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ESTABLISHMENT OF EMBRYOGENIC CELL SUSPENSION CULTURES AND PLANTLET REGENERATION IN ASHWAGANDHA (*WITHANIA SOMNIFERA* L.)

A. JHANKARE[1], M.K. TRIPATHI[1], G. TIWARI[2], G. N. PANDEY[3], B. S. BAGHEL[4] AND S. TIWARI [5]

[1]Horticultural Biotechnology Laboratory, [2]Department of Medicinal and Aromatic Plants, [3]Department of Plant Pathology, [4]KNK-College of Horticulture, Mandsaur 458 001, India

[5]Biotechnology Centre, J.N. Agricultural University, Jabalpur 482 004, India

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SUMMARY

The establishment of embryogenic cell suspension culture from the embryogenic callus culture of *Withania somnifera* (L.) was performed by transferring mature embryo and hypocotyl embryogenic callus cultures in liquid media. The cultures obtained were swamped with clumps of proliferating globular embryos with modest non-embryogenic tissues. The number and size of somatic embryos/clumps was recorded to compute growth of embryogenic tissues under various conditions. Initiation and proliferation of embryogenic suspension culture was influenced by genotypes and various exogenous plant growth regulators fortified to the culture medium at variable magnitude. For the establishment of suspension cultures, MS medium fortified with 1.0 mgl^{-1} 2,4-D with 0.5 mgl^{-1} BAP was found to be the most effective. For subsequent subculturing, the reduced level of 2,4-D (0.5 mgl^{-1}) in combination with 0.5 mgl^{-1} BAP promoted somatic embryogenesis at a faster rate. Frequent and efficient plantlet regeneration occurred on MS medium with 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} each of NAA and kinetin. For culture efficiency genotype JA-20 responded well as compared to MWS-100. A combination of 65% relative humidity and 28°C temperature regime exhibited higher survival of regenerated plantlets (~90%) followed by 60% RH and 30°C (~85%). Later approximately 80% plants survived field transplantation.

Keywords: **Cell suspension culture, plantlet regeneration, somatic embryogenesis, *Withania somnifera***

INTRODUCTION

Ashwagandha [*Withania somnifera* (L.) Dunal], also known as Indian ginseng, is an important medicinal crop and widely cultivated throughout the world. Due to over-exploitation of natural resources for the extraction of these compounds this species is becoming endangered under natural habitat. Ashwagandha is propagated by seeds and the variability generated by out crossing and recombination is very high. In *Withania*, likewise other crop species, efficient and frequent plant regeneration through embryogenic cell suspension cultures is imperative for novel crop improvement techniques such as somaclonal variation, *in vitro* selection, protoplast fusion and genetic transformation.

In *Withania* an array of explants have been employed to establish regenerable callus culture system such as leaf disc (Rani *et al*. 2003), cotyledon (Rani *et al*. 2003), shoot tips (Supe *et al.* 2006), hypocotyl (Anjali *et al*. 2000, Rani *et al*. 2003), root (Rani *et al*. 2003), apical bud (Sivanesan 2007), nodal segments (Anjali *et al*. 2000, Sivanesan and Murugesan 2008), auxiliary bud (Saritha and Naidu 2007), embryo (Anjali *et al*. 2000) including cell suspension culture (Ciddi 2006) with varying degree of success, however, till date no encouraging

*Corresponding author, E-mail: tiwarig_111@yahoo.co.in

report has been documented for efficient plantlet regeneration through embryogenic cell suspension cultures of *Withania*. During the present investigation efforts have been made to ascertain the effects of plant growth regulators, genotypes and culture conditions on initiation of embryogenic suspension culture followed by plantlet regeneration of *Withania*.

MATERIALS AND METHODS

Experimental materials: Two cultivars of ashwagandha *viz*: Jawahar Asgandh -20 (JA-20) and Mandsaur *Withania somnifera* - 100 (MWS-100) were selected to carry out the present study.

Culture media: During the preliminary investigation with varied fortifications of two basal media *viz*: MS (Murashige and Skoog 1962) and B_5 (Gamborg *et al*. 1968), MS basal medium exhibited a better response. Consequently, MS was used as basal medium for all later experimentations. Apart from MS basal micro and macro salts, vitamins, and agar powder, three auxins, namely: 2,4-D, NAA and 2,4,5-T and three cytokinins *viz*: BAP, kinetin and TDZ in varying concentrations were added to fortify MS media for initiation of callus cultures from mature embryo and hypocotyls explants and subsequently raising of embryogenic cell suspension cultures. Based on the initial experiment, main experiment comprises of varying concentrations of BAP and Kn in combination with NAA and 2,4-D.

Establishment of callus cultures: Mature embryo explants were excised from pre-soaked surface sterilised seeds for 24 hours in sterilised double distilled water and hypocotyl from 4-7 days-old seedlings. Cultured Petri dishes with 7-8 explants were incubated under complete darkness at 25±2°C for 1 week. Later the cultures were subjected to 16 h 2000 lux luminance provided with cool white fluorescent lamps.

