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Somatic Embryogenesis in *Withania somnifera* (L.) Dunal

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Abstract

Somatic embryos were formed from calli obtained from axillary shoots (raised from nodal segments of glasshouse-grown plants under aseptic conditions), internodal segments (from *in vitro*-raised plants), and root and cotyledonary leaf segments (from *in vitro*-raised seedlings) after 8 weeks of initial culture. Embryo formation was the highest (97.33%) from cotyledonary leaf callus on Murashige and Skoog's (MS) medium containing kinetin (KN) (3 mg/L). Somatic embryo induction was lesser with different combinations of auxins while it increased to 100% in internodal segment and cotyledonary leaf calli with 6-benzyladenine (BA) (2 mg/L) along with 2,3,5-triiodobenzoic acid (TIBA) (2 mg/L). The shoots were induced from somatic embryos raised from root, cotyledonary leaf and internodal segment calli grown on MS medium containing BA in combination with indole-3-acetic acid (IAA). Maximum of 66.67% cultures formed shoots on MS medium containing BA (1 mg/L) in combination with IAA (2 mg/L). The shoots raised from somatic embryos were rooted on MS medium supplemented with indole-3-butyric acid (IBA) (2 mg/L). The plantlets transferred to the field showed 70% survival rate after one year.

Key words: Embryogenic callus, somatic embryogenesis, *Withania somnifera*

Introduction

Withania somnifera (L.) Dunal, belonging to the family Solanaceae, is one of the important medicinal plants mentioned in 'Charaka Samhita' - an ancient medical treatise of India. It possesses immense therapeutic value against a large number of ailments, such as mental diseases, asthma, inflammation, arthritis, rheumatism, tuberculosis, infections, fever, male sexual disorders, and a variety of other diseases, including cancer (Asthana and Raina 1989). It is also called 'Indian Ginseng' for its rejuvenating properties. Its micropropagation, employing different explants, such as shoot tips (Sen and Sharma 1991), nodal segments (Tiwari and Singh 1991; Kulkarni et al. 2000), axillary meristems (Roja et al. 1991), leaf explants (Baburaj and Gunasekaran 1995; Kulkarni et al. 1996), meristems (Teli et al. 1999), axillary shoots, and hypocotyls and root segments (Rani and Grover 1999), has been reported. Among different micropropagation techniques, somatic embryogenesis has tremendous potential for large-scale production of plant material (Ammirato and Styer 1985). Since there is no report on somatic embryogenesis in *W. somnifera*, an attempt was made to develop a micropropagation technique via somatic embryogenesis using different explants.

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Materials and Methods

Callus induction

The plants of *Withania somnifera* grown in glasshouse of Botanic Garden, Guru Nanak Dev University, Amritsar, Punjab (India) were used as experimental material. The explants obtained from various sources, *viz.* leaf and internodal segments (from glasshouse-grown plants), axillary shoots (raised from nodal segments of glasshouse-grown plants under aseptic conditions), hypocotyl, root and cotyledonary leaf segments (from *in vitro*-raised seedlings), and internodal and nodal segments (from *in vitro*-sourced plants) of *W. somnifera*, were used for callus induction (Rani and Grover 1999; Rani et al. 2003).

Induction of somatic embryos

The calli (5×5 mm) raised from different explants were transferred to MS medium (Murashige and Skoog 1962) containing various concentrations and combinations of cytokinins [6-benzyladenine (BA), N^6-(2-isopentenyl)adenosine (2-iP) and kinetin (KN)], auxins [indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA)], and BA and 2,3,5-triiodobenzoic acid (TIBA), alone or in combination, to study their effectiveness on the induction of somatic embryogenesis. For each treatment, 25 tubes were inoculated at 25± 1℃ with 16 h photoperiod and 40 μmole/m^2/s provided by cool-white fluorescent tubes. After an interval of 60 days, the data with respect to number of cultures producing somatic embryos were recorded.

Germination of somatic embryos

Mature somatic embryos were transferred on MS medium containing various concentrations of BA and IAA for shoot induction. The shoots emerged from somatic embryos were transferred to MS medium containing different concentrations of auxins (IAA, IBA and NAA) for rooting. The experiments were also conducted for direct root induction from somatic embryos obtained from calli raised from different explants using IAA and IBA alone or in combinations.

