BRIEF COMMUNICATION

Indirect regeneration of *Withania somnifera* and comparative analysis of withanolides in *in vitro* and greenhouse grown plants

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Abstract

The present study reports an efficient protocol for indirect shoot organogenesis and plantlets regeneration of *Withania somnifera* (L.) Dunal. Leaf explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). The highest callus induction rate (89.5 %) and shoot regeneration rate (92 %) were obtained when 2 mg dm^{-3} BAP was combined with 0.5 mg dm^{-3} IAA. Three major withanolides (withaferine A, 12-deoxywithastramonolide and withanolide A) were investigated in different plant organs from *in vitro* and greenhouse grown plants. Leaves contained higher contents of withanolides and phenolics than roots or stems, whereas roots contained the highest contents of flavonoids and polysacharides. *In vitro* grown plants contained greater contents of phenolics, flavonoids and polysaccharides while lower contents of withanolides than greenhouse grown plants.

Additional key words: DPPH, flavonoid, phenolics, polysaccharides, shoot organogenesis, withaferine.

Withania somnifera (L.) Dunal, *Solanaceae*, is a reputed medicinal shrub recognized as Indian ginseng. The medicinal properties are attributed to a group of steroids called withanolides which are ergostane type steroids with atoms C-22 and C-26 bridged by a δ-lactone and an oxidized C-1 position. Besides a thin-layer chromatography (TLC) method for the quantification of withaferin A (Gupta *et al*. 1996), few HPLC methods are described in literature (Bessalle and Lavie 1987, Hunter *et al*. 1979, Banerjee *et al*. 1994). Recently, a quantification of withanolide D and withaferine A was developed using HPLC (Ganzera *et al*. 2003).

Withania propagation by seeds is difficult because of low seed germination percentage even after stratification. Previous reports on *in vitro* propagation of *Withania* include multiple shoot formation from axillary buds (Roja *et al*. 1991), micropropagation from shoot tips and nodal explants (Sen and Sharma 1991), androgenesis (Vishnoi *et al*. 1979), direct regeneration from leaf explants (Kulkarni *et al*.1996) and from node, internode, hypocotyl and embryo (Kulkarni *et al*. 2000) and indirect shoot regeneration from internode explants (Manickam *et al*. 2000). However, these studies reported low regeneration frequency not exceeding 65 %. The aim of present study was to elaborate an efficient protocol for indirect shoot regeneration of *Withania* using leaf explants and to apply liquid chromatography-mass spectrometry (LC-MS) for the detection of three major withanolides (withaferin A, 12-deoxywithastramonolide and withanolide A). We investigated the distribution of withanolides, total contents of polyphenolic, flavonoids, and polysaccharides in different parts from plants grown *in vitro* and in greenhouse. We also examined the antioxidant capacity employing 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging in order to under-stand the usefulness of *Withania* in medicine.

Withania somnifera (L.) Dunal seeds were collected from local nurseries in western India. Seeds were surface

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Abbreviations: BAP - 6-benzylaminopurine; DPPH - 1,1-diphenyl-2-picrylhydrazyl; HPLC - high-pressure liquid chromatography; IAA - indole-3-acetic acid; LC-MS - liquid chromatography-mass spectrometry; TLC - thin-layer chromatography.
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sterilized and inoculated in Petri dishes each containing 20 cm^{-3} of Murashige and Skoog (1962; MS) medium with 2 % (m/v) sucrose and 0.8 % (m/v) agar. The pH of the medium was adjusted to 5.8 before autoclaving for 20 min at 121 °C. The cultures were incubated at temperature of 25 °C and 16-h photoperiod with irradiance of 40 μmol(photon) m^{-2} s^{-1} provided by cool white fluorescent tubes. Young leaves of *in vitro* grown *Withania* seedlings were cut into segments (0.5 × 0.5 cm) with a portion of mid rib. The leaf segments were cultured on MS medium supplemented with 3 % sucrose and various concentrations and combinations of 6-benzyl-aminopurine (BAP; 0.2, 0.5, 1.0 and 2.0 mg dm^{-3}) and indole-3-acetic acid (IAA 0.0, 0.5 and 1.0 mg dm^{-3}). All media were solidified with 0.8 % agar and the pH was adjusted to 5.8 before autoclaving. Explants were cultured in 100 ×15 mm sterile Petri dishes containing 20 cm^{-3} medium and maintained for 6 weeks at irradiance of 40 μmol m^{-2} s^{-1} (16 h d^{-1}). The callus induction rate, characteristics of developed callus and shoot regeneration rate were recorded after 6 weeks of culture. *Withania* shoots were separated and cultured on MS medium without hormones for 4 weeks for their subsequent growth and rooting. Plantlets at the 3 - 4 leaf stage were transplanted into culture pots filled with sterilized peatmoss. The plantlets were grown in growth chamber for 1 week before their transfer to the greenhouse. The environment in the growth chamber was adjusted to a 25 ± 2 °C air temperature, 40 - 50 % relative humidity and irradiance of 100 μmol m^{-2} s^{-1} (16 h d^{-1}).