Initiation of cell suspension culture: For raising suspension cultures three to four weeks-old embryogenic calli (3 g fresh mass) obtained from mature embryo and hypocotyl cultures were transferred to 250 ml Erlenmeyer flasks containing 50ml of MS liquid medium. Callus pieces were strained through a stainless steel mesh (1mm) and were agitated on a horizontal shaker (140 rpm) at 25±2°C under complete darkness. After 15 days the cultures were sieved aseptically to remove large clumps and 10 ml filtrate was added with 40 ml of fresh medium of the old suspension for subculturing. Relative growth rate was calculated on the basis of increment in fresh weight after culturing of embryogenic calli on different liquid medium after 4 weeks of initial culture. Cell cultures were examined microscopically within 15 to 30 days for somatic embryo induction and determination of developmental stages.

Plantlet regeneration: Cell clumps/embryoids 2.5 to 5 mm in diameter acquired from 4-6 weeks old liquid suspension cultures were placed onto fortified solid MS regeneration medium supplemented with different concentrations and combinations of plant growth regulators (BA and kinetin alone as well as BAP and kinetin in combination with NAA), 20 gl^{-1} sucrose and 7.5 gl^{-1} agar (Table 4). Cultures were subjected to 25±2°C and 16 h photoperiod regimes of 2000 lux luminance provided by PAR lamps. Frequency of plantlet regeneration was calculated as percentage of cell clumps/embryoid with plantlets from total cell clumps/ embryoid plated. Observations were recorded for percentage of shoots proliferating clumps/embryoid, average number (s) of shoots per clumps/embryoid and mean shoot length. Factorial Completely Randomised Design (CRD-factorial) was used to find out the significance of genotype, culture media combinations and their interactive effect. Each treatment consisted of two replications. Per replication approximately 500-800 clumps/embryoid were cultured on each media of each genotype. The data was analysed as per method suggested by Snedecor and Cochran (1967).

In vitro rooting of regenerants: At times root formation was not attained on regeneration medium, plantlets were subsequently transferred to MS + 20 gl^{-1} sucrose + 7.5 gl^{-1} agar fortified with varying strength of IBA, NAA, and Kn alone or IAA in combination with BA and Kn. Observations were recorded for percentage of root proliferating shoots, average number (s) of root(s) per shoot and mean root length.

Acclimatisation of regenerants: Rooted plants were thoroughly washed under running tap water to remove the adhering agar and were planted in 2.5 cm root

trainers filled with 1:1:1 sand, soil and FYM sterilized mixture. Root trainers with transplanted plants were placed under 30±2°C and 60±5% RH for 15-20 days in a greenhouse for acclimatisation. Finally, acclimatised plants were transferred to the field.

RESULTS AND DISCUSSION

The first response of cultured mature cotyledons and hypocotyls was similar after 5-7 days and mostly independent from explant and culture media. All explants became swollen and no callus proliferation was evident during the first few days. After 5-7 days of culture, callus initiation was observed from most of the explants. Callus initiation usually started from the cut edges from cultured mature cotyledons, whereas, from hypocotyl segment, callus initiation usually started from the wounded ends and spread towards the middle region. The induced calli were light to moderate yellow - greenish in colour, small to medium in size and friable to compact in texture. Friable calli initiated non-morphogenic calli from both explants. On the other hand compact callus with green nodules were not found suitable for initiating cell suspension cultures.

For initiating suspension cultures, inoculation of ~3.0 g embryogenic calli was beneficial for continued maintenance and proliferation of the embryogenic suspension cultures. Higher response from low explants inoculation frequency during the present experiments is in accordance with the reports of wild cotton (Finer *et al*. 1987) and onion (Tiwari *et al*. 2007). To obtain a fine suspension culture, 21-28 days old pale yellow friable calluses were used. Increased friability of the callus facilitated the separation of cells. During incubation, the biomass in suspension increased due to cell division and enlargement. This continued for a limited period as the growth stopped due to the exhaustion of some factors or the accumulation of certain toxic metabolites in the culture medium. Cell growth revived after the subculture by transferring small aliquot of the cell suspension to a fresh medium.

During an initial experiment, it was observed that in liquid culture, calli could not disintegrate to form suspension of cells or small cell clumps. Tiwari *et al*. (2007) reported similar behaviour for onion callus in liquid medium. To obtain small cell clumps, calluses were agitated mechanically. Friable calli when agitated were easily broken and dispersed into clumps of 1.0-5.0 mm in diameter. Further, agitation fragmented these clumps into small cell aggregates (Fig.1 A-B). Genotype JA-20

