initiation, regeneration and alkaloid accumulation from *in vivo* stem and shoot tip explants of *P. nodiflora*. In addition, this study deals with the development of a rapid regeneration system, the quantification of alkaloids from stem and shoots tip callus (indirect organogenesis) and the subsequent transplantation of the plantlets to natural environmental conditions.

Material and methods

Plant material and sterilization

Healthy, young stem (shoot internodal segment without buds) and shoot tips of *P. nodiflora* were collected from field grown plants and maintained in the Department of Plant Science Garden at Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. First, both type of explants were washed thoroughly in running tap water 3-5 times, including 2% (v/v) Teepol (Reckitt Benckiser, India) for 10 min; then washed with 70% ethanol for 3 min followed by another wash with 0.1% $HgCl_2$ for 2 min. Prior to inoculation, explants were washed three times with sterile distilled water.

Callus induction

Stem and shoot tip explants of *P. nodiflora* were grown in MS medium (Murashige and Skoog, 1962), SH medium (Schenk and Hildebrandt, 1972), B5 medium (Gamborg *et al.,* 1968) and WPM (woody plant medium) (Lloyd and McCown, 1980) supplemented with 1.0-2.5 mg L^{-1} of NAA (naphthalene acetic acid), 2,4-D (2,4-dicholorophenox acetic acid), IBA (indole butyric acid) and IAA (indole acetic acid); 1.0-3.0 mg L^{-1} of BA (benzyl adenine) and KN (kinetin) were used for callus induction. The callus culture was maintained at 25 ± 2 °C, 16/8 h (light/dark) of photoperiod with 25 µmol m^{-2} s^{-1} of light intensity. The role of media on the nature and biomass of callus was studied in stem and shoot tip explants of *P. nodiflora*.

In vitro callus growth curve

Callus cultures were optimized and evaluated quantitatively for their nature, biomass and alkaloid content at

the end of their respective growth cycle based on a previous protocol (Ahmed *et al.,* 2010). By treating with various concentrations of auxins, fresh and dry weights of the calli were determined at 15, 25, 35, 45 and 55 days. At regular interval for all the treatments, each callus was harvested by careful separation from media using metal spatulas, and fresh and dry weight was promptly recorded.

Extraction and isolation of alkaloids

For the extraction of alkaloids, 5 g of dried intact stem and shoot tips of *P. nodiflora* and *in vitro* regenerated calli were extracted with methanol 5 times (Rehman *et al.,* 2003). The collected methanol extract was centrifuged at 5000 ×g for 10 min at room temperature, and the methanol supernatant carefully pipetted out into fresh eppendorf tubes and subjected to the determination of alkaloids.

For gravimetric analysis, 5 g of the intact stem, shoot tip and callus were taken in 250 mL beaker individually, then 200 mL of ethanol containing 10% acetic acid was added, covered and allowed to stand for 4 h. After filtration, the filtered extract was concentrated on a water bath and then concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was completed. The precipitate was washed with dilute ammonium hydroxide, filtered once again and dried. The dried residue was weighed and used as alkaloid standard (Harborne, 1973).

TLC and HPTLC analysis

Methanol extract of intact plant and regenerative callus raised from stem and shoot tip explants were analyzed by TLC (thin layer chromatography) and HPTLC (high performance thin layer chromatography). TLC was performed using silica gel G with MeOH:$CHCl_3$ (20:80) as solvent system. After loading the extract, Dragendorff's reagent was applied to the TLC plate for the determination of alkaloids. The isolated gravimetric alkaloid residue standard was compared with respectable R_f (ratio-to-front) value of the sample. In addition, methanol extracts (20 µL) were applied in the Camag (Switzerland) HPTLC system assisted with sample applicator Linomat IV for quantification of alkaloids. The mobile phase of chloroform:methanol:acetic acid (8:1.5:0.5) was suitable for separation of alkaloids and scanned at 200 nm by the use of TLC scanner III and the supporting software CAT 4.0.

In vitro callus regeneration

For organogenesis, fresh stem and shoot tip calli were placed in respective culture tubes containing MS medium supplemented with different concentrations of BA (1.0-3.0 mg L^{-1}) or KN (1.0-3.0 mg L^{-1}) with NAA (1.5 mg L^{-1}) and 2,4-D (2.0 mg L^{-1}) was used. During the experiment, to avoid phenolic excretion from the callus, 100 mg L^{-1} of ascorbic acid was added to the MS media and subcultured every week up to callus regeneration.

