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Full Length Research Paper

Development of efficient micropropagation protocol for *Withania coagulans* (Stocks) Dunal

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The present study describes the development of efficient micropropagation protocol for a commercially important medicinal plant *Withania coagulans.* Nodal segments were immersed in various concentrations of cefotaxime, viz 100, 250, 500 and 750 mgl^{-1} for 5 min and implanted on Murashige and Skoog medium (MS) medium fortified with 6-benzyladenine (BA) (2 to 4 mgl^{-1}) or indolebutyric acid (IBA) (0.25 to 0.5 mgl^{-1}) either alone or in combination with different concentrations. Of the four concentrations of cefotaxime tested, the 250 mgL^{-1} showed nonphytotoxic effect on cultures and completely eliminated bacterial infection. Direct multiple shoots differentiation occurred in cultured explants without intervening callus phase and the maximum number of shoots (7.2 ± 1.0 per explant) and elongation (7 ± 1.4 cm) were achieved on MS media containing 2 mgl^{-1} BA + 0.5 mgl^{-1} IBA. For induction of stout root system, the shoot buds were cultured on ½MS medium fortified with different concentrations of IBA (1 to 4 mgl^{-1}), indole-3-acetic acid (IAA) (0.25 to 1.0 mgl^{-1}) and kinetin (Kin) (1 to 2 mgl^{-1}). MS medium with 2 mgl^{-1} IBA was found most effective for the induction of stout root system. Well-rooted plantlets were transferred to outside pots containing sterile soil, and sand mixture (2:1) showed 75% survival.

Key words: *In vitro*, medicinal plant, propagation, *Withania coagulans.*

INTRODUCTION

Among the twenty-three known species of *Withania*, only two (*Withania somnifera* (L.) Dunal and *Withania coagulans* Dunal) are economically significant and widely cultivated (Mirjalili et al., 2009). *W. coagulans* is commercially important for its milk coagulating properties (Negi et al., 2006; Hemalatha et al., 2008; Ali et al., 2009). It is well known in the indigenous system of medicine for the treatment of ulcers, dyspepsia, rheumatism, dropsy, consumption and sensile debility (Hemalatha et al., 2008). It has received much attention in recent years due to the presence of a large number of steroidal alkaloids and lactones known as withanolides.

Three withanolides, viz coagulins P, Q and R, were isolated from the whole plant of *W. coagulans* (Atta-ur-Rahman et al., 1999). One of the most important withanolides is withaferin A, which is isolated from *Withania* extracts has anticancer properties (Yang et al., 2007). *Withania* is distributed in the east of the Mediterranean region and South Asia (Negi et al., 2006; Atta-ur-Rahman et al., 1999). It was abundant until a few decades ago, but ruthless collection for medicinal purposes, habitat destruction and climate changes makes the species to become endangered in their natural habitats. The erosion of plant biodiversity is a matter of global concern. Therefore, it is important to propagate and conserve them to meet up with future demand. The conventional propagation of this species is performed through seeds and cuttings of stem since root is too slow and labourious. *In vitro* propagation technique may be the best solution for its rapid multiplication and reestablishment in nature.

In vitro techniques have been found to be useful in the propagation of a large number of threatened and endangered plants (Sarasan et al., 2006). This technique

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Abbreviations: BA, 6-Benzyladenine; **IBA,** indolebutyric acid; **IAA,** indoleacetic acid; **Kin,** kinetin; **PGRs,** plant growth regulators.

are more advantageous over a single shoot formation for rapid clonal multiplication as well as for conservation (Saritha and Naidu, 2007). The induction of multiple shoots through axillary branching is now recognized as a useful technique for micropropagation and *in vitro* conservation of threatened plants (Constable, 1990). Micropropagation has many advantages over conventional methods of vegetative propagation, which suffer from several limitations. Protocols for *in vitro* regeneration in *W. somnifera* were developed (Rani and Grover, 1999), but there is still insufficient data for *in vitro* micropropagation of *W. coagulans*. Researches have tended to focus on *W. somnifera* rather than *W. coagulans*. Therefore, the present studies were undertaken with a view to evaluate the potential of nodal explant with the possibility of developing an efficient protocol for the *in vitro* micropropagation of *W. coagulans*.