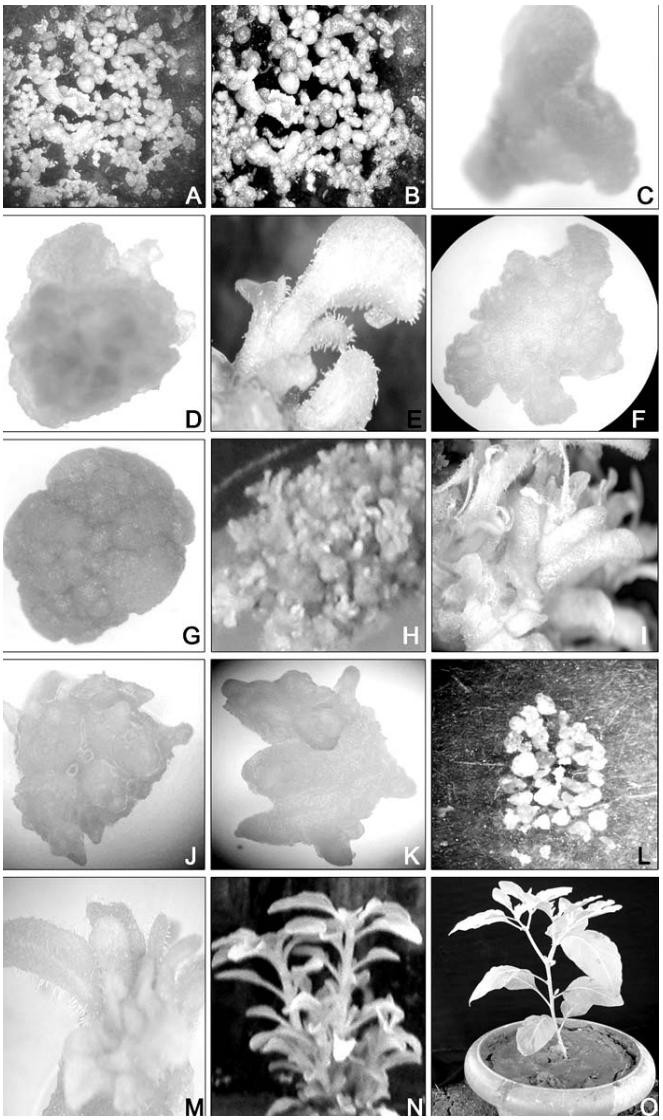

Fig. 1. Suspension culture of *Withania*: A. Initiation of cell clumps and embryoid formation from suspension culture raised from embryogenic calli of hypocotyls; B. Initiation of cell clumps and embryoid formation from suspension culture raised from embryogenic calli of mature embryos; C. Typical bipolar embryoid; D. Germination of embryoid; E. Shoot initiation from embryoids; F-G. Multiple embryoid formation; H. Germination of multiple embryoids; I. Shoot initiation from multiple embryoids; J-K.Typical multi-polar embryoid; L. Embryoid/cell clumps transferred into regeneration medium; M-N. Regenerated plantlets; O. Regenerant transferred in pot.

responded better as compared to other genotypes for higher growth rate and clump formation leading to *in vitro* embryogenesis. Cells obtained from the suspension cultures, when viewed under the microscope were undifferentiated meristem-like with dense cytoplasm and prominent nuclei.

Suspension cultures raised from embryogenic callus produced embryoids that enlarged and germinated after maturation (Fig. 1C-E). In succession to embryogenesis *in vitro*, embryogenic cultures proliferated multiple embryoids from subcultured primary embryoids (Fig. 1F-H). Developing globular embryos were characteristically yellowish-green and varied 1.0 to 5 mm in diameter. Clumps were multi-lobed with each lobe representing an early-stage globular embryo (Fig. 1F-G). Embryos were apparently attached to the base of the cultures and clearly indicated that the embryos arose from the primary embryos. This finding was in accordance to the findings of Kageyama *et al*. (1991) for muskmelon and Tiwari *et al*. (2007) for onion embryogenic suspension cultures. Proliferation of primary embryos gave rise to concentric layering of the proliferating secondary embryos until the clumps enlarged and separated into the liquid culture medium. Usually, embryoids gave rise to single plant regeneration, however, few embryoids turned into multi-polar structures exhibiting multiple gammogenesis from single embryoid (Fig. 1I-J).

Auxin 2,4-D has proven extremely useful for raising embryogenic cultures (Smith and Street 1974, Vasil and Vasil 1981). In many instances, elimination or change of auxin concentration is necessary for somatic embryo development. The effect of various auxins at different levels on initiation of suspension culture is presented in Table 1. Although, at lower levels all the auxins have been found to initiate embryoid proliferation and increase fresh mass, however, such embryos fail to produce

Table 1. Effect of auxins fortified in varying concentrations to MS medium on growth of cell suspension cultures derived from callus of mature embryos and hypocotyls

