Indian Journal of Experimental Biology
Vol. 53, March 2015, pp. 177-183

Effect of carbon and nitrogen sources on *in vitro* flower and fruit formation and withanolides production in *Withania somnifera* (L.) Dunal

Ganeshan Sivanandhan[1]*, Jeevaraj Theboral[1], Gnanajothi Kapil Dev[1], Natesan Selvaraj[2], Markandan Manickavasagam[1] & Andy Ganapathi[1]*

[1]Department of Biotechnology and Genetic Engineering, School of Life Sciences, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India
[2]Government Arts College, Kulithalai 639 120, Tamil Nadu, India

Received 06 November 2013; revised 21 February 2014

We studied the influence of sucrose and nitrogen concentration on *in vitro* flowering and fruit setting in elongated shoots of *Withania somnifera.* BA (1.5 mg/l) and IAA (0.3 mg/l) on MS medium supplemented with 4% sucrose showed 67% of *in vitro* flower induction frequency, 9 flowers/shoot, 4 fruits/shoot and 11 seeds/fruit in elongated-shoots. Different concentrations of nitrogen sources (L-glutamine, adenine sulphate, ammonium nitrate, potassium nitrate and sodium nitrate 5-25 mg/l) were tested in combination with 4% sucrose and BA at 1.5 mg/l and IAA at 0.3 mg/l. Highest number of flowers (20 flowers/shoot; 2.2-fold) and fruits (16 fruits/shoot; 3.39-fold), fruit setting (12 seeds/fruit; 1.08-fold) at a higher frequency (88 %) were achieved on MS medium augmented with 15 mg/l adenine sulphate with same PGRs and sucrose concentration. The maximum production of withanolide A (0.68 mg/g DW) and withanolide B (0.77 mg/g DW) was recorded in i*n vitro* fruits. Highest accumulation of withaferin A (2 mg/g DW) was quantified from *in vitro* flowers, whereas, it was low in *in vitro* fruits (0.49 mg/g DW withaferin A). However, withanone (0.23 mg/g DW) was found accumulated uniformly in both *in vitro* flowers and fruits compared to control.

Keywords: Ashwagandha, Indian ginseng, Withaferin A, Withanone

Withania somnifera (L.) Dunal ("Indian ginseng") is an important medicinal plant in the Indian traditional medicinal system. Withanolide A, withanolide B, withaferin A and withanone, the major bioactive constituents of this plant, have remarkable activities in physiological and metabolic restoration, anti-arthritic, anti-aging, anti-cancer, cognitive function improvement in geriatric states and recovery from neurodegenerative disorders[1].These major withanolides are biosynthesized in leaves, roots, flowers and fruits[2]. Since *W. somnifera* flowers and fruits possess pharmacological activities, *in vitro* culture of flowers and fruits is considered as a viable option for uniform bioactive component synthesis. In addition, knowledge on *in vitro* flowering, development of fruits and seeds is precious in understanding the floral biology and prompt breeding of *W. somnifera*[3].

Although, Saritha and Naidu[4] reported *in vitro* flowering in *W. somnifera* with a low frequency of flowering and fruit setting, they did not account seed germination ability and the ploidy level in the progenies. Also, they did not record withanolides content in *in vitro* flowers and fruits. Although, many reports are available on withanolides production in shoot culture, adventitious root culture, hairy root culture and cell suspension culture[5-9], there are no reports so far on withanolides production in *in vitro* flowers and fruits.

The formation of flowers *in vitro* depends on numerous factors, which comprise the environment and stage of the explants, composition of the medium (PGRs, nitrogen and carbon sources) and environmental conditions[10]. The type and concentration of carbohydrate source are important in *in vitro* flowering and fruit setting, and its effect may differ depending up on the plant species[10]. Sucrose is the most favoured carbon source for flower induction and fruit setting in *Solanaceae* members[11]. Nitrogen bearing compounds at optimal level have played a desicive part in influencing the flower induction and fruit setting[12-14]. In the present study, we explored potential carbon and nitrogen sources to improve *in vitro* flower formation, fruit setting and withanolides production in *W. somnifera*.