MATERIALS AND METHODS

Plant material and surface sterilization

Nodal segments obtained from 5 year-old plants of *W. coagulans* between April and May 2008, in Saravan region (Sistan and Baluchestan Province, Iran) were used as explant. These were washed thoroughly under running tap water and disinfected first in 70% ethanol for 30 s and then in 2% (v/v) NaClO for 10 min. After rinsing several times with sterile distilled water, 1 to 2 cm sized nodal segments were excised aseptically and used for culture.

Immersion of the surface sterilized explants in cefotaxime antibiotic

Although initially surface sterilization was successful, endogenous microbial contamination at the base of the explants was a major problem after inoculation. We examined the problem of endogenous bacterial contamination in the micropropagation of *W. coagulans.* In this test, the surface sterilized explants were immersed in various concentrations of filter sterilized cefotaxime: 100, 250, 500 and 750 mgl^{-1} for 5 min to ensure a contamination free culture.

Shoot multiplication and elongation medium

After the treatment with cefotaxime antibiotic, the explants were implanted vertically onto culture tubes (23 × 150 mm) containing Murashige and Skoog (1962) medium with 3% (w/v) sucrose, 0.8% (w/v) agar, supplemented with different concentrations of 6-benzyladenine (BA, 0.5 to 4 mgl^{-1}) and indolebutyric acid (IBA, 0.25 to 0.5 mgl^{-1}) alone or in combination (Table 2). The pH of the medium was adjusted to 5.8 prior to the addition of agar. Culture medium was autoclaved at 120°C for 20 min at 1.2 kg/cm^2 or 15 lb/in^2. Cultures were maintained at 25 ± 2°C under 16/8 h light/dark cycle with 40 $\mu molm^{-2}s^{-1}$ provided by cool white fluorescent light. For shoots elongation, the shoots with no detectable signs of bacterial contamination were transferred onto culture medium without cefotaxime. For browning prevention in cut surfaces of the explants, these were transferred every 15 days on the fresh medium. The percentage of bud break, number of differentiated shoots per explant and shoot lengths were recorded after 8 weeks of culture.

Rooting and acclimatization

To induce strong and stout root system, the isolated micro-shoots (4 to 5 cm) with 4 to 6 leaves were separated from the clumps of multiple shoot buds and transferred to half strength MS medium supplemented with 2% (w/v) sucrose and various concentrations of IBA (1, 2, 4 and 6 mgl^{-1}), indoleacetic acid (IAA) (0.25 to 1.0 mgl^{-1}) or kinetin (Kin) (0.5 to 2 mgl^{-1}) used individually. Observations on percent rooting, number of roots per shoot and root length were recorded after 6 to 8 weeks. The rooted plantlets were washed softly with tap water to remove agar and traces of the medium from root surface and transplanted into pots containing sterile soil and sand mixture (2:1). After 25 days, the hardened plants were transferred to a greenhouse, maintained under partial shade and irrigated daily. The percentage of survival was recorded at 2-week intervals.

Statistical analysis

For all experiments, four replicates per treatment were maintained and each experiment was repeated twice. Observations based on percentage of culture response with regard to the number of shoots per explant, shoot length, percentage of shoot rooted and roots length were recorded after 6 to 8 weeks. The data were analyzed statistically by analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at 5% probability level. Data analysis was performed using MSTATC software.

RESULTS

Effect of cefotaxime on endogenous contamination

Explants collected from field-grown plants are usually contaminated by various microorganisms. Besides surface contaminants, there may be some internal infectants that can be expressed even after a long time in culture due to some endophytic bacteria or fungi; eradication has not been achieved so far.

For *in vitro* micropropagation of *W. coagulans*, endogenous bacterial contamination is a great problem. Microbial contamination at the base of the explant was observed within 10 to 15 days after inoculation. Bacterial growth was also observed around the explants in the culture media and the majority of the explants (>80%) were destroyed in the culture due to endogenous bacteria. The smaller size of explants was chosen due to the fact that these explants provide less chance of contamination. When treated explants with 100 mgl^{-1} cefotaxime were grown for three weeks on multiplication medium, bacterial growth was inhibited, but not completely eliminated (