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In vitro Callus Induction and Plant Regeneration From *Withania coagulans*: A Valuable Medicinal Plant

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Abstract: Leaf and internode explants of *Withania coagulans* Dunal were used to evaluate the effect of growth regulators on the *in vitro* callus induction and plant regeneration. Two independent experiments were performed to establish callus and plant regeneration. In the first experiment, leaf segments were cultured on MS basal medium fortified with 2, 4-dichlorophenoxyacetic acid (2, 4-D, 2-4 mg L^{-1}) with combination of 6-benzyladenine (BA, 0.5-1 mg L^{-1}) or kinetin (Kin, 0.5-1 mg L^{-1}). Callus initiation was observed best in all media after 14-16 days (100%). Highest callus growth in terms of dry weight (76±5.34 mg) was observed in MS medium fortified with 2 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} Kin. In the second experiment, callusing also obtained from internode segments cultured on MS medium with the presence of 2, 4-D (2-4 mg L^{-1}) in association with BA (0.25-0.5 mg L^{-1}). Best callusing rates (42%) and dry weight (86±3.68 mg) occurred in the presence of 4 mg L^{-1} 2, 4-D with combination of 0.25 mg L^{-1} BA. Shoot induction was obtained just from callus induced from internode explants on MS medium containing BA (2 mg L^{-1}) with IBA (0.5 mg L^{-1}), but the yield of shoot regeneration was unsatisfactory (18-33%). Regenerated shoots were rooted best (100%) on half strength MS medium containing IBA (2 mg L^{-1}) and produced 23 roots with an average root length of 6.5±0.41 cm. Rooted plantlets transferred to soil and sand mixture (2:1) showed 75% survival when transferred to outdoor. The procedure reported here *in vitro* culture of *W. coagulans* for first time.

Key words: Callus, medicinal plant, tissue culture, *Withania coagulans*

INTRODUCTION

The genus *Withania* is an important member of the family Solanaceae. Twenty three species of the genus *Withania* have been reported (Negi *et al*., 2006). Plants of *Withania* genus are distributed in the east of the Mediterranean region and South Asia (Negi *et al*., 2006; Atta-ur-Rahman *et al*., 1999). *Withania coagulans* is commercially important because of the ability possessed by its berries to coagulate milk (Negi *et al*., 2006; Hemalatha *et al*., 2008). This plant is well known in the indigenous system of medicine for the treatment of ulcers, rheumatism, dropsy, consumption and sensile debility (Atta-ur-Rahman *et al*., 1999). It has received much attention in recent years due to the presence of a large number of steroidal alkaloids and lactones known as withanolides (Hemalatha *et al*., 2008).

Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as selection, multiplying, *in vitro* regeneration, genetic transformations and conserve the critical genotypes of medicinal plants (Tripathi and Tripathi, 2003; Rout *et al*., 2000). The production of withanone and withanolide A from *Withania somnifera* by undifferentiated callus cultures and cell suspensions induced using the natural variations of cells (different explants) and Plant Growth Regulators (PGRs) is noteworthy (Sharada *et al*., 2007). Tissue cultures having different morphology analyzed for withanolide production showed that the inherent biosynthetic capability of the donor plant was retained in cultures and they produced withanolides *in vitro* (Sharada *et al*., 2007). *In vitro* culture is more advantageous over a single shoot formation for rapid clonal multiplication as well as for its conservation (Saritha and Naidu, 2007). Protocols for *in vitro* regeneration in *W. somnifera* were developed by Rani and Grover (1999). However, according to present evidence, there is no report about *W. coagulans* tissue culture.

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Therefore, the present study was undertaken in order to examine the potential of different explants in responding to *in vitro* conditions with the possibility of developing a protocol for the *in vitro* culture of *W. coagulans*.

MATERIALS AND METHODS

Production of plantlets as the explant source: Mature fruits of *W. coagulans* were collected from the medicinal plant garden of Agriculture and Natural Resources of Saravan College, Saravan, Iran, in the summer 2008. Each fruit (berry) contains many seeds. After removing the pulp, endocarp was manually broken to release the intact seeds. Seeds were thoroughly washed in running tap water for 30 min to remove any adherent particles and were rinsed in 70% (v/v) ethyl alcohol for 30 sec followed by 2% (v/v) sodium hypochlorite for 10 min. Seeds were then washed under sterile conditions with sterile distilled water and imbibed overnight (for 16 h) in sterile distilled water at 25±2°C. Two seeds were inoculated in each glass culture tube (2.5×15 cm) containing 20 mL of MS (Murashige and Skoog, 1962) basal medium with 2% (w/v) sucrose and 0.8% (w/v) agar (Merck) without growth regulators. The pH of the medium was adjusted to 5.8 before autoclaving for 10 min at 121°C. The cultures were incubated at 25±2°C, with 24 h light (30 μmol/m^2/sec) provided by cool white fluorescent tubes in growth chamber (Kulkarni *et al*., 2000). Leaf and internode segments excised from 1 month old aseptic seedlings were used as explants.