*Correspondence:
Tel.: 91 0431 2407086; Fax: 0431-2407045
aganapathi2003@rediffmail.com (AG); gsivabdu@gmail.com (GS)

Materials and Methods

Plant material and shoot elongation—Twelve-weeks old nodal explants were collected from *W. somnifera* grown at Kolli hills, Eastern Ghats (1000 m), Tamil Nadu, India and surface sterilized by our previous report[5]. The nodal explants (10 mm) were inoculated onto MS[15] solid medium containing BA (1.5 mg/l) and IAA (0.3 mg/l) for multiple shoot induction and elongation. This hormonal composition was selected on the basis of our earlier reports[5,7].

Media and growth conditions—The growth medium consisting of MS mineral salts, and vitamins with 3% (w/v) sucrose (SRL, India) and 0.2% (w/v) phytagel (Sigma, USA) / 0.8% (w/v) agar (Himedia, India) was applyed in the tests. The MS medium was added with ideal concentrations of cytokinin, and auxin. The pH was regulated to 5.75 by 1 N NaOH or 1 N HCl prior to autoclaving at 1.06 kg cm^{-2} (121 °C) pressure for 20 min. The medium was distributed in 15 ml aliquot in culture tubes (Borosil, India), respectively capped with cotton plugs. The cultures were kept at 25±2 °C under a 16/8 h photoperiod with 40 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR) provided with cool-white fluorescent tubes (4 tubes, 40 W, Philips, India) and 60 % of relative humidity (RH).

Effect of sucrose and nitrogen sources on flower induction, fruit setting and withanolides production—Different concentrations (1–5%) of sucrose in combination with BA (1.5 mg/l), and IAA (0.3 mg/l) on MS medium were tested to find out their effect on flower induction, and fruit setting from elongated shoots. The MS medium devoid of sucrose was employed as a control. To find out the most optimum concentration of nitrogen source, the elongated shoots (5–6 cm length) were transferred to MS medium containing various concentrations (0, 5, 10, 15, 20 and 25 mg/l) of L-glutamine, adenine sulphate, ammonium nitrate, potassium nitrate and sodium nitrate and sucrose (4%) in combination with BA (1.5 mg/l), and IAA (0.3 mg/l) to find out their effect on flower induction, and fruit setting. The MS medium devoid of nitrogen source was treated as a control. The number of flowers were documented and the fruit setting verified after 3 weeks. Seeds were collected after 2 weeks and tested for seed viability. After the culture periods, data were collected, recorded and tabulated.

In vitro seed sowing, germination and development—The *in vitro*-derived seeds were collected from plants reared on MS medium containing 15 mg/l adenine sulphate, BA (1.5 mg/l), IAA (0.3 mg/l) and 4% sucrose. About 100 seeds were sown in paper cups containing autoclaved vermiculite: garden sand: soil (2: 1: 1) under diffused light (16 h photoperiod) and maintained in the greenhouse.

Withanolides extraction and HPLC analysis—*In vitro*-raised plant materials (flowers, and fruits) and their respective controls were dried and crushed into fine powder (1 g DW). Extraction and HPLC analysis were done as depicted by Sivanandhan *et al.*[7]. The levels of withanolides were estimated by matching their peak areas with standard curve created employing various levels of standards. Withanolides data were expressed as mg/g dry wt. All the samples were run in triplicate for consistency. Standards of withanolide A and B were procured from Chromadex Inc. (Laguna Hills, CA, USA) and withaferin A and withanone were obtained from Natural Remedies (Bangalore, Karnataka, India).

Experimental design and statistics—All experiments were done in a fully randomized block with three replicates. Outcomes were stated as mean±SE of three experiments. The results were evaluated statistically utilizing SPSS version 17 (SPSS Inc., Chicago, IL, USA) and substantial variances among the means were measured by DMRT at $P < 0.5$.