Auxins	**mgl^{-1}**	**Mature embryo derived**				**Hypocotyl derived**			
Cultivar		**JA20**		**MWS100**		**JA20**		**MWS100**	
		FW (g)	**RG (%)**	**FW (g)**	**RG (%)**	**FW (g)**	**RG (%)**	**FW (g)**	**RG (%)**
Control	0.0	3.00 ±0.12	100.00	3.00 ±0.11	100.00	3.00 ±0.10	100.00	3.00 ±0.08	100.00
2, 4-D	0.5	3.62 ±0.24	120.66	3.58 ±0.20	119.30	3.82 ±0.29	127.33	3.72 ±0.24	124.00
	1.0	3.80 ±0.26	126.66	3.74 ±0.28	124.66	3.94 ±0.38	131.33	3.88±0.30	129.33
	2.0	3.98 ±0.34	132.66	3.92 ±0.36	130.66	4.12 ±0.42	137.33	3.98 ±0.38	132.66
	3.0	4.12 ±0.38	137.33	3.96 ±0.38	132.00	4.26 ±0.44	142.00	4.14 ±0.39	138.00
	4.0	CM	-	CM	-	3.88 ±0.24	129.33	3.83 ±0.25	127.66
	5.0	CM	-	CM	-	3.60 ±0.26	120.00	3.54 ±0.24	118.00
NAA	0.5	3.60 ±0.22	120.00	3.44 ±0.20	114.66	3.48 ±0.22	116.00	3.42 ±0.21	114.00
	1.0	3.68 ±0.24	122.66	3.53 ±0.22	117.66	3.54 ±0.24	118.00	3.49 ±0.24	116.33
	2.0	3.70 ±0.28	123.33	3.66 ±0.26	122.00	3.60 ±0.24	120.00	3.46 ±0.22	115.33
	3.0	3.86 ±0.30	128.66	3.80 ±0.28	126.66	3.79 ±0.26	126.33	3.76 ±0.24	125.33
	4.0	3.89 ±0.32	129.66	3.82 ±0.30	127.33	3.82 ±0.29	127.33	3.74 ±0.28	124.66
	5.0	3.38 ±0.17	112.66	3.26 ±0.15	108.66	CC	-	CC	-
2, 4, 5-T	0.5	3.44 ±0.18	114.66	3.28 ±0.16	112.66	3.46 ±0.19	115.33	3.41 ±0.15	113.66
	1.0	3.46 ±0.20	115.33	3.40 ±0.18	113.33	3.48 ±0.17	116.00	3.44 ±0.18	114.66
	2.0	3.58 ±0.22	119.33	3.56 ±0.20	118.66	3.55 ±0.20	118.33	3.50 ±0.24	116.66
	3.0	3.64 ±0.24	121.33	3.70 ±0.22	116.66	3.59 ±0.21	119.66	3.53 ±0.28	117.66
	4.0	3.72 ±0.24	124.00	3.70 ±0.22	123.33	3.68 ±0.22	122.66	3.60 ±0.19	120.00
	5.0	3.82 ±0.26	127.33	3.79 ±0.24	126.33	3.84 ±0.24	128.00	3.80 ±0.24	126.66

CM: Cell Mortality, **CC**: Compact callus; **FW**: Fresh Weight; **RG**: Relative growth.

normal plants. At higher level (>3.0 mgl^{-1} 2, 4-D and >4.0 mgl^{-1}NAA) growth of the embryogenic suspension cultures stagnated. On the other hand, 2, 4, 5-T in fact a lower-activity auxin promoted embryo proliferation even at higher strength (~5.0 mgl^{-1}) in contrast to 2,4-D and NAA.

After initiating two types of cell suspensions from mature embryos and hypocotyls it was possible to distinguish the origin of cultures. Cell suspensions initiated from hypocotyls directly produced yellow-green chlorophyllous cells with rapid growth rate (Fig.1 B), while cell suspensions acquired from mature embryos (Fig. 1A) exhibited a slower growth rate with yellow-brown colour. As compared to the suspension cultures raised from hypocotyls, the initiation and growth response was totally different with all culture media combinations. Even after 3-4 subculturing, cell growth declined and suspension became necrotic. Several subcultures with culture medium containing higher concentrations of 2,4-D resulted in decline of cell growth and plasmolysis of the cells, which was mainly due to the gradual increase in the absolute concentration of 2,4-D per cell (Oridate and Oosawa 1986, Kageyama *et al*. 1991, Tiwari *et al*. 2007). The initiation responses of cell cultures were found to be auxin-specific as growth regulator NAA showed beneficial effect on the cell growth on suspension cultures raised from hypocotyls that kept it's fast growing phenol-free status for long-term. All the regenerants proved to be phenotypically stable under field-testing. This finding was in accordance to Jelaska (1974) who observed the necessity of auxins especially IBA and NAA for initiation of embryogenesis in pumpkin.

The effect of cytokinin on growth of the *Withania* embryogenic suspension is presented in Table 2. The lower level of cytokinins (0.5-2.0 mgl^{-1} BA, 0.5-3.0 mgl^{-1} kinetin and 0.1-0.5 mgl^{-1} TDZ) exhibited a slightly better

Table 2. Effect of cytokinins in varying concentrations on growth of cell suspension cultures derived from callus of mature embryos and hypocotyls

Cytokinins	**mgl^{-1}**	**Mature embryo derived cultures**				**Hypocotyl derived cultures**			
Cultivar		**JA20**		**MWS100**		**JA20**		**MWS100**	
		FW (g)	**RG (%)**	**FW (g)**	**RG (%)**	**FW (g)**	**RG (%)**	**FW (g)**	**RG (%)**
Control	0.0	3.00 ±0.10	100.00	3.00 ±0.12	100.00	3.00 ±0.12	100.00	3.00 ±0.14	100.00
BAP	0.5	3.20±0.16	106.66	3.16 ±0.15	105.33	3.42 ±0.22	114.00	3.36 ±0.22	112.00
	1.0	3.44 ±0.20	114.66	3.38 ±0.21	112.66	3.82 ±0.26	127.33	3.68 ±0.25	122.66
	2.0	3.18 ±0.13	106.00	3.14 ±0.12	104.66	3.34 ±0.16	111.33	3.24 ±0.14	108.00
	3.0	CC	-	CC	-	CC	-	CC	-
	4.0	CC	-	CC	-	CC	-	CC	-
	5.0	CC	-	CC	-	CC	-	CC	-
Kinetin	0.5	3.38 ±0.18	112.66	3.30 ±0.20	110.00	3.56 ±0.18	118.66	3.48 ±0.16	116.00
	1.0	3.52 ±0.22	117.33	3.47 ±0.18	115.66	3.74 ±0.22	124.66	3.69 ±0.20	123.00
	2.0	3.58 ±0.24	119.33	3.51 ±0.22	117.00	3.80 ±0.24	126.66	3.81 ±0.22	127.00
	3.0	3.66 ±0.28	122.00	3.60 ±0.36	120.00	3.78 ±0.28	126.00	3.72 ±0.26	124.00
	4.0	3.42 ±0.20	114.00	3.37±0.19	112.33	3.52 ±0.16	117.33	3.45 ±0.18	115.00
	5.0	FNMC	-	FNMC	-	FNMC	-	FNMC	-
TDZ	0.1	3.14 ±0.11	104.66	3.10 ±0.10	103.33	3.28±0.15	109.33	3.25±0.14	108.33
	0.2	3.22 ±0.16	107.33	3.12 ±0.12	104.00	3.36±0.19	112.00	3.32±0.18	106.66
	0.3	3.34 ±0.20	111.33	3.26 ±0.16	108.66	3.49±0.22	116.33	3.44±0.20	114.66
	0.5	3.44 ±0.24	114.66	3.36 ±0.18	112.00	3.57±0.24	119.00	3.51±0.22	117.00
	1.0	CC	-	CC	-	CC	-	CC	-
	2.0	CC	-	CC	-	CC	-	CC	-