Induction of rooting and acclimatization

Callus regeneration plantlets (60-70 mm) were kept in either quarter or half or full strength MS medium with IAA and IBA (0.1-3.0 mg L^{-1}) for *in vitro* rooting. Six-week-old well-rooted plantlets were removed from the culture tubes and washed to make it free of agar. The plantlets were transferred to plastic pots (5 cm diameter) containing red soil and vermiculite (1:3) and maintained at 25 ± 2 °C, 16 h day length (35-50 m EM^{-2} s^{-1}) and at 75-80% relative humidity. Then the grown plants were transplanted to earthenware pots (10 cm diameter) containing natural soil, kept under shade for 2 weeks and finally moved to the garden.

Statistical analysis

All the experiments were repeated thrice and used 30 replicates. The effect of different treatments was quantified as mean ± SE and the data were subjected to statistical analysis using Duncan's multiple range test (DMRT) at 5% level significance (Gomez and Gomez, 1976).

Results and discussion

Callus induction and growth curve analysis

In all media tested, the callus initiation did not occur without PGRs (plant growth regulators, control) in stem and shoot tip explants (data not shown). The suitable callus induction was obtained in 2,4-D and NAA on MS, B5, SH and WPM which induced GCC (green compact callus), GFC (green friable callus), WFC (white friable callus), WWC (white watery callus) and BFC (brown friable callus) in stem and shoot tip explants (Fig. 1a-b and Fig. 2a-h). For a successful callus

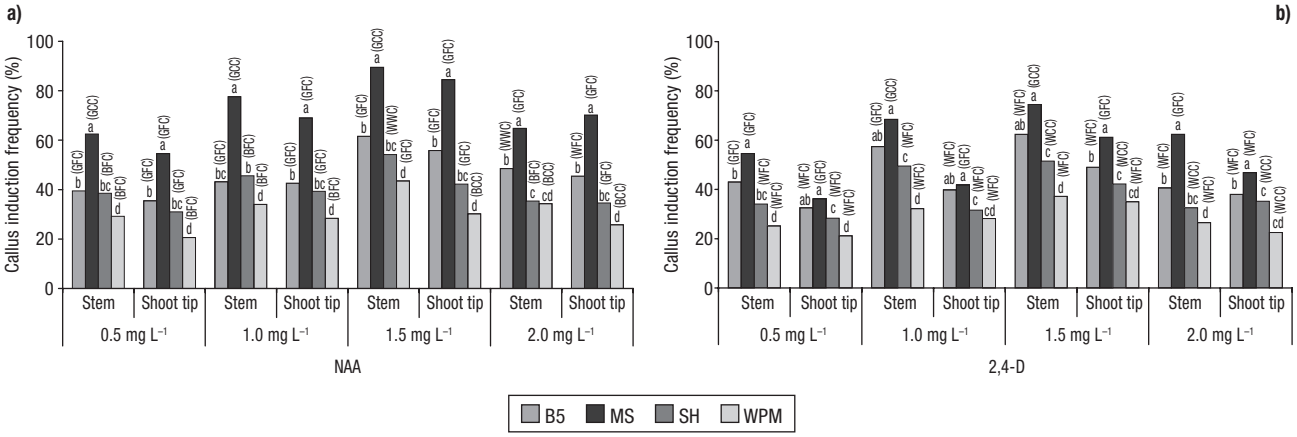

Figure 1. Effect of different media (B5, MS, SH, WPM) supplemented with a) NAA (naphthalene acetic acid) and b) 2,4-D (2,4-dichlorophenoxy acetic acid) on induction of different callus nature and their frequency on stem and shoot tip explants of *Phyla nodiflora* after 45 days incubation. BCC: brown compact callus; BFC: brown friable callus; GCC: green compact callus; GFC: green friable callus; WFC: white friable callus; WWC: white watery callus.