Results and Discussion

Various sucrose concentrations combined with PGRs (BA 1.5 mg/l and 0.3 mg/l IAA) revealed varied effects on *in vitro* flowering, and fruit setting (Table 1). Maximum production of *in vitro* flowers (9/shoot) and fruit setting (4/shoot) was observed with the culture medium augmented with 4% sucrose. An average of 4 out of 9 flowers produced fruits after 3 weeks of flower initiation and these fruits set an average of 11 seeds/fruit. These seeds exhibited 84% seed viability. The seeds obtained from *in vitro* plants took 25 days more to germinate compared to the parent plant's seed germination (15 days). Combinations of cytokinins/auxins or individual cytokinins has been stated to improve in the apical/axillary meristems in floral transition, development of flower, or both, in several species such as *Arabidopsis thaliana*[16], *Doritaenopsis* and *Phalaenopsis* orchids[17]. Lindsay *et al.*[18] reported that cytokinins influence flower growth by synchronizing genes that organize roles in the shoot apical/axillary meristems. Patil and Jeyanthi[19] have shown that the

exogenous cytokinins induced flowering by activating endogenous cytokinins in ascending xylem sap. In the present study, exogenous hormonal supply has possibly triggered flowering process along with endogenous contents. Among a variety of carbon sources, sucrose is the best carbon source in growth medium for initiation and improvement of flowers[20]. Lunn and MacRae[21] hypothesized that sucrose is not only a source of carbon and energy for plant growth and development, but it also has a signaling role and controls gene expression that encode enzymes, transporters and storage proteins for flower formation. Additionally, sucrose can act as a long-distance signal in flower recreation as observed by Lejeune *et al.* [22] in *Salix alba*, where an improved sucrose amount in the phloem attaining the apex, induced flowering[23]. In this study too, we have confirmed that 4% sucrose influenced *in vitro* flowers as well as fruits induction.

Nitrogen sources play an important role in flower formation and fruit setting in the *W. somnifera*. Addition of adenine sulphate in MS medium enhance flower and fruit settings in shoots derived from nodal explants of *W. somnifera*. Flowering is influenced by the source and/or quantity of nitrogen supplied to plants[24]. In our study, of the various types of nitrogen sources tested, shoots reared on MS medium augmented with 15 mg/l adenine sulphate**,** 4% sucrose, 1.5 mg/l BA, and 0.3 mg/l IAA achieved maximum flowers (20/shoot) and fruits (16/shoot) after 3 weeks of culture (Table 2 and Fig. 1). An

Table 1—Effect of different sucrose concentrations on *in vitro* flowering and fruit setting of *W. somnifera* shoots cultured on MS medium supplemented with 1.5 mg/l BA and 0.3 mg/l IAA.

[Values represent the mean±standard error. Mean followed by same letters within a column are not significantly different according to Duncan's multiple test at 5% level]

Sucrose concentration (%)	Frequency of flower induction (%)	No. of flowers/shoot	No. of fruits/shoot	No. of seeds/fruit	Seed viability (%)
0	-	-	-	-	-
1	44.3e	4.4±0.24e	1.2±0.12e	10.6±0.17b	68.40c
2	52.2d	6.2±0.22d	1.8±0.10d	10.8±0.16b	70.1b
3	60.2c	7.4±0.27c	2.6±0.15c	11.4±0.18a	77.6b
4	67.5a	9.8±0.25a	4.8±0.18a	11.6±0.13a	84.3a
5	65.3b	8.6±0.22b	3.4±0.16b	11.2±0.11a	80.4a

Fig. 1—Effect of nitrogen sources on *in vitro* flower formation and fruit setting on MS medium supplemented with BA 1.5 mg/l, IAA 0.3 mg/l and 4% sucrose in *W. somnifera*. (a) An elongated shoot. Arrows indicate floral primordium and subsequent flower formation; (b) *In vitro* flower formation on medium containing adenine sulphate (15 mg/l). Arrows indicate *in vitro* flowers; (c) *In vitro* flower formation at terminal region of non-elongated shoot (Rosette flower formation); (d) *In vitro* fruit formation on medium augmented with adenine sulphate (15 mg/l); (e) Seeds collected from *in vitro* fruits; (f) T_1 progenies from *in vitro* seeds in paper cups; (g) Axillary meristems in elongated shoot. Arrows indicate axillary meristems; (h) Floral primordium initiation from axillary meristem. Arrows indicate floral bud primordium from upper view; (i) Floral bud initiation; (j) Floral bud formation: 1. Flower bud, 2. Initiation of flower bud, and 3. Initiation of floral bud primordium. All bars represent 40 mm (a-c), 1 cm (d-f) and 600 µm (g-j).