Acclimatization and transfer of plantlets to field

The plantlets were taken out from the rooting medium and washed thoroughly to remove the adhering agar. The plantlets were then transferred to plastic pots containing a mixture of sterilized sand and garden soil (1:1). After 20-25 days, the plants were shifted to earthen pots containing garden soil and transplanted to the field.

Scanning electron microscopy (SEM)

For scanning electron microscopy, the embryos were fixed in formaldehyde, acetic acid and absolute alcohol in the ratio of 1:1:18 overnight, and dehydrated in ethanol series. Fur further dehydration, the embryos were placed in a solution of absolute alcohol and amyl acetate in the ratio of 1:1 for 15 min, followed by 100% amyl acetate for 10 min. These dehydrated embryos were dried with critical point drier (Polaron CPDE 3000). The samples were then loaded on aluminium stabs and sputtered with gold (Fine Coat Ion Sputter JFC 1100, Jeol, Japan). Each stab was then examined under scanning electron microscope (ISM 6100, Jeol, Japan) operated at 20 KV.

Statistical analysis

For each treatment, 25 tubes were inoculated and the experiments were performed in triplicate. The data pertaining to number of cultures producing shoots/roots, number and height of shoots, and number and length of roots in each such culture were recorded after 30 days. The Chi-square (χ^2) test was used to find out statistically significant differences among the per cent cultures producing somatic embryos, shoots and roots. The data for number and height of shoots, and number and length of roots produced per culture were statistically analyzed by one-way of variance of analysis (ANOVA) test with HSD $\leq$ 0.05 multiple range test.

Results and Discussion

Somatic embryos were induced from calli derived from axillary shoots (raised from nodal segments of glasshouse-grown plants under aseptic conditions), root and cotyledonary leaf segments (from *in vitro*-raised seedlings), and internodal segments (from *in vitro*-sourced plants). However, the calli raised from leaf and internodal segments (from glasshouse-grown plants), and nodal (from *in vitro*-sourced plants) and hypocotyl (from *in vitro*-raised seedlings) segments did not show any somatic embryogenesis. Embryogenic callus differed distinctly from non-embryogenic callus and was often covered with shining globular somatic embryos which gradually became enlarged to a detectable size within 6-8 weeks.

Among the cytokinins tested for the induction of somatic embryos, KN was found to be positive for all the four types of calli (Table 1). However, 2-iP failed to induce somatic embryos in any of these calli while BA at its various concentrations was effective in inducing somatic embryogenesis in internodal, root and cotyledonary leaf segment calli. Maximum somatic embryogenesis was observed in 97.33% cultures of cotyledonary leaf segment calli grown on MS medium supplemented with 3 mg/L of KN. Embryogenic calli of white colour developed after 4, weeks which then turned green and hard with increase in time duration (Figure 1a). As is evident from Table 2, different concentrations and combinations of auxins could induce somatic embryogenesis in axillary shoot base calli but failed to induce somatic embryogenesis in internodal, root and cotyledonary leaf segment calli. Maximum somatic embryogenesis was seen in 76% cultures of axillary shoot-raised calli grown on MS medium supplemented with IBA (2 mg/L) and NAA (2 mg/L). When TIBA alone or in combination with BA was used, axillary shoot callus did not show somatic embryo induction (Table 3). Somatic embryo induction in calli raised from root segments occurred only in 16% of cultures with TIBA (2 mg/L) while it increased to 52% when TIBA (2 mg/L) was

Table 1. Effect of cytokinins on induction of somatic embryos from axillary shoot, and internodal, root and cotyledonary leaf segment-derived calli on MS medium after 8 weeks of initial culture.

Plant growth regulators (mg/L)			Percent cultures showing somatic embryo induction			
			Axillary shoot callus	Internodal callus	Root callus	Cotyledonary leaf callus
KN	BA	2-iP	$\bar{X} \pm SE$	$\bar{X} \pm SE$	$\bar{X} \pm SE$	$\bar{X} \pm SE$
1	-	-	12.00 ± 0.58	22.67 ± 0.33	44.00 ± 0.58	64.00 ± 0.58
2	-	-	16.00 ± 0.58	30.67 ± 0.33	50.67 ± 0.33	84.33 ± 0.58
3	-	-	18.67 ± 0.33	33.33 ± 0.33	57.33 ± 0.88	97.33 ± 0.88
4	-	-	14.67 ± 0.67	29.33 ± 0.33	48.00 ± 0.58	86.67 ± 0.67
			df=3; χ^2=1.130	df=3; χ^2=2.093	df=3; χ^2=3.086	df=3; χ^2=4.374
-	1	-	-	17.33 ± 0.33	14.67 ± 0.67	13.33 ± 0.33
-	2	-	-	28.00 ± 0.58	21.33 ± 0.33	22.67 ± 0.33
-	3	-	-	20.00 ± 0.58	10.67 ± 0.33	10.67 ± 0.33
-	4	-	-	14.67 ± 0.67	-	5.33 ± 0.33
				df=3; χ^2=3.733	df=2; χ^2=2.800	df=3; χ^2=9.103[a]
-	-	1	-	-	-	-
-	-	2	-	-	-	-
-	-	3	-	-	-	-
-	-	4	-	-	-	-