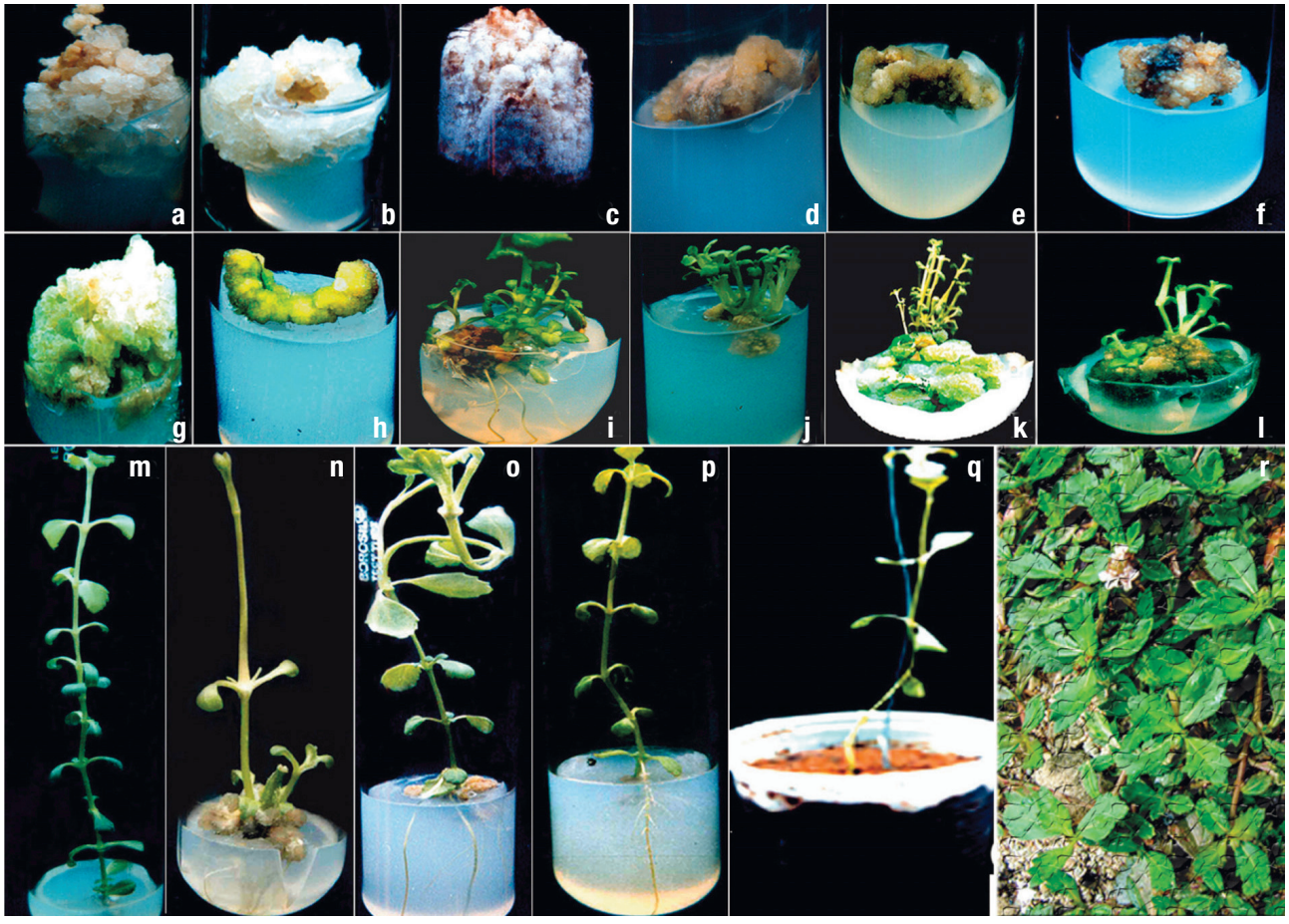

Figure 2. a-b) White watery callus (WWC). c-d) White friable callus (WFC). e-f) Brown friable callus (BFC) in stem-shoot tip explants. g) Green friable callus (GFC) in shoot tip explant. h) Green compact callus (GCC) in stem explant. i-j) Shoot regeneration in shoot tip explant. k-l) Shoot regeneration in stem explant. m) Shoot elongation in stem explant. n) Rooting in MS with IBA (indole-3-butyric acid). O) ½ MS with IBA. p) ¼ MS with IBA in stem explant. q) Hardening. r) Field grown plants.