Table 2—Effect of nitrogen sources on *in vitro* flowering and fruit setting of *W. somnifera* shoots cultured on MS medium supplemented with 1.5 mg/l BA, 0.3 mg/l IAA and 4% sucrose
[Values represent the mean±standard error. Mean followed by same letters within a column are not significantly different according to Duncan's multiple test at 5% level]

Nitrogen sources (mg/l)	Frequency of flower induction (%)	No. of flowers/shoot	No. of fruits/shoot	No. of seeds/fruit	Seed viability (%)
Control (1.5 mg/l BA and 0.3 mg/l IAA)	67.5[c]	9.8±0.25[c]	4.8±0.18[e]	11.6±0.13[b]	84.3[a]
Adenine sulphate					
5	52.6[d]	8.7±0.34[d]	6.2±0.12[d]	10.3±0.10[c]	85.43[a]
10	64.3[c]	11.4±0.37[b]	9.4±0.17[b]	11.5±0.14[b]	85.21[a]
15	88.8[a]	20.2±0.28[a]	16.3±0.14[a]	12.6±0.12[a]	85.34[a]
20	76.9[b]	16.3±0.21[b]	13.2±0.11[a]	10.8±0.18[c]	80.55[a]
25	73.5[b]	14.1±0.25[b]	9.3±0.10[b]	10.2±0.12[c]	79.41[b]
L-glutamine					
5	37.6[f]	2.4±0.26[h]	1.2±0.16[h]	10.7±0.19[c]	78.50[b]
10	43.3[e]	4.3±0.22[f]	2.4±0.17[g]	10.6±0.12[c]	78.30[b]
15	56.5[d]	8.1±0.31[d]	4.3±0.14[e]	10.8±0.17[c]	84.21[a]
20	63.8[c]	11.6±0.29[b]	6.7±0.18[d]	11.4±0.13[b]	80.50[a]
25	51.3[e]	5.7±0.25[e]	2.4±0.11[g]	10.4±0.11[c]	78.57[b]
Ammonium nitrate					
5	54.1[d]	7.1±0.27[e]	3.3±0.13[f]	10.8±0.16[c]	79.62[b]
10	63.4[c]	10.3±0.27[b]	8.6±0.17[c]	10.4±0.11[c]	79.80[b]
15	74.5[b]	14.6±0.26[b]	9.6±0.12[b]	11.8±0.16[b]	84.92[a]
20	65.3[c]	8.4±0.21[d]	4.1±0.19[e]	11.2±0.17[b]	82.05[a]
25	49.7[e]	4.2±0.29[f]	1.4±0.11[h]	10.8±0.18[c]	77.77[b]
Potassium nitrate					
5	33.3[f]	2.2±0.22[h]	-*	-**	-***
10	41.9[e]	3.4±0.28[g]	-*	-**	-***
15	47.3[e]	7.8±0.24[e]	2.3±0.15[g]	10.4±0.19[c]	81.48[a]
20	44.8[e]	4.2±0.27[f]	-*	-**	-***
25	37.2[f]	1.6±0.26[la]	-*	-**	-***
Sodium nitrate					
5	13.2[h]	1.1±0.21[i]	-*	-**	-***
10	23.6[g]	2.7±0.24[h]	-*	-**	-***
15	34.8[f]	4.9±0.29[f]	1.3±0.11[h]	11.3±0.19[b]	84.95[a]
20	26.4[g]	1.8±0.27[i]	-*	-**	-***
25	18.3[h]	1.0±0.21[i]	-*	-**	-***

*Nil response in fruit setting; **Nil response in seed setting; ***Nil response in seed viability.