CC: Compact callus; **FW**: Fresh Weight; **RG**: Relative growth, **FNMC:** Friable non-mrophogenic calli.

effect on growth of embryogenic tissues. Beyond these levels, higher cytokinin resulted in initiation of compact and non-embryogenic cultures. Culture media containing only cytokinins have been found to be ineffective for growth and development of suspension cultures of *Withania* since rapid growth and development of embryogenic cultures can be achieved without their presence. This phenomenon is in accordance with the findings of Ranch *et al*. (1985) in soybean and Tiwari *et al*. (2007) in onion as they documented that it is not necessary or beneficial to add cytokinins for raising embryogenic suspension cultures. However, in a number of other species cytokinins have been found suitable for induction of somatic embryogenesis (Nagarajun *et al*. 1986).

Since, fortification of culture media with auxins and cytokinins in separately did not support the initiation of suspension cultures in *Withania*, various combination of an auxin with a cytokinin were tested subsequently (Table 3). For the establishment of suspension cultures, the liquid media containing 1.0 mgl^{-1} 2,4-D in combination with lower concentration of BAP (0.5 mgl^{-1}) have been found to be the most effective. During initial two subculturing this media gave rise to loose, friable cell aggregates from the cultured embryogenic calli, which formed a fine and light cream cell suspension. For subsequent sub-culturing 2,4-D concentration was reduced to 0.5 mgl^{-1}, which supported growth and embryogenesis *in vitro*. Medium containing 2,4-D or NAA with kinetin proved unresponsive for efficient embryogenesis *in vitro* from mature embryo and formed large filamentous brown calli, failing to form suspension. However, this combination proved reasonable for hypocotyl culture.

Plant cell cultures are normally established and maintained on media containing an auxin and a cytokinin. Removal of either hormone from the medium would normally result in culture death (Stafford, 1996). Higher cell growth rates were obtained in a liquid medium containing BAP and 2,4-D. On the other hand a much lower cell division rate was observed without plant growth regulators. This is in accordance with the findings of Oridate and Oosawa (1986) and Kageyama *et al*. (1991) for muskmelon and Tiwari *et al*. (2007) for onion cell suspension cultures. However, the optimal concentration and combination of 2,4-D and BAP varied greatly from genotype to genotype and nature of explants indicating a role of genotypic variation in morphogenesis *in vitro*.

After 4 to 6 weeks cell clumps/embryoids were placed on various media for regeneration from embryoids (Fig. 1K-L). Growth regulator 2,4-D used in *Withania* does not support whole plants regeneration. During present experimentations, a combination of 0.5-1.0 mgl^{-1} cytokinin with 0.5 mgl^{-1} auxin supported higher shoot proliferating ability and number(s) of shoots (Table 4). MS basal medium supplemented with two cytokinin and one auxin *i.e*. 0.5 mgl^{-1} each of BAP, kinetin and NAA enhanced all plantlet regeneration attributes (shoot proliferating ability, number(s) of shoots and shoot length) as compared to medium with a cytokinin even at the concentration of 1.0 $mg.l^{-1}$ and an auxin responded scantily. Thus, it was concluded that plantlet formation is determined by quantitative interaction, *i.e*. ratios rather than absolute concentration of substances participating in growth and development. This is in accordance with the findings of Tiwari *et al*. (2007) for onion and Sivanesan (2007) and Kannan *et al*. (2005) for *Withania somnifera* tissue cultures.