Leaf, stem and roots of *Withania* collected 6 months after transfer of *in vitro* regenerated plants to the greenhouse were used for this study. Finely powdered plant material (1 g) was extracted three times with 3.0 cm^{-3} of 80 % methanol for 10 min by sonication. After centrifugation (5 min at 4 000 *g*), the extracts adjusted to the final volume 10 cm^3 with MeOH. Three additional calibration levels were prepared by diluting this solution with MeOH. Standard solutions were stored at 4 - 8 °C and were stable for at least 30 d (confirmed by re-assaying the solution). HPLC analysis was performed on a *Waters Alliance 2795* HPLC system, equipped with a photodiode array detector (*Waters*, Milford, MA, USA). For all separations a *XTerra MSC*$_{18}$ (21 × 150 mm, 5 mm particle size) was used. The mobile phase consisted of acetonitrile + 0.1 % phosphoric acid (flow rate 0.2 cm^{-3} min^{-1}). Peaks were assigned according to authentic samples of withaferin A, 12-deo-xywithastramonolide and withanolide A, and comparison of the UV-spectra and retention times. For peak confirmation an LC-MS experiment, using a *Waters Micromass ZQ*, together with the HPLC system was performed. Best results were obtained in positive ESI mode, with ionization voltage set to 30 V, source voltage to 3.0 kV and probe temperature to 250 °C. With this instrumentation the separations had to be performed at ambient temperature.

The content of total phenolics was analyzed spectrophotometrically according to Folin and Ciocalteu (1927) and total flavonoid content was determined according to Jia *et al*. (1999). After withanolides extraction with methanol, the sediment was collected and desiccated in an oven at 60 °C. The sediment of 0.2 g was resuspended in 5 cm^{-3} 5 % (v/v) sulphuric acid and placed for 2 h in boiling water. After acidic hydrolysis, the liquid-solid mixture was diluted to 50 cm^3 with distilled water. The supernatant was separated by sedimentation, and the polysaccharide in the supernatant assayed according to the carbazole reaction as follows. A sample of 0.2 cm^3 taken from the above supernatant was mixed with 6 cm^3 concentrated sulphuric acid, held in a boiling water bath for 20 min, and cooled. Then, 0.2 cm^3 carbazole in absolute ethanol (0 - 15 %, v/v) was added and the contents mixed vigorously. After a reaction time of 2 h in dark at room temperature, a purplish red colour developed and absorbance was measured at 530 nm. *D*-galacturonic acid (0, 50, 100, 200, 400 and 600 mg cm^{-3}) was used as a standard. The antioxidant activity of each extract was determined by using DPPH radicals according to Hatano *et al*. 1988. Experiments were set up in a completely randomized design and repeated twice. Data were subjected to Duncan's multiple range test using *SAS* program (version *6.12*, *SAS Institute, Inc.*, Cary, USA).