induction, factors such as type of explants, PGRs, culture media and cultural conditions are very important (Yeoman and Yeoman, 1996). The stem explants produced GCC with higher induction frequency (92%) than shoot tip explants (83%) (Fig. 1a-b). Among the various concentrations of auxins tried in callus induction, NAA (1.5 mg L^{-1}) and 2,4-D (2.0 mg L^{-1}) significantly induced GC natured callus with a maximum biomass at 45 days in terms of fresh and dry weight (Fig. 2h; Fig. 3a-b). In our study, callus biomass progressively increased with an increase in the NAA concentration (>0.5-3.0 mg L^{-1}) and especially when the culture medium contained 3.5-5.0 mg L^{-1} NAA, WFC and WWC were induced significantly (data not shown). In addition, it was observed that IAA and IBA drastically reduced the callus biomass resulting in WFC natured calli all the media. These results are well authenticated with the previous reports of Ahmed *et al.* (2011), who demonstrated that NAA and 2,4-D were suitable in the callus induction of *P. nodiflora*.

P. nodiflora callus growth curve was sigmoid type and four growth phases can be distinguished in different days (15, 25, 35, 45 and 55 days). In the lag phase (15-25 days), callus initiation and proliferation were observed by profound cell division (Leticia *et al.,* 2010). At 25-35 days (exponential phase), biomass of the GCC natured was significantly increased. The high level of callus biomass in the stationary phase (45 days) of the callus growth curve suggests the cellular membrane stabilization. It has been previously reported that the stationary phase callus evidently demonstrated an increase in the accumulation of gaggaminine in the callus (GC) of *Cynanchum wilfordii* (Shin *et al.,* 2003). At the decline phase (55 days) the callus biomass was drastically reduced as compared to other phases.

Gravimetric, TLC and HPTLC analysis

The identification and quantification of alkaloids was done in gravimetric, TLC and HPTLC studies. Gravimetric analysis showed that the alkaloid content (26.2 mg g^{-1}) was higher in the media containing NAA (1.5 mg L^{-1}) with BA (2.0 mg L^{-1}), which was greater than intact stem explants followed by shoot tip explants (Table 1). However, the alkaloid content was significantly reduced in other PGRs treatments for callus (Table 1). Methanol extracts of intact and regenerative stem and shoot tip explants showed an additional band (0.75) with a Rf value nearing the standard alkaloid (0.78) (figure not shown). The above Rf value was conformed in the presence of yellow fluorescence, when the TLC plate was sprayed with Dragendorff's reagent. On the other hand, intact and regenerative callus from stem and shoot tip explants were further analyzed by HPTLC for the quantification alkaloids and compared with the alkaloids in gravimetry (Table 1). In HPTLC, the sample curve for the alkaloid content was linear, where the correlation coefficient indicated good linearity between concentration and area. To ascertain the purity of sample peaks, the test sample was analyzed in reflectance spectra. The alkaloid content was significantly increased in regenerative callus of stem explants (5.5 mg g^{-1}) followed by shoot tip explants

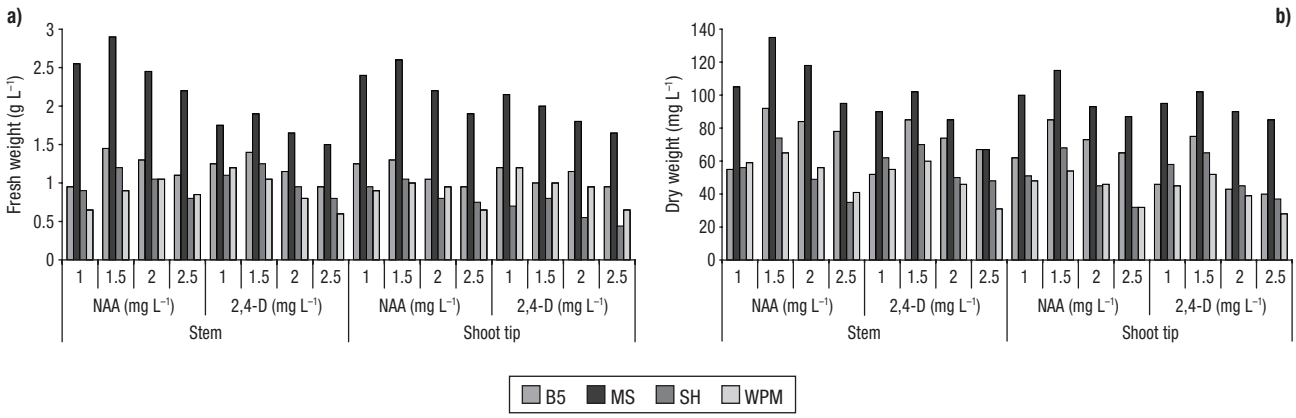

Figure 3. Effect of different media (B5, MS, SH, WPM) supplemented with 2,4-D (2,4-dichlorophenoxy acetic acid) and NAA (naphthalene acetic acid) on induction of callus biomass [(a) fresh and b) dry weight] on stem and shoot tip explants of *Phyla nodiflora* after 45 days incubation.