During the present investigation, *in vitro* rooting frequency was found to be higher when were shoots transfered to rooting medium (Table 5). In *Withania*, auxins like IBA (Rani and Grower 1999, Govindraju *et al*. 2003, Manikam *et al*. 2000), IAA (Rani and Grower 1999, Supe *et al*. 2007) as well as NAA (Rani and Grower 1999) were found to be effective in inducing *in vitro* rooting. In the present study, full strength MS medium supplemented with IBA alone at the concentration of 1.0 mgl^{-1} was found to be optimum for proliferating 94.86% of shoots with an average of 7.32 roots per shoot. Higher root proliferation ability and number(s) of roots was exhibited by culture medium MSI as compared to MSN/MS2N, suggesting suitability of IBA for this purpose. Similar results have been reported by Govindraju *et al*. (2003) and Rani and Grover (1999) in *Withania*. Culture media supplemented with IBA alone respond better as compared to NAA even at higher levels suggesting effectiveness of IBA for *in vitro*

Table 3. Effects of cytokinins and auxins fortified in varying concentrations and combinations to MS medium on growth of embryogenic cell suspension cultures derived from mature embryo and hypocotyl culture

Culture	Growth regulators mgl^{-1}				Mature embryo derived cultures				Hypocotyl derived cultures			
Medium	2,4-D	NAA	BA	Kn	JA-20		MWS-100		JA-20		MWS-100	
					FW (g)	RG (%)	FW (g)	RG (%)	FW (g)	RG (%)	FW (g)	RG (%)
Basal MS	-	-	-	-	3.00 ±0.13	100.00	3.00 ± 0.11	100.00	3.00±0.13	100.00	3.00±0.11	100.00
MS5D5B	0.5	-	0.5	-	4.12 ±0.24	137.33	4.08 ± 0.22	136.00	4.30±0.25	143.33	4.12±0.23	137.33
MSD5B	1.0	-	0.5	-	4.86 ±0.38	162.00	4.65 ± 0.36	155.00	5.98±0.44	166.00	5.94±0.42	164.66
MS2D5B	2.0	-	0.5	-	4.44 ±0.32	148.00	4.32 ± 0.30	144.00	4.68±0.32	156.00	4.62±0.30	154.00
MS3D5B	3.0	-	0.5	-	4.26 ±0.26	142.00	4.21 ± 0.25	140.33	4.46±0.28	148.66	4.40±0.26	146.66
MS4D5B	4.0	-	0.5	-	CM	-	CM	-	CM	-	CM	-
MS.5D5Kn	0.5	-	-	0.5	3.98 ±0.22	132.66	3.90± 0.21	130.00	4.10±0.26	136.66	4.02±0.25	134.00
MSD5Kn	1.0	-	-	0.5	4.23 ±0.28	141.00	4.12± 0.26	137.33	4.30±0.28	143.33	4.22±0.26	140.66
MS2D5Kn	2.0	-	-	0.5	4.13 ±0.24	137.66	4.06± 0.28	135.33	4.43±0.30	147.66	4.36±0.29	145.33
MS3D5Kn	3.0	-	-	0.5	4.12 ±0.22	137.33	3.98± 0.28	132.66	4.24±0.28	141.33	4.18±0.26	139.33
MS4D5Kn	4.0	-	-	0.5	3.86 ±0.20	128.66	3.77± 0.24	125.66	3.94±0.24	131.33	3.87±0.22	129.00
MS.5N5B	-	0.5	0.5		3.96 ±0.22	132.00	3.90± 0.21	130.00	4.16±0.24	138.66	3.90±0.22	130.00
MSN5B	-	1.0	0.5		4.46 ±0.30	148.66	4.40± 0.29	146.66	4.68±0.34	156.00	4.56±0.32	152.00
MS2N5B	-	2.0	0.5		4.22 ±0.28	140.66	4.10± 0.28	136.66	4.44±0.32	148.00	4.36±0.30	145.33
MS3N5B	-	3.0	0.5	-	3.94 ±0.24	131.33	3.80± 0.22	126.66	3.98±0.28	132.66	3.90±0.28	130.00
MS4N5B	-	4.0	0.5	-	3.78 ±0.20	126.00	3.72± 0.18	124.00	3.89±0.26	129.66	3.82±0.24	127.33
MS5N5Kn	-	0.5	-	0.5	3.12 ±0.12	104.00	3.18± 0.11	106.00	3.59±0.17	119.66	3.48±0.16	116.00
MSN5Kn	-	1.0	-	0.5	3.58 ±0.18	119.33	3.48± 0.16	116.00	3.68±0.24	122.66	3.62±0.22	120.66
MS2N5Kn	-	2.0	-	0.5	3.66 ±0.20	122.00	3.60± 0.19	120.00	3.86±0.26	128.66	3.80±0.25	126.66
MS3N5Kn	-	3.0	-	0.5	3.78 ±0.22	126.00	3.56± 0.20	118.66	3.82±0.25	127.33	3.74±0.23	124.66
MS4N5Kn	-	4.0	-	0.5	3.66 ±0.19	122.00	3.52± 0.17	117.33	3.79±0.24	126.33	3.74±0.22	124.66

CM: Cell Mortality, **FW**: Fresh Weight; **RG**: Relative growth.

Table 4. Effects of growth regulators fortified in varying concentrations and combinations to MS medium on shoot proliferating ability of embryogenic cell suspension cultures arising from mature embryo and hypocotyl culture of two genotypes JS20 and MWS100