average of 16 out of 20 flowers produced fruits after 3 weeks of flower initiation and these fruits set an average of 12 seeds/fruit. Seed viability and seed germination were similar to our earlier results[5]. Seeds derived from *in vitro* plants showed 85% viability when compared to control. The *in vitro*-derived seeds resembled normal seeds in terms of size and color (Fig. 1). However, these *in vitro* flowers were smaller than the field-grown flowers. Irrespective of sucrose and nitrogen treatments, from the observations under stereo microscope, it was clear that axillary meristem in the regenerated-shoots had organized into a flower bud primodium, then into flower formation and subsequently fruits (Fig. 1). But in *in vivo* plants, single axillary meristem had organized into a cluster of 3-8 flower bud primordia which developed into 3-8 flowers and subsequently 3-8 fruits. Saritha & Naidu[4] reported that KN at 2 mg/l and IAA at 0.1 mg/l produced 10 flowers/shoot and 5% of shoots only fruited with one fruit in each. They observed that when KN concentration was increased to 4 mg/l + 0.1 mg/l IAA, a shoot formed 8 flowers and finally only one shoot produced one fruit in *W. somnifera*[4].

Nandagopal and Kumari[25] reported that exogenous adenine sulphate along with BA and IAA produced flowers in *Cichorium intybus*. Lovatt *et al.*[13] reported that nitrogenous compounds may directly affect

flowering and fruit setting via regeneration of internal nitrogen content and polyamines levels in the flower bud, which play a desicive role in the mersitematic activity involved in flower bud differentiation and subsequent fruit setting. Similarly, Bichsel *et al.*[14] stated that nitrogen at optimal level is required for constant flower initiation and improvement as reproductive organs growth is a robust nutrient drop.

Next to adenine sulphate, ammonium nitrate and L-glutamine influenced the flower formation and fruit setting in *W. somnifera*. Ammonium nitrate (15 mg/l) along with 4% sucrose, 1.5 mg/l BA, and 0.3 mg/l IAA induced formation of flower and fruits in *in vitro*, however, with moderate production of flowers (14 flowers/shoot) [Table 2]. In the same medium, 74% of regenerated shoots flowered and set seeds. A medium containing higher concentration of ammonium nitrate (25 mg/l) showed only 49% of flowering, thereby suggests that higher concentrations of ammonium nitrate are inhibitory to *in vitro* flowering. Similar results have been observed in green pea[26]. The flowers were self-fertilized and most of them set fruits with viable seeds. All the fruits contained 11 seeds, resembling normal seeds in size and color. Other nitrogen sources tested also produced flowering, fruiting and seed setting at moderate levels compared to the control (Table 2).

In the present study, shoot elongation played an important role in *in vitro* flowering of *W. somnifera* (Fig. 1). The shoots that were not elongated in the medium exhibited rosette flower formation; although the flowers appeared normal (Fig. 1), they failed to develop viable fruits and seeds (data not presented). *In vitro*-raised shoots continuously produced flowers, fruits and seeds even after the shoots were subjected to acclimatization and subsequent transfer to the field. During *in vitro* culture, the axillary meristem resulted in the formation of a single flower whereas the same shoots after transferred to field produced three flowers and three fruits. It implies that the field/natural conditions favours normal flowering in *W. somnifera*.

In the present investigation, major withanolides were quantified in *in vitro* flowers and fruits for the first time. Fig. 2 and table 3 illustrate withanolides