Table 1. Effect of plant growth regulators on shoot regeneration and alkaloid content from induced calli of stem and shoot tip explants of *Phyla nodiflora* cultured on MS medium after 60 days

Plant growth regulators (mg L^{-1})[1]	Percentage of response (%)		Regenerated shoot number Mean ± S.E		Shoot length (cm) Mean ± S.E		Alkaloid content			
							Gravimetric analysis (mg g^{-1})		HPTLC (mg g^{-1})	
	Stem	Shoot tip	Stem	Shoot tip	Stem	Shoot tip	Stem	Shoot tip	Stem	Shoot tip
NAA + BA										
1.0 + 1.0	61.5^{b}	44.2^{b}	6.6 ± 0.42^{ab}	6.2 ± 0.24^{bc}	2.5 ± 0.26^{b}	2.4 ± 0.16^{c}	–	–	–	–
2.0	77.4^{a}	56.8^{a}	8.6 ± 0.19^{a}	8.4 ± 0.18^{a}	3.0 ± 0.19^{a}	3.0 ± 0.22^{ab}	19.75	16.20	3.94	2.89
3.0	59.5^{bc}	40.0^{bc}	4.2 ± 0.22^{c}	7.0 ± 0.20^{b}	2.0 ± 0.24^{bc}	3.4 ± 0.17^{a}	–	–	–	–
NAA + BA										
1.5 + 1.0	67.2^{bc}	59.6^{bc}	8.7 ± 0.17^{b}	5.4 ± 0.14^{c}	3.0 ± 0.14^{bc}	2.2 ± 0.14^{c}	–	–	–	–
2.0	85.6^{a}	82.4^{a}	12.3 ± 0.14^{a}	9.2 ± 0.22^{a}	4.2 ± 0.19^{a}	3.5 ± 0.19^{a}	26.26	19.10	5.52	4.16
3.0	72.4^{b}	68.2^{b}	7.2 ± 0.26^{bc}	8.0 ± 0.18^{ab}	3.5 ± 0.22^{b}	3.2 ± 0.10^{ab}	–	–	–	–
NAA + BA										
2.0 + 1.0	58.1^{c}	74.8^{ab}	6.1 ± 0.26^{ab}	5.2 ± 0.19^{b}	2.6 ± 0.10^{b}	2.8 ± 0.14^{b}	–	–	–	–
2.0	69.4^{a}	80.0^{a}	7.4 ± 0.18^{a}	6.8 ± 0.28^{a}	3.4 ± 0.24^{a}	3.5 ± 0.19^{a}	15.21	12.78	3.29	1.48
3.0	67.5^{ab}	66.4^{c}	4.8 ± 0.32^{c}	5.0 ± 0.22^{bc}	2.0 ± 0.16^{bc}	2.2 ± 0.16^{bc}	–	–	–	–
NAA + KN										
1.0 + 1.0	72.5^{b}	72.2^{a}	2.8 ± 0.16^{b}	5.8 ± 0.20^{ab}	2.5 ± 0.12^{b}	1.8 ± 0.14^{bc}	–	–	–	–
2.0	76.4^{a}	68.8^{ab}	5.6 ± 0.19^{a}	7.0 ± 0.14^{a}	3.6 ± 0.22^{a}	3.4 ± 0.10^{a}	7.89	5.45	0.94	0.58
3.0	69.3^{bc}	52.2^{c}	2.5 ± 0.22^{bc}	4.4 ± 0.19^{c}	2.2 ± 0.16^{bc}	2.0 ± 0.16^{b}	–	–	–	–
NAA + KN										
1.5 + 1.0	68.4^{ab}	60.0^{b}	4.2 ± 0.12^{bc}	4.0 ± 0.14^{c}	1.8 ± 0.14^{bc}	1.9 ± 0.10^{c}	–	–	–	–
2.0	72.2^{a}	68.4^{a}	6.8 ± 0.10^{a}	5.2 ± 0.16^{a}	2.2 ± 0.20^{b}	3.6 ± 0.14^{a}	5.26	4.11	0.75	0.86
3.0	64.5^{c}	52.6^{bc}	5.6 ± 0.21^{b}	4.8 ± 0.19^{ab}	2.8 ± 0.19^{a}	3.2 ± 0.22^{ab}	–	–	–	–
NAA + KN										
2.0 + 1.0	44.5^{c}	45.4^{b}	6.1 ± 0.14^{b}	4.8 ± 0.21^{ab}	1.2 ± 0.14^{bc}	2.2 ± 0.19^{b}	–	–	–	–
2.0	67.4^{ab}	62.8^{a}	7.4 ± 0.10^{a}	5.4 ± 0.14^{a}	1.8 ± 0.22^{a}	2.8 ± 0.20^{a}	7.34	5.60	0.82	0.90
3.0	78.5^{a}	36.2^{bc}	5.8 ± 0.14^{bc}	3.2 ± 0.18^{c}	1.4 ± 0.19^{b}	1.6 ± 0.22^{bc}	–	–	–	–