Culture	mgl^{-1}			Shootlet initiation efficiency (%)						Shootlet regeneration (%)					
Media	BAP	Kin	NAA	Mature embryo derived			Hypocotyl derived			Mature embryo derived			Hypocotyl derived		
Genotypes				JA20	MWS	Mean	JA20	MWS	Mean	JA20	MWS	Mean	JA20	MWS	Mean
MS1B	0.1	-	-	62.43	56.37	**59.40^{f}**	65.36	61.52	**63.44^{g}**	3.39	3.69	**3.54^{i}**	3.90	3.81	**3.85^{kl}**
MS5B	0.5	-	-	70.94	66.90	**68.92^{bc}**	76.65	71.32	**73.98^{c}**	4.38	4.10	**4.24^{hi}**	4.82	4.60	**4.71^{k}**
MSB	1.0	-	-	73.36	67.24	**70.30^{b}**	77.87	72.80	**75.33^{c}**	6.11	6.05	**6.08^{h}**	6.32	6.25	**6.28^{j}**
MS2B	2.0	-	-	68.25	60.19	**64.22^{d}**	73.32	63.20	**68.26^{ef}**	7.11	7.07	**7.90^{fg}**	7.87	7.69	**7.88^{i}**
MS3B	3.0	-	-	63.92	57.88	**60.90^{f}**	71.18	60.80	**65.99^{fg}**	6.12	6.10	**6.11^{gh}**	6.57	6.40	**6.48^{j}**
MS1Kn	-	0.1	-	18.08	14.04	**16.06^{j}**	26.35	17.76	**22.05^{j}**	2.22	2.14	**2.18^{i}**	2.40	2.30	**2.35^{l}**
MS5Kn	-	0.5	-	26.78	22.73	**24.75^{g}**	31.67	25.92	**28.79^{h}**	3.27	3.22	**3.24^{i}**	3.47	3.51	**3.49^{l}**
MSKn	-	1.0	-	22.44	18.40	**20.42^{hi}**	27.41	21.47	**24.44^{i}**	4.10	4.07	**4.08^{i}**	4.30	4.25	**4.27^{k}**
MS2Kn	-	2.0	-	23.54	19.50	**21.52^{h}**	26.10	20.96	**23.53^{ij}**	5.12	5.09	**5.11^{h}**	5.37	5.27	**5.32^{jk}**
MS3Kn	-	-	-	20.51	16.37	**18.44^{ij}**	22.12	19.87	**20.99^{j}**	5.10	5.04	**5.07^{h}**	5.21	5.31	**5.26^{k}**
MS5B5N	0.5	-	0.5	74.30	66.20	**70.25^{b}**	79.17	71.39	**75.28^{c}**	15.61	11.57	**13.59^{e}**	16.50	12.46	**14.48^{f}**
MSB5N	1.0	-	0.5	78.94	70.90	**74.92^{a}**	84.30	75.68	**79.99^{b}**	19.61	15.57	**17.59^{d}**	20.16	18.12	**19.14^{d}**
MS2B5N	2.0	-	0.5	71.93	63.87	**67.90^{d}**	76.24	71.65	**73.94^{c}**	15.37	12.33	**13.85^{e}**	17.47	16.35	**16.98^{e}**
MS3B5N	3.0	-	0.5	68.69	64.65	**66.67^{cd}**	71.14	69.15	**70.14^{de}**	10.48	85.42	**9.45^{f}**	12.37	11.13	**11.75^{g}**
MS5Kn5N	-	0.5	0.5	65.77	61.73	**63.75^{de}**	70.86	64.72	**67.79^{f}**	7.68	5.62	**6.65^{g}**	8.12	7.10	**7.61^{i}**
MSKn5N	-	1.0	0.5	68.57	60.53	**64.55^{d}**	73.66	63.48	**68.57^{e}**	10.70	6.66	**8.68^{f}**	12.60	10.14	**11.37^{gh}**
MS2K5N	-	2.0	0.5	65.54	61.48	**63.51^{e}**	72.56	62.56	**67.56^{f}**	10.55	8.51	**9.53^{f}**	12.58	10.46	**11.52^{g}**
MS3K5N	-	3.0	0.5	65.27	57.19	**61.23^{ef}**	69.84	60.68	**65.26^{g}**	7.65	7.53	**7.59^{g}**	10.70	8.64	**9.67^{h}**
MS5B5K5N	0.5	0.5	0.5	75.39	71.35	**73.37^{a}**	90.92	79.96	**85.44^{a}**	25.78	21.63	**23.70^{c}**	30.68	24.89	**27.78^{c}**
MSB5K5N	1.0	0.5	0.5	78.28	74.23	**76.25^{a}**	88.68	73.85	**81.26^{b}**	36.65	31.50	**34.07^{a}**	38.75	34.71	**36.73^{a}**
MSK5B5N	0.5	1.0	0.5	70.84	62.80	**66.82^{c}**	72.94	68.82	**70.88^{d}**	32.52	29.80	**31.16^{b}**	34.70	30.92	**32.81^{b}**
Mean				**58.75^{a}**	**53.07^{b}**		**64.20^{a}**	**57.02^{b}**		**11.4^{a}**	**9.79 ^{b}**		**12.61^{a}**	**11.15^{b}**	
CD (0.05)															
Genotypes						**1.07**			**0.93**			**0.80**			**0.63**
Medium						**2.89**			**2.53**			**2.18**			**1.70**
G x M						**5.00**			**4.38**			**3.77**			**2.95**

Values within column followed by different letters are significantly different at 5% probability level

Table 5. Effect of different plant growth regulators (PGR) on *in vitro* root proliferation of shooltets acquired from cell suspension cultures arising from mature embryo and hypocotyl culture of two genotypes JS20 and MWS100