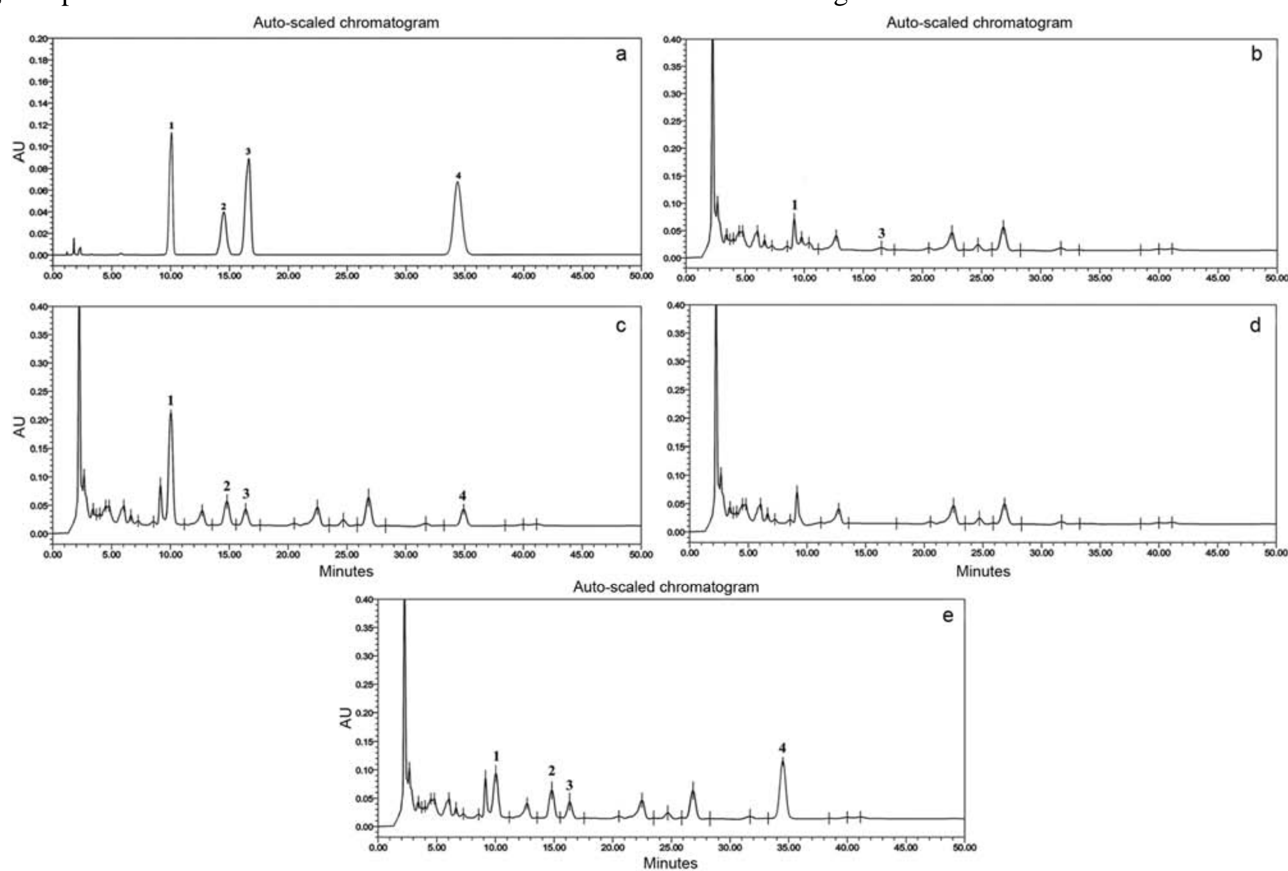

Fig. 2—HPLC analysis of withanolides in methanolic extracts of *in vitro* flowers, fruits and its respective field-grown parent plant parts of *W. somnifera*. (a) Withanolides standards; (b) Extract from *in vivo* flowers; (c) Extract from *in vitro* flowers; (d) Extract from *in vivo* fruits; and (e) Extract from *in vitro* fruits (1. Withaferin A, 2. Withanolide A, 3. Withanone and 4. Withanolide B).