[1] NAA: naphthalene acetic acid; BA: benzyl adenine; KN: kinetin. Values are mean of 30 replicates per treatment and repeated trice. Values with the same superscript are not significantly different at 5% probability level according to Duncan.

(4.1 mg g^{-1}); the results were compared to the intact stem and shoot tip explants (Table 1; Fig. 4a-d). However, few callus extracts showed the addition peaks which deviated from intact plant extracts and that have been identified for the presence of other compounds. These results were correlated with the gravimetric, HPTLC and HPLC analysis of poly-herbal combinations and compared with respective biomarker compounds (Joshi *et al.,* 2007).

In vitro callus regeneration

Successful callus regeneration was obtained by the use of BA (2.0 mg L^{-1}) and NAA (1.5 mg L^{-1}), which increased the shoot number (12.3 shoots $stem^{-1}$) and shoot length (4.20 cm) followed by shoot tip explants (9.6 shoots $shoot\ tip^{-1}$) and shoot length (3.5 cm) derived callus from stem of *P. nodiflora* after 60 days (Table 1; Fig. 2i-k). However, BA + 2,4-D and KN + 2,4-D combinations drastically reduced the callus regeneration in stem and shoot tip explants (data not shown). It was observed that the frequency of regenerated shoots per culture varied by 29.2-85.6% in stem and 17.4-82.4% in shoot tip derived calluses respectively (Table 1). In addition, various combinations of BA and KN were tried for callus regeneration, which resulted in less shoot numbers and stunted shoot length than with other auxins and cytokinins combination (figure not shown). During the callus regeneration, the