Culture	PGR (mg l^{-1})				Root proliferating shoots (%)			Number of roots			Root length (cm)		
Medium	**IBA**	**Kn**	**BAP**	**NAA**	**JA20**	**MWS**	**Mean**	**JA20**	**MWS**	**Mean**	**JA20**	**MWS**	**Mean**
MS5I	0.5	-	-	-	82.88	78.84	**80.86^{d}**	5.33	4.51	**4.92^{e}**	4.88	4.72	**4.80^{c}**
MSI	1.0	-	-	-	96.88	92.84	**94.86^{a}**	7.40	7.24	**7.32^{a}**	5.16	5.08	**5.12^{b}**
MS2I	2.0	-	-	-	87.77	81.71	**84.74^{c}**	5.98	4.94	**5.46^{cd}**	4.92	4.84	**4.88^{c}**
MS3I	3.0	-	-	-	88.78	84.74	**86.76^{bc}**	4.91	4.85	**4.88^{e}**	4.38	4.22	**4.30^{d}**
MS5N	-	-	-	0.5	78.32	68.28	**73.30^{ef}**	4.72	4.65	**4.68^{ef}**	3.26	3.16	**3.21^{f}**
MSN	-	-	-	1.0	80.25	76.21	**78.23^{de}**	5.14	5.10	**5.12^{e}**	3.72	3.64	**3.68^{e}**
MS2N	-	-	-	2.0	66.36	62.32	**64.34^{g}**	5.19	5.05	**5.12^{de}**	2.89	2.83	**2.86^{g}**
MS5Kn	-	0.5	-	0.5	71.84	69.80	**70.82^{f}**	4.88	2.84	**3.86^{g}**	2.37	2.27	**2.32^{h}**
MSKn	-	1.0	-	0.5	76.27	72.23	**74.25^{ef}**	4.90	4.86	**4.88^{e}**	3.82	3.70	**3.76^{e}**
MS2Kn	-	2.0	-	0.5	67.36	63.32	**65.34^{g}**	3.61	3.55	**3.58^{gh}**	2.30	2.18	**2.24^{h}**
MS5I5B	0.5	-	0.5	-	72.94	68.90	**70.92^{f}**	3.45	3.35	**3.40^{h}**	2.28	2.18	**2.23^{h}**
MSI5B	1.0	-	0.5	-	82.85	76.79	**79.82^{d}**	5.80	5.67	**5.73^{c}**	5.32	5.24	**5.28^{a}**
MS2I5B	2.0	-	0.5	-	82.82	78.78	**80.80^{d}**	5.35	5.05	**5.20^{d}**	5.24	5.12	**5.18^{ab}**
MS3I5B	3.0	-	0.5	-	70.38	64.34	**67.36^{fg}**	3.35	3.23	**3.29^{h}**	2.89	2.83	**2.86^{g}**
MS.5I5Kn	0.5	0.5	-	-	78.34	72.28	**75.31^{e}**	4.50	4.20	**4.35^{f}**	3.17	3.07	**3.12^{f}**
MSI5Kn	1.0	0.5	-	-	90.97	86.93	**88.95^{b}**	7.50	5.40	**6.45^{b}**	4.96	4.88	**4.92^{c}**
MS2I5Kn	2.0	0.5	-	-	78.93	75.87	**77.40^{de}**	5.72	5.64	**5.68^{c}**	3.28	3.22	**3.25^{f}**
MS3I5Kn	3.0	0.5	-	-	74.87	70.83	**72.85^{ef}**	3.30	3.26	**3.28^{h}**	2.35	2.25	**2.30^{h}**
Mean					**79.04^{a}**	**74.44^{b}**		**5.05^{a}**	**4.63^{b}**		**3.73^{a}**	**3.63^{b}**	
CD (0.05)													
Genotypes							**1.41**			**0.19**			**0.05**
Media							**3.81**			**0.47**			**0.13**
G x M							**6.60**			**0.81**			**0.22**

Values within column followed by different letters are significantly different at 5% probability level.

rooting of shoots arising from cell suspension cultures in *Withania*.

Plant regeneration ability varied considerably between two genotypes (Table 4). Genotype JA-20 was found consistently superior as compared to MWS-100 for all attributes investigated. Genotype JA-20 exhibited higher shoot regeneration capability i.e. 58.75% from mature embryo and 64.20% from hypocotyl derived embryoids. Tiwari *et al*. (2007) have also reported strong genotypic effects in shoot regeneration from cell suspension culture in *Allium cepa*.

Regenerated plantlets were successfully acclimatised with an average 85-90% survival under greenhouse conditions. An arrangement of 28°C and 65% RH exhibited higher survival (~90%) followed by 30°C and 60% RH (~85% survival rate). Approximately 80% plants survived field transplantation (data not presented). The regenerated plants *via* embryogenesis appeared phenotypically normal and true to the type. Embryogenic suspension cultures sustained over 8 months with fortnightly subculture without any loss in embryogenic and regenerative ability.

During the present investigation, we used mature embryo and hypocotyl obtained from mature seed instead of immature seeds to obtain embryo and cotyledon explants. The liquid culture system in the present study improves somatic embryogenesis and in turn plant regeneration in *Withania*. It is considered that the liquid culture system may be more efficient in cell selection, for micropropagation and for production of secondary metabolites. The suspension cultures may be a good source for the isolation of totipotent protoplasts for potential use in genetic manipulations. Furthermore, somatic embryos produced in suspension culture will be more accessible for *Agrobacterium*-mediated or mechanical transformation.

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