Callus initiation: The leaf explants (0.5×0.5 cm) and internode (0.5 cm long) from *W. coagulans* plantlet inoculated on agar-solidified MS medium. In the first experiment, leaf segments were cultured on MS medium supplemented with 2, 4-dichlorophenoxyacetic acid (2 ,4-D, 2-4 mg L^{-1}) with combination of 6-benzyladenine (BA, 0.5-1 mg L^{-1}) or kinetin (Kin, 0.5-1 mg L^{-1}). For the callus induction from internode segments, explants were cultured on MS medium in the presence of 2, 4-D (2-4 mg L^{-1}) in association with BA (0.25-0.5 mg L^{-1}). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. All chemicals were purchased from Merck. Cultures were maintained at 25±2°C in a 16/8 h light/dark cycle with a light intensity of 40 μmol/m^2/sec provided by cool white fluorescent. Callus was subcultured after 15 days on the original callus-inducing medium. The frequency of callus induction and callus dry weight was determined 8 weeks after culture initiation.

Shoot induction and rooting: For shoot induction, calli were cultured on MS medium containing 2 mg L^{-1} BA with 0.5 mg L^{-1} IBA and kept under the same conditions employed in callus initiation for 8 weeks. After 8 weeks, the number of shoots per treatment was recorded. To induce roots, elongated shoots were excised and transferred into culture tubes (23×150 mm) containing 20 mL of half strength MS medium supplemented with IBA or Kin (1-2 mg L^{-1}) either alone. A set containing MS medium without growth regulators served as control. Data were recorded after 4 weeks of culture. The rooted plantlets were washed with tap water to remove rooting medium debris. The young plants were transplanted into boxes containing a soil and sand mixture (2:1) autoclaved at 120°C for 20 min under non sterile conditions and gradually exposed to ambient humidity. After 10 days, the acclimated plants were transferred to a greenhouse, maintained under partial shade and irrigated daily. The percentage of survival was recorded at 2 weeks intervals.

Statistical analysis: All experiments had five replicates per treatment and each experiment was repeated twice. Observations based on the percentage of culture response with regard to leaf and internodes induction, dry weight of callus, number of shoots and roots per explant and roots length were recorded. Results were subjected to Analysis of Variance (ANOVA) and mean values were separated according to Duncan's multiple range test at $p = 0.05$. The statistical program used was MSTATC. The results are expressed as the Mean±SD of experiments.

RESULTS

Callus initiation: Callus initiation appeared for both explants after 14-16 days and callus was creamish-white, light green, compact or friable (Fig. 1A, B). Table 1 shows callus induction for leaves and internode segments. Leaf explant produced 100% callus induction in all media. Callus was also creamish and friable (Fig. 1A). Highest callus growth in terms of dry weight (76±5.34 mg) was observed in MS medium fortified with 2 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} Kin. Concentrations of BA (1 mg L^{-1}) enhanced the growth response in leaf explants. Kin at 1 mg L^{-1} showed a lower growth response in explants.

In second experiment, Internode explants showed an initial swelling at the cut end in all medium after the second week. The percentage culture response, callus color and texture varied according to the type of medium used (Table 1). Internode explants produced significantly very low percentage of callus formation (25-42%) (Table 1). The comparison of callusing potential of different explants showed that leaf explant is the best one, as its callusing capacity was 100%, but highest dry weight

Fig. 1: Callus induction and plantlet regeneration from leaf and internode segment explant of *W. coagulans*. (A) Induction of callus from leaf, (B) internode segment drived callus, (C) induction of multiple shoot from internode derived callus in MS+2 mg L^{-1} BA with 0.5 mg L^{-1} IBA, (D) shoot elongation, (E) rooting of regenerated shoot and (F) establishment in soil

Table 1: Callus induction from leaves and internode segments of *W. coagulans* on MS medium supplemented with various growth regulators

Source of explant	2, 4-D	BA	Kin	Percentage of callus formation	Color	Texture of callus	Dry weight of callus (mg)	Cultures producing shoots (%)
Internode	2	0.25	-	25	LG	C	75.20±8.23	18
	2	0.50	-	32	LG	C	82.00±11.19	33
	4	0.25	-	42	CW	C	86.00±3.68	-
	4	0.50	-	36	CW	C	70.20±11.51	-
Leaf	2	0.50	-	100	LG	F	52.56±3.64	-
	2	1.00	-	100	LG	F	58.80±3.24	-
	4	0.50	-	100	LG	F	59.50±5.53	-
	4	1.00	-	100	LG	F	64.60±5.87	-
	2	-	0.5	100	LG	F	76.00±5.34	-
	2	-	1.0	100	LG	F	54.40±5.87	-

–: No response. LG: Light green, CW: Creamish-white, C: Compact, F: Friable. Values represent Means±SD of 5 explants per treatment in five repeated experiments. Means followed by the same letter(s) are not significantly different by the DMRT at 5% probability level

Table 2: Effect of various concentrations of IBA and Kin on rooting after 6 weeks of incubation in half strength MS medium

Growth regulators (mg L^{-1})	No. of shoots/ treatment	Response	Shoots rooted (%)	No. of roots/ plant	Root length (cm)
Control	20	S	-	-	-
1 IBA	20	R	100	12	4.2±0.24
2 IBA	20	R	100	23	6.5±0.41
1 Kin	20	C	-	-	-
2 Kin	20	C	-	-	-

Values represent Means±SD. S: Swelling, R: Rooted, C: callusing, -: No response

(86±3.68 mg) occurred in callus derived from internode explant in the presence of 4 mg L^{-1} 2, 4-D with combination of 0.25 mg L^{-1} BA. Moreover, though leaf segment was best in callusing potential, but all callus be brown after subculture even by using 15 days intervals.