Table 3—Withanolides production in *in vivo* and *in vitro* reproductive organs of *W. somnifera*.
[Data was recorded after 3 weeeks of flower formation and 3 weeks of fruit formation.
Data represents mean ± standard error of three replicates]

Reproductive organs	withanolide A (mg/g DW)	withanolide B (mg/g DW)	withaferin A (mg/g DW)	Withanone (mg/g DW)
In vivo flower	*TL	*TL	0.04±0.08c	0.01±0.09b
In vitro flower	0.45±0.07b	1.38±0.04a	2.03±0.06a	0.23±0.06a
In vivo fruit	*TL	*TL	*TL	*TL
In vitro fruit	0.68±0.02a	0.77±0.07b	0.49±0.05b	0.23±0.03a

*Trace level

contents in both *in vitro* and *in vivo* flowers and fruits. Withanolide A and B were detected at trace level in the methanolic extracts of field-grown parent plant's flowers and withanolide A, withanolide B, withaferin A and withanone were detected in field-grown parent plant's fruits (Table 3). Maximum production of withanolide A (0.68 mg/g DW) and withanolide B (0.77 mg/g DW) was observed in i*n vitro* fruits. Highest accumulation of withaferin A (2 mg/g DW) was quantified from *in vitro* flowers, whereas it was low in *in vitro* fruits (0.49 mg/g DW withaferin A). Withanone (0.23 mg/g DW) was found accumulated uniformly in both *in vitro* flowers and fruits (Table 3).

This study confirms that addition of sucrose (4%) and adenine sulphate (15 mg/l) along with 1.5 mg/l BA and 0.3 mg/l IAA trigger highest flower formation and fruit setting in *in vitro* elongated shoots and subsequent withanolides production in them. The protocol can be used for *in vitro* flower and fruit formation in mass cultures and extraction of withanolides from flowers and fruits through out the year without depending on field-grown plants.

Acknowledgments

Prof. A. Ganapathi is thankful to University Grants Commissions (UGC), Government of India, for the award of UGC-BSR (Basic Scientific Research Fellowship). The first author (GS) gratefully acknowledges the Council of Scientific and Industrial Research (CSIR), New Delhi, for the award of CSIR-SRF. The authors thank Dr R Boopathy, HOD and Dr S Girija, Associate Professor, Department of Biotechnology, Bharathiar University, Coimbatore for providing HPLC facility.

References

1 Mishra LC, Singh BB & Dagenais S, Scientific basis for the therapeutic use of *Withania somnifera* (ashwagandha): a review. *Alternat Med Rev*, 5 (2000) 346.

2 Singariya P, Kumari P & Mourya KK, Comparative pharmacological study of fruits and flowers extract of *Withania somnifera*. *Int J Pharm Phytopharmacol Res,* 5 (2012) 282.

3 Sharma MM, Singh A, Verma RN, Ali DZ & Batra A, Influence of PGRs for the *in vitro* plant regeneration and flowering in *Portulaca oleracea* (L.): A medicinal and ornamental plant. *Int J Bot,* 7 (2011) 107.

4 Saritha KV & Naidu CV, *In vitro* flowering of *Withania somnifera* Dunal.—an important antitumor medicinal plant. *Plant Sci*, 172 (2007) 851.

5 Sivanandhan G, Mariashibu TS, Arun M, Rajesh M, Kasthurirengan S, Selvaraj N & Ganapathi A, The effect of polyamines on the efficiency of multiplication and rooting of *Withania somnifera* (L.) Dunal and content of some withanolides in obtained plants. *Acta Physiol Plant,* 33 (2011) 2288.

6 Sivanandhan G, Arun M, Mayavan S, Rajesh M, Jeyaraj M, Kapil Dev G, Manickavasagam M, Selvaraj N & Ganapathi A, Optimization of elicitation conditions with methyl jasmonate and salicylic acid to improve the productivity of withanolides in the adventitious root culture of *Withania somnifera* (L.) Dunal. *Appl Biochem Biotechnol,* 168 (2012b) 696.

7 Sivanandhan G, Rajesh M, Arun M, Jeyaraj M, Kapil Dev G, Arjunan A, Manickavasagam M, Muthuselvam, M, Selvaraj N & Ganapathi A, Effect of culture conditions, cytokinins, methyl jasmonate and salicylic acid on the biomass accumulation and production of withanolides in multiple shoot culture of *Withania somnifera* (L.) Dunal using liquid culture. *Acta Physiol Plant,* 35 (2013a) 728.