Data shown are Mean±SE of three experiments; each experiment consisted of 25 replicates.
[a] Significant at $p \leq 0.05$.

Table 2. Effect of auxins on induction of somatic embryos from axillary shoot, and internodal, root and cotyledonary leaf segment-derived calli on MS medium after 8 weeks of initial culture.

Plant growth regulators (mg/L)			Percent cultures showing somatic embryo induction			
			Axillary shoot callus	Internodal callus	Root callus	Cotyledonary leaf callus
IBA	IAA	NAA	$\bar{X} \pm SE$	$\bar{X} \pm SE$	$\bar{X} \pm SE$	$\bar{X} \pm SE$
1	2	-	48.00 ± 0.58	-	-	-
2	2	-	58.67 ± 0.67	-	-	-
3	2	-	50.67 ± 0.33	-	-	-
4	2	-	45.33 ± 0.33	-	-	-
			df=3; χ^2=2.829			
1	-	2	61.33 ± 0.33	-	-	-
2	-	2	76.00 ± 0.58	-	-	-
3	-	2	69.33 ± 0.33	-	-	-
4	-	2	60.00 ± 1.00	-	-	-
			df=3; χ^2=1.880			

Data shown are Mean±SE of three experiments; each experiment consisted of 25 replicates.

employed in combination with BA (2 mg/L). Internodal and cotyledonary leaf segment calli showed somatic embryo induction in 78.67% and 69.33% cultures, respectively, when TIBA (2 mg/L) was added to the medium, whereas it increased to 100% for both of these calli when TIBA (2 mg/L) and BA (2 mg/L) were used jointly (Table 3). A similar combination of hormones, *viz.* BA and TIBA, was considered favourable for somatic embryogenesis in *Beta vulgaris* (Tetu et al. 1987; Kulshreshtha and Coutts 1997). Somatic embryogenesis was also observed in *Panax ginseng* on MS medium supplemented with TIBA (Choi et al. 1997) and *Eragrostis tef* on MS medium containing TIBA (2 mg/L) in combination with 2,4-D (2 mg/L) (Kebebew et al. 1998). Somatic embryogenesis was reported in many other plants on MS medium containing 2,4-D alone

Table 3. Effect of BA and TIBA on induction of somatic embryos from axillary shoot, and internodal, root and cotyledonary leaf segment-derived calli on MS medium after 8 weeks of initial culture.

Plant growth regulators (mg/L)		Percent cultures showing somatic embryo induction			
		Axillary shoot callus	Internodal callus	Root callus	Cotyledonary leaf callus
BA	TIBA	$\bar{X} \pm$ SE	$\bar{X} \pm$ SE	$\bar{X} \pm$ SE	$\bar{X} \pm$ SE
-	1	-	69.33 ± 0.33	5.33 ± 0.33	62.67 ± 0.33
-	2	-	78.67 ± 0.33	16.00 ± 0.58	69.33 ± 0.33
-	3	-	66.67 ± 0.33	-	57.33 ± 0.88
-	4	-	61.33 ± 0.33	-	48.00 ± 0.58
			df=3; χ^2=1.715	df=1; χ^2=4.000*	df=3; χ^2=0.863
2	1	-	74.67 ± 0.33	45.33 ± 0.33	78.67 ± 0.33
2	2	-	100.00 ± 0.00	52.00 ± 0.58	100.00 ± 0.00
2	3	-	72.00 ± 1.00	44.00 ± 0.58	76.00 ± 0.58
2	4	-	64.00 ± 0.58	33.33 ± 0.33	62.67 ± 0.33
			df=3; χ^2=7.017	df=3; χ^2=3.076	df=3; χ^2=6.773

Data shown are Mean±SE of three experiments; each experiment consisted of 25 replicates.
*Significant at $p \leq 0.05$.