Shoot induction and rooting: Leaf explants failed to respond morphogenetically to a growth regulator and non shoot regeneration yield. Shoot induction was obtained just from callus induced from internodes explant 6 weeks after transferring the callus to MS medium containing 2 mg L^{-1} BA with 0.5 mg L^{-1} IBA (Fig. 1C), but shoot regeneration yield was unsatisfactory. The regeneration frequency varied from 18 to 33% and, by day 45, on average of 3-5 shoots were regenerated per explant up to two subcultures (Fig. 1D), average length of shoots per culture was 3.5 cm.

Regenerated shoots were rooted best (100%) on half strength MS medium containing 1-2 mg L^{-1} IBA (Fig. 1E). Medium with 2 mg L^{-1} IBA produced highest number of roots (23 roots/plant) with an average root length of 6.5±0.41 cm (Table 2). Rooted plantlets transferred to soil and sand mixture (2:1) autoclaved at 120°C for 20 min (Fig. 1F), showed 90% survival when transferred to outdoor.

DISCUSSION

Withania coagulans is normally propagated through seeds, but these methods are not efficient in producing large numbers of planting stock due to poor germination rate with low viability of seeds. Biotechnological interventions for *in vitro* regeneration, mass micro-propagation and gene transfer methods in medicinal species have been practiced with success especially in the last decade (Tripathi and Tripathi, 2003; Rout *et al*., 2000). The induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control of conditions in the culture medium (Tripathi and Tripathi, 2003). Protocols for *in vitro* regeneration in *W. somnifera* were developed (Rani and Grover, 1999) but the present study describes for the first time callus induction and plant regeneration from *W. coagulans*.

In vitro response of *W. coagulans* was varying depending on the explants. The comparison of callusing potential of different explants showed that leaf explant is the best one, as its callusing capacity was 100%, but no shoots could be regenerated from this source of callus and callus were turned brown after three subcultures. These results corroborate with the findings of Rani and Grover (1999) on *W. somnifera*. They reported callus induction from the leaves, hypocotyls, roots and cotyledonary leaf segments of *W. somnifera*. Maximum callusing (100%) was obtained from root and cotyledonary leaf segments grown on the medium supplemented with a combination of 2 mg L^{-1} 2, 4-D and 0.2 mg L^{-1} kinetin.

In the present investigation, it was observed that average of 3-5 shoots were regenerated per explant up to two subcultures. A similar finding of was reported in *W. somnifera* (Siddique *et al*., 2004; Sivansean and Murugesan, 2008). Present results suggest that the ability to form multiple shoots is dependent on explant source. Rani and Grover (1999) reported Maximum shoot multiplication on the medium containing 2 mg L^{-1} BA, also reported that Regenerated shoots rooted best on MS medium containing IBA (2 mg L^{-1}) alone and IBA (2 mg L^{-1}) with IAA (2 mg L^{-})^{1} in *W. somnifera*. For rooting, the various levels of IBA and Kin were tested. Result showed that 2 mg L^{-1} IBA proved to be most effective, the maximum number of rootlets with 6.5±0.41 root length (cm) obtained with 2 mg L^{-1} IBA (Table 2). It seems that Cytokinens (Kin) have no effect on rooting in *W. coagulans* regenerated microshoots. These results corroborate with the findings of Sivansean and Murugesan (2008) on *W. somnifera*. A successful tissue culture method of propagation must result in re-establishment in soil of a high frequency of tissue culture derived plant (Sivansean and Murugesan, 2008). In this study, regenerated plantlet of *W. coagulans* showed 75% of survival rate when transferred to outdoor. Rani and Grover (1999) and Sivansean and Murugesan (2008) obtained 83 and 87% survival rate in the *W. somnifera*, respectively.

CONCLUSION

The present study describes here *in vitro* culture of *W. coagulans* for first time. In summary, present experiment show that Use of internodes for micro propagation was beneficial than leaf explants. It will also be of use in conservation and genetic transformation studies aimed at improving the plant. However, the yield of shoot regeneration was unsatisfactory (18-33%). Regenerated shoots were rooted best (100%) on half strength MS medium containing IBA (2 mg L^{-1}) and produced 23 roots with an average root length of 6.5±0.41 cm. Since, the success rate of vegetative propagation in *Withania* sp. is very low (Kulkarni *et al*., 2000), It is hoped that a standard protocol to induce multiple shoots in culture may achieve.

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