8 Sivanandhan G, Kapil Dev G, Jeyaraj M, Rajesh M, Arjunan A, Muthuselvam M, Manickavasagam M, Selvaraj N & Ganapathi A, Increased production of withanolide A, withanone, and withaferin A in hairy root cultures of *Withania somnifera* (L.) Dunal elicited with methyl jasmonate and salicylic acid. *Plant Cell Tiss Organ Cult,*114 (2013c) 129.

9 Sivanandhan G, Kapil Dev G, Jeyaraj M, Rajesh M, Muthuselvam M, Selvaraj N, Manickavasagam M, & Ganapathi A, A promising approach on biomass accumulation and withanolides production in cell suspension culture of *Withania somnifera* (L.) Dunal. *Protoplasma*, 250 (2013b) 898.

10 Taylor NJ & Marnie EL, Monosaccharides promote flowering in *Kniphofia leucocephala in vitro. Plant Growth Regulator,* 52 (2007) 79.

11 Tiburcio AF, Kaur-Sawhney R & Galston AW, Polyamine biosynthesis during vegetative and floral bud differentiation in thin layer cultures. *Plant Cell Physiol,* 29 (1988) 1249.

12 Klein I & Weinbaum SA, Foliar application of urea to olive: Translocation of urea nitrogen as influenced by sink demand and nitrogen deficiency. *J Amer Soc Hort Sci*, 109 (1984) 360.

13 Lovatt CJ, Zheng Y & Hake KD, A new look at the Kraus-Kraybill hypothesis and flowering in *Citrus*. *Proc 6th Int Citrus Congress*, 1 (1988) 483.

14 Bichsel RG, Starman TW, & Wang YT, Nitrogen, phosphorus, and potassium requirements for optimizing growth and flowering of the nobile dendrobium as a potted orchid. *HortSci,* 43 (2008) 332.

15 Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*, 15 (1962) 497.

16 Corbesier L, Prinsen E, Jacqmard A, Lejeune P, Van Onckelen H, Périlleux C & Bernier G, Cytokinin levels in leaves, leaf exudate and shoot apical meristem of *Arabidopsis thaliana* during floral transition. *J Expt Bot,* 54 (2003) 2517.

17 Blanchard MG & Runkle ES, Temperature during the day, but not during the night, controls flowering of *Phalaenopsis* orchids. *J Exp Bot,* 57 (2006) 4049.

18 Lindsay DL, Sawhney VK & Bonham-Smith PC, Cytokinin-induced changes in *CLAVATA1* and *WUSCHEL* expression temporally coincide with altered floral development in *Arabidopsis*. *Plant Sci*, 170 (2006) 117.

19 Patil VM & Jayanthi M, Micropropagation of two species of *Rauvolfia* (Apocynaceae). *Curr Sci*, 72 (1997) 965.

20 Scorza R, *In vitro* flowering. *Hort Rev*, 4 (1982) 127.

21 Lunn LE & MacRae E, New complexities in the synthesis of sucrose. *Curr Opin Plant Biol,* 6 (2003) 214.

22 Lejeune P, Bernier G & Kinet JM, Sucrose levels in leaf exudates as a function of floral induction in the long day plant *Sinapsis alba*. *Plant Physiol Biochem,* 29 (1991) 157.

23 Bernier G & Périlleux C, A physiological overview of the genetics of flowering time control. *Plant Biotech J,* 3 (2005) 16.

24 Vidal EA & Gutierrez RA, A systems view of nitrogen nutrient and metabolite responses in *Arabidopsis*. *Curr Opin Plant Biol,* 11 (2008) 529.

25 Nandagopal S, & Ranjitha Kumari BD, Adenine sulphate induced high frequency shoot organogenesis in callus and *in vitro* flowering of *Cichorium intybus* L. cv. Focus-a potent medicinal plant. *Acta Agr Slov,* 87 (2006) 425.

26 Franklin G, Pius PK & Ignacimuthu S, Factors affecting *in vitro* flowering and fruiting of green pea (*Pisum sativum* L.). *Euphytica,* 115 (2000) 73.