Callus induction and shoot regeneration occurred when explants were cultured on medium containing BAP alone or in combination with IAA (Table 1). However, the frequency of callus induction and shoot regeneration was decreased when BAP was applied alone. The highest callus induction rate (89.5 %) and shoot regeneration rate (92 %) were obtained when 2 mg dm^{-3} BAP was combined with 0.5 mg dm^{-3} IAA. The frequency of shoot regeneration decreased and root formation increased with increased IAA concentration to 1.0 mg dm^{-3}. This may be due to high endogenous auxin levels which lead to a decrease in the percentage response of explants to form shoot buds. Similar findings were also reported by Kulkarni *et al*. 1996. Nodular calli were observed on the leaf segments after 2 weeks of culture on MS medium supplemented with 2.0 mg dm^{-3} BAP and 0.5 mg dm^{-3} IAA. Small green meristems were visible on the surface of calli and were later developed into shoot clusters upon their subculture to hormone-free MS medium (Fig. 1*A*,*B*). The regenerated shoots *via* indirect shoot organogenesis (1 - 2 cm in length) were separated from the explants and cultured for 4 weeks on hormone-free MS medium for their rooting and 100 % of plantlets were rooted (Fig. 1*C*). *Withania* plantlets (3 - 5 cm) at the 3 - 4 leaf stage were then grown in growth chamber for 1 week before transfer to the greenhouse. The plantlets were acclimatized successfully (Fig. 1*D*) with 100 % survival rate.

Three major withanolides (withaferin A, 12-deoxy-withastramonolide and withanolide A) were separated and all compounds could be baseline separated in less than 35 min after all separation parameters were carefully assessed. Additionally, an LC-MS experiment was performed to confirm the identity of the peaks of interest. With modifications in the solvent composition and flow

Table 1. Callus induction rate, characteristics of developed callus and shoot regeneration rate of *Withania somnifera* after 6 weeks in culture. Mean separation within columns by Duncan's multiple range test at 5 % level. PGR - plant growth regulators.

PGR [g dm^{-3}] BAP	IAA	Callus induction [%]	Callus colour	consistency	growth	rooting	Shoot regeneration [%]
0.2	0	21.0 d	whitish-green	soft	+	-	10.3 e
0.5	0	32.0 d	whitish-green	soft	+	-	12.9 e
1.0	0	59.9 c	whitish-green	soft	+	-	28.9 d
2.0	0	56.3 c	whitish-green	soft	++	-	29.8 d
0.2	0.5	54.3 c	whitish-yellow	soft	++	+	43.6 c
0.5	0.5	59.0 c	yellow-green	soft-hard	++	-	65.8 d
1.0	0.5	89.0 a	green	soft-loose	+++	-	87.0 a
2.0	0.5	92.0 a	green	soft-loose	+++	-	89.5 a
0.2	1.0	78.0 b	green-yellow	soft-hard	++	++	45.0 c
0.5	1.0	87.0 a	green-yellow	soft-hard	++	++	42.1 c
1.0	1.0	56.0 c	green-brown	very hard	++	+	21.4 d
2.0	1.0	78.0 b	green-brown	very hard	++	-	5.4 e

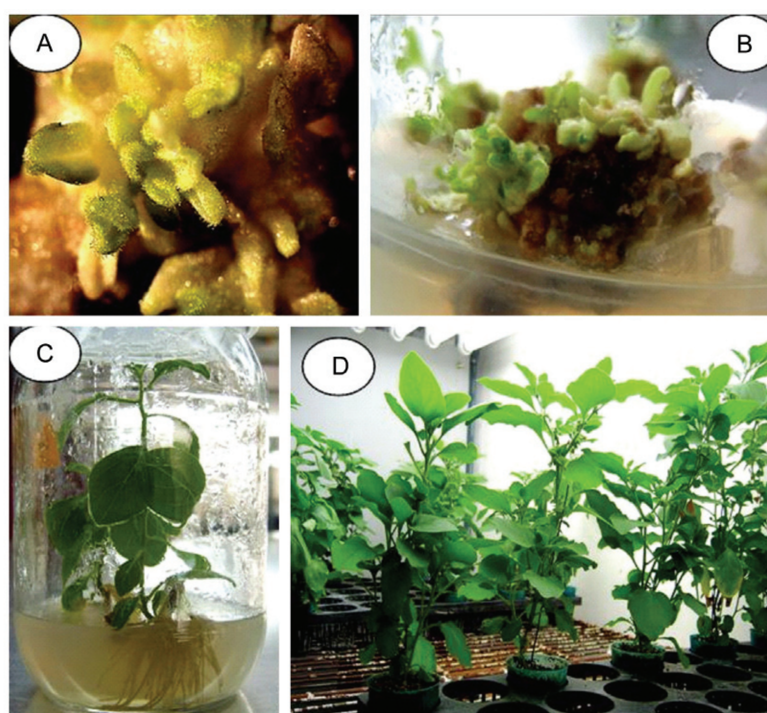

Fig. 1. Indirect shoot organogenesis in *Withania somnifera*: *A* - organogenic callus formation from leaf segments, *B* - greenish organogenic clusters forming shoots, *C* - plantlets rooting *in vitro*, *D* - acclimatized plants in the greenhouse.