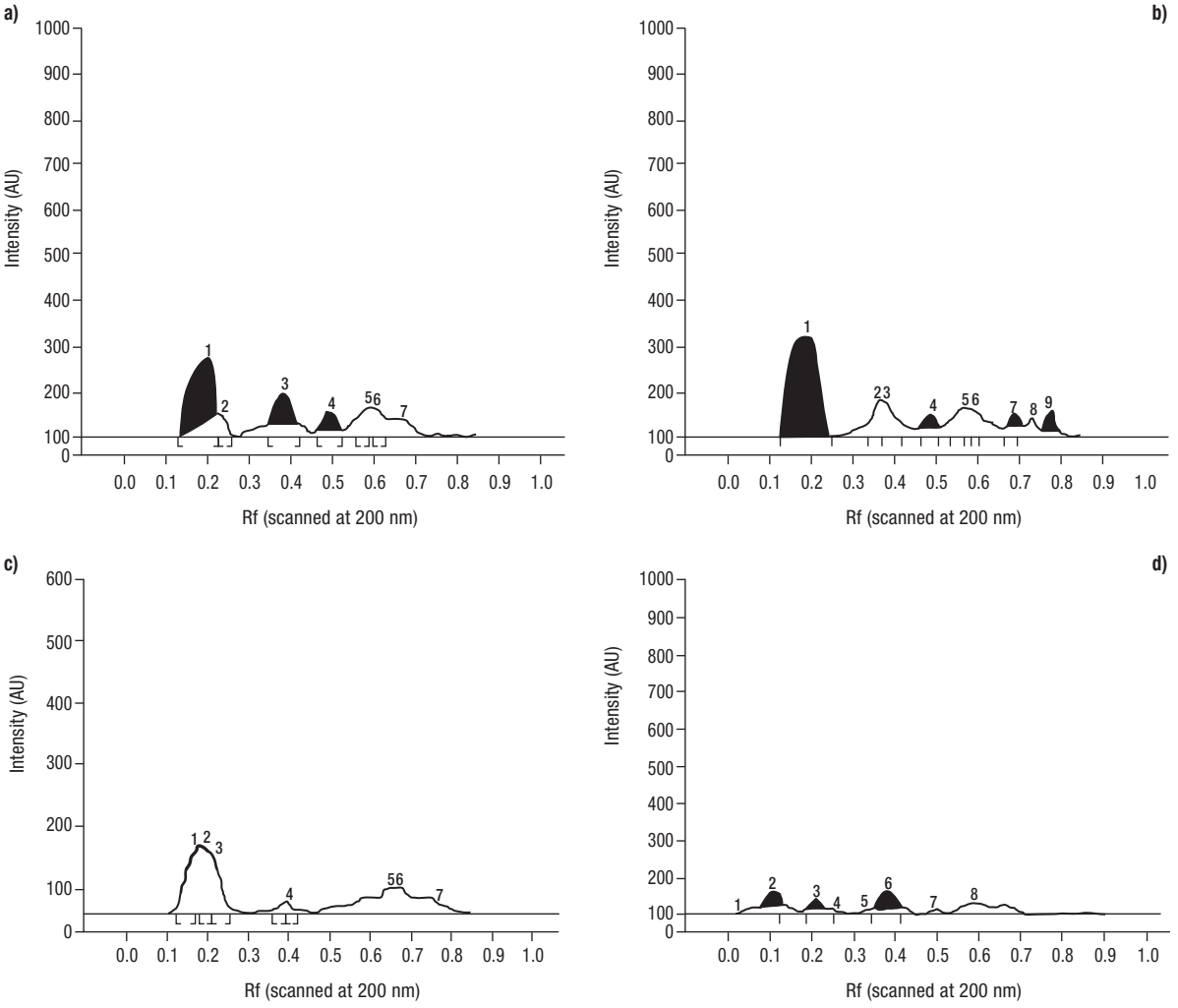

Figure 4. Alkaloids determined by HPTLC (high performance thin layer chromatography) in intact and *in vitro* regenerated callus from stem and shoot tip explants of *Phyla nodiflora*: a) intact stem explants; b) *in vitro* regenerated callus (MS + 1.5 mg L^{-1} NAA + 2.0 mg L^{-1} BA) from stem explants; c) intact shoot tip explants; d) *in vitro* regenerated callus (MS + 1.5 mg L^{-1} NAA + 2.0 mg L^{-1} BA) from shoot tip explants. BA: benzyl adenine; NAA: naphthalene acetic acid; Rf: ratio-to-front; AU: absorption unit.

batch callus culture was continuously examined by taking a subculture at weekly intervals to prevent the cell death and browning of media. These findings are well supported by Patel and Shah (2009), who reported the induced callus regeneration by NAA with BA in the nodal explants of *Stevia rebaudiana*.

In vitro rooting and acclimatization

Regenerated leafy shoots derived from callus of both stem and shoot explants did not show any root induction, when they were transferred to MS media without PGRs (data not shown). Roots were formed on excised shoots grown on half-strength MS media containing either IAA or IBA (Fig. 5). A high percentage of root induction (72.7%) was obtained in MS medium with 1.0 mg L^{-1} IBA in stem explants after 35 days (Fig. 2o; Fig. 5a-b), in comparison to IAA. The present results indicate that the stem explants showed better root induction than shoot tip explants when the culture medium was supplemented with 0.4-1.4 mg L^{-1} of IAA and IBA. Further, the root length of the stem explants was higher in half-strength MS medium containing 1.0 mg L^{-1} IBA, when compared to full and quarter strength MS medium (Fig. 2n-p; Fig. 5c). However, a stronger inhibition of root length by IAA treatment may be responsible for the poorer rooting responses respect to