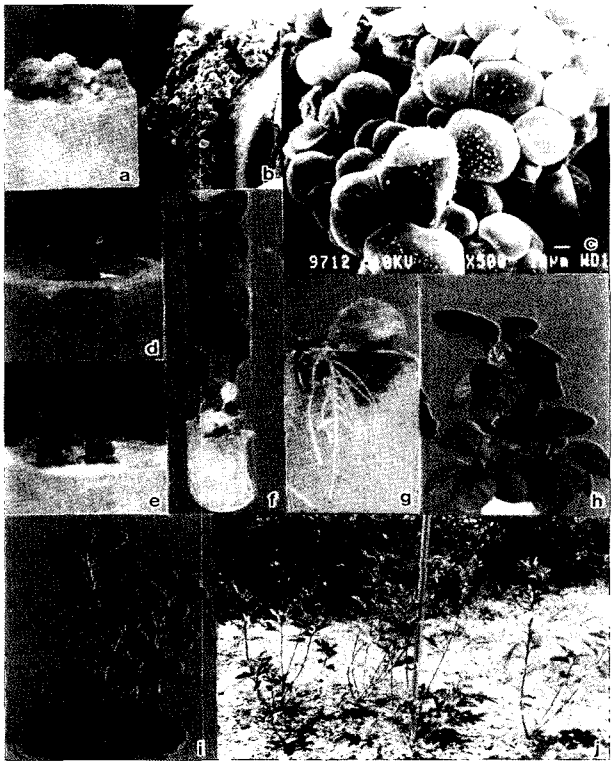

Figure 1. Somatic embryogenesis in *Withania somnifera*: (a) Embryogenic callus obtained from cotyledonary leaf segment-derived calli on MS medium containing BA (2 mg/L) and TIBA (2 mg/L); (b) Globular, heart and cotyledonary-shaped somatic embryos (X55); (c) Enlarged view of various shapes of somatic embryos (X500); (d) Cotyledonary stage somatic embryo transferred to MS medium containing BA (2 mg/L) and IAA (2 mg/L) for shoot induction; (e) Shoot induction from cotyledonary somatic embryo; (f) Rooting of shoot derived from somatic embryo on MS medium containing IBA (2 mg/L); (g) Direct root induction from embryogenic callus on MS medium containing IAA (2 mg/L) and IBA (2 mg/L); (h) Transplanted plants in plastic pot; (i) Plant transferred to earthen pot; and (j) Plants in the field.

or in combination with BAP (Jeya-Mary and Jayabalan 1997; Wakhlu and Sharma 1998; Anbazhagan and Ganapathi 1999; Canhoto et al. 1999; Sarasan et al. 2001). Embryonic calli showed all stages of embryogenesis, *viz.*, globular, heart and cotyledonary-shaped structures. The various shapes of embryos were confirmed with scanning electron microscopy (Figure 1b,c). Cotyledonary stage (Figure 1d) embryos were transferred to the shoot induction medium.

Shoot induction did not occur from somatic embryos raised from axillary shoot calli when they were cultured on MS medium containing different concentrations of BA (1-4 mg/L) and KN (1-4 mg/L), either alone or in various combinations, and also with various combinations of BA and IAA. In some earlier studies also, shoot growth from somatic embryos was either slow or absent, e.g., in *Petroselinum hortense* (Watin and Bigot, 1989), *Arachis hypogaea* (Wetzstein and Baker 1993) and *Manihot glaziovii* (Joseph et al. 2000). As many as 66.67% of cultures raised from internodal calli showed shoots when grown on MS medium containing BA (1 mg/L) in combination with IAA (2 mg/l) (Table 4, Figure 1e). The number and height of shoots formed from these somatic embryos were also greater with a similar treatment of BA in combination with IAA. BA in combination with IAA was found to be effective in plantlet formation from somatic embryos in *W. somnifera*, though, in certain other plants, only BA was sufficient for the formation of plantlets from somatic embryos (Sarasan et al. 2001; Vesco and Guerra 2001). The shoots raised from somatic embryos were rooted on MS medium supplemented with various concentrations of auxins (IAA, IBA and NAA). Among these, maximum root induction was achieved on MS medium supplemented with IBA (2 mg/L) (Figure 1f). The auxin/cytokinin ratio had an influence on the intensity of embryo formation, germination, and capability to regenerate plants.