Table 2. Contents of secondary metabolites in different plant parts of *Withania somnifera* from *in vitro* and greenhouse grown plants. Mean separation within columns by Duncan's multiple range test at 5 % level. 12-deo - 12-deoxywithastramonolide.

Plants	Organs	Withaferin A [mg g^{-1}(d.m.)]	12-deo [mg g^{-1}(d.m.)]	Withanolide A [mg g^{-1}(d.m.)]	Phenolics [mg g^{-1}(d.m.)]	Flavonoids [mg g^{-1}(d.m.)]	DPPH [%]	Polysaccharides [%]
In vitro	leaf	0.606 b	0.034 b	0.013 e	16.77 a	0.16 b	42.94 d	0.60 de
	stem	0.487 c	0.028 c	0.034 b	7.33 d	0.17 b	72.85 b	0.63 c
	root	0.013 d	0.024 d	0.019 d	14.99 b	0.33 a	78.64 a	0.99 a
Greenhouse	leaf	0.904 a	0.049 a	0.018 d	13.47 b	0.10 c	49.94 c	0.72 b
	stem	0.604 b	0.048 a	0.044 a	5.17 d	0.07 d	28.63 e	0.59 e
	root	0.018 d	0.032 b	0.025 c	7.83 c	0.07 d	46.50 cd	0.61 cd

rate, the MS signals were readily assignable. In positive ESI mode, the spectra of withaferin A and 12-deoxy-withastramonolide showed signals at m/z of 471.2 and 490.8, a signal at m/z 490.8 was observed for withanolide A. The analysis of *Withania* root, stem and leaf (*in vitro* and greenhouse material) confirmed the presence of withaferin A, 12-deoxywithastramonolide and withanolide A in all parts of the plants, but with significant differences in their ratio (Table 2). Withaferin A was most dominant in the leaves from both greenhouse and *in vitro* grown plants and this compound was only minor in roots. Stems contained the lowest amount of total withanolides (withaferin A, 12-deoxywithastramonolide and withanolide A). Greenhouse grown plants contained higher amount of withanolides compared to *in vitro* grown plants. This could be explained by different environmental conditions that may influence the synthesis of withanolides.

Among different plant parts of *Withania*, leaf extracts had the highest total phenolic content, followed by root, and stem. *In vitro* grown plants had higher phenolic content than greenhouse grown plants. *In vitro* roots had higher content of total flavonoids than greenhouse materials. Flavonoids possess one or more of the following structural elements that are considered important to their antioxidant activities: an *o*-diphenol group in ring B; a 2 - 3-double bond conjugated with the 4-oxo function, and hydroxyl groups at positions 3 and 5 (Rice-Evans *et al*. 1996). Flavonoids are also described as scavengers of reactive oxygen species, *via* inhibition of oxido-reductases (Carlo *et al*. 1999). *In vitro* roots exhibited the greatest content of polysaccharides, followed by leaf of greenhouse grown plants. Plant polysaccharides also have strong antioxidant activities and can be explored as novel potential antioxidants (Jiang *et al*. 2005). *In vitro* grown root and stem exhibited the greatest scavenging activity of DPPH free radical, followed by greenhouse material. In general, extracts or fractions with a high radical scavenging activity showed a high phenolic content as well, but good correlations could not be found among them (Table 2). Our results indicate that *Withania* extract possess several antioxidant substances which scavenge the free radicals.

In conclusion, the present study has established a protocol for indirect organogenesis of *Withania somnifera* through leaf-derived callus. The method described herein represents a significant improvement in the analysis of withaferin A, 12-deoxy withastramonolide and withanolide A. Also, this study reports on the presence of phenolics, flavonoids, polysaccharides and DPPH activity in *Withania*.

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