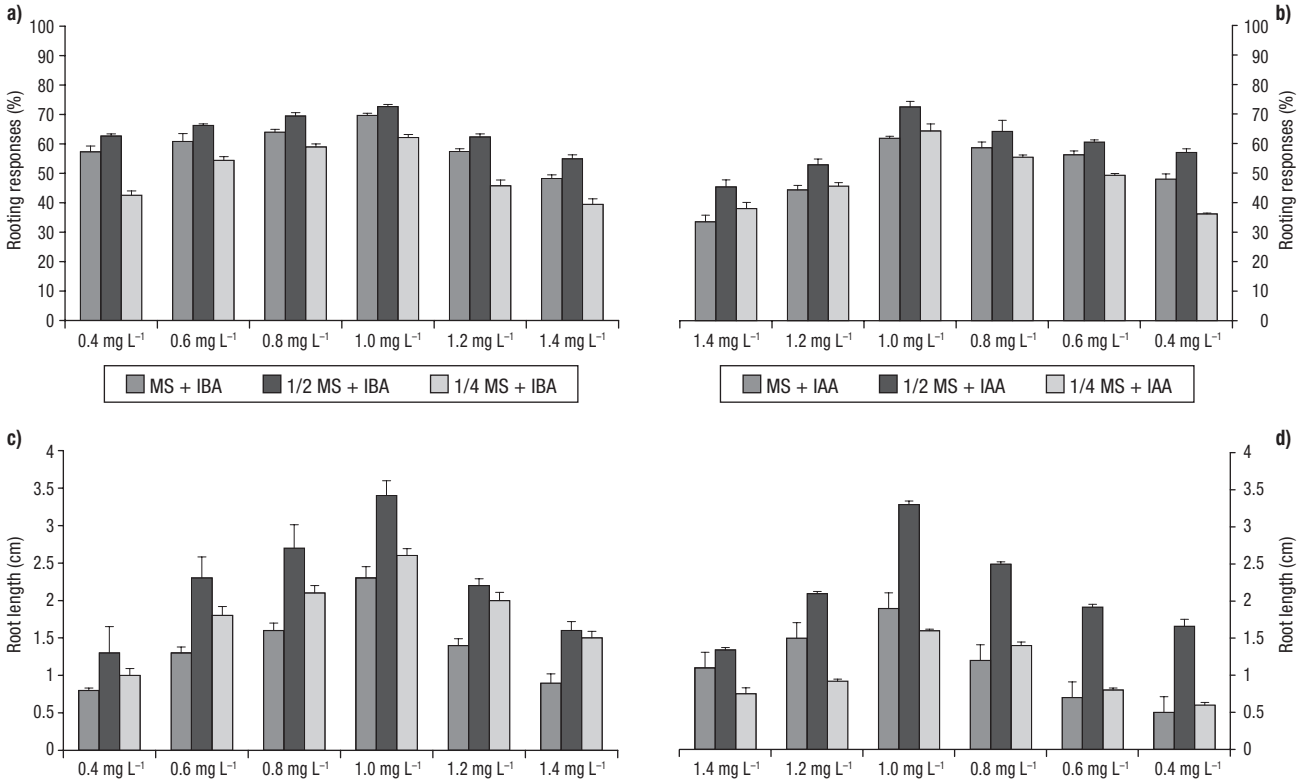

Figure 5. Effect of MS, ½ MS and ¼ MS medium with IBA (a and c) and IAA (b and d) on rooting response and root length per explant in the stem explants of *Phyla nodiflora.* IBA: indole-3-butyric acid; IAA: indole acetic acid.

IBA treatment (Fig. 5d). Balaraju *et al.* (2008) observed successfully rooted plantlets of *Vitex agnus-castus* (Verbenaceae) on half-strength MS medium containing IBA (0.1 mg L^{-1}). The present findings of rooting and acclimatization were similar to our previous studies of *P. nodiflora* (Ahmed *et al.,* 2005). After one month of transfer, the rooted plantlets were transferred into pots containing soil, sand and well rotted cow-dung manure, and shifted to a green house for acclimatization (Fig. 2q), where they grew normally (Fig. 2r).

Conclusions

Successful callus regeneration was optimized from stem explants in the combinations of BA and NAA. The best GCC induction on MS medium with NAA (1.5 mg L^{-1}) in the stem explants were optimum for plant regeneration. In this study, the alkaloids content was superior in regenerative callus than intact stem, shoot tip explants, as confirmed by gravimetric and HPTLC. Our data on the effects of PGRs in *P. nodiflora* batch culture will be useful in enhancing the accumulation of alkaloid compounds in such cells and can also be further extended to other plant culture models.

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