Table 4. Shoot induction of somatic embryos raised from axillary shoot, and internodal, root and cotyledonary leaf segment-derived calli on MS medium containing BA and IAA after 30 days of initial culture.

Callus	Plant growth regulators (m5g/L)		Number of cultures producing shoots**	Percent shoot induction	Number of shoots	Height of shoots (cm)
	BA	IAA		$\bar{X}$ ± SE	$\bar{X}$ ± SE	$\bar{X}$ ± SE
Axillary shoots	1	2	-	-	-	-
	2	2	-	-	-	-
	3	2	-	-	-	-
	4	2	-	-	-	-
Internodal segments	1	2	50	66.67 ± 0.88	3.62 ± 0.99a	1.77 ± 0.11a
	2	2	39	52.00 ± 0.58	2.77 ± 0.09b	1.41 ± 0.08b
	3	2	35	46.67 ± 0.33	2.43 ± 0.08b	1.33 ± 0.13c
	4	2	25	33.33 ± 0.88	2.20 ± 0.10bc	1.22 ± 0.07d
				df=3; χ^2=8.611*	F(df 3,145)=47.59*; HSD=0.350	F(df 3,145)=206.72*; HSD=0.064
Root segments	1	2	37	49.33 ± 0.33	2.43 ± 0.08a	1.19 ± 0.07a
	2	2	28	37.33 ± 0.33	2.11 ± 0.11a	1.06 ± 0.08b
	3	2	26	34.66 ± 0.88	1.85 ± 0.10ab	0.98 ± 0.07c
	4	2	23	30.66 ± 0.33	1.74 ± 0.09ab	0.91 ± 0.07d
				df=3; χ^2=3.825	F(df 3,110)=10.79*; HSD=0.365	F(df 3,110)=82.46*; HSD=0.052
Cotyledonary leaf segments	1	2	46	61.33 ± 0.88	2.46 ± 0.08a	1.52 ± 0.08a
	2	2	41	54.67 ± 0.33	2.29 ± 0.09a	1.36 ± 0.09b
	3	2	34	45.33 ± 0.33	2.15 ± 0.10a	1.22 ± 0.07c
	4	2	26	34.66 ± 0.67	1.92 ± 0.11ab	1.03 ± 0.09d
				df=3; χ^2=6.170	F(df 3,143)=4.93*; HSD=0.368	F(df 3,143)=195.54*; HSD=0.054

Data shown are Mean±SE of three experiments; each experiment consisted of 25 replicates.
*Significant at $p \leq 0.05$; **Out of 75 cultures inoculated.
Means followed by the same letter are not significantly different using HSD multiple comparison test.

Table 5. Direct root induction of somatic embryos raised from axillary shoot callus on MS medium containing various concentrations of IAA and IBA after 30 days.

Plant growth regulators (mg/L)		Number of cultures producingroots**	Percent root induction	Number of roots	Root length (cm)
IAA	IBA		$\bar{X}$ ± SE	$\bar{X}$ ± SE	$\bar{X}$ ± SE
1	2	36	48.00 ± 0.58	9.11 ± 0.23^b	2.59 ± 0.10^b
2	2	38	50.67 ± 0.88	11.50 ± 0.21^a	3.03 ± 0.11^a
3	2	34	45.33 ± 0.33	32 8.65 ± 0.16^b	2.52 ± 0.09^c
4	2	27	36.00 ± 0.58	8.33 ± 0.16^{bc}	2.30 ± 0.08^d
			df=3; χ^2=2.037	F(df 3,131)=54.06*; HSD=0.744	F(df 3,131)=328.53*; HSD=0.063

Data shown are Mean±SE of three experiments; each experiment consisted of 25 replicates.
*Significant at p £ 0.05; **Out of 75 cultures inoculated.
Means followed by the same letter are not significantly different using HSD multiple comparison test.

Embryogenic callus raised from axillary shoot base showed direct root induction within 10-15 days when transferred to MS medium containing IAA along with IBA while no root induction was observed from calli raised from other explants (Table 5). Root induction occurred in 50.67% cultures with a combination of IAA and IBA (2 mg/L each) (Figure 1g). The average number and length of roots were also maximum with a similar concentration of IAA and IBA in combination.

The plantlets derived from somatic embryos were removed from test tubes, freed from agar and transferred to the field by the procedure mentioned in 'Materials and Methods'. The plants transferred to the field showed 70% survival (Figure 1h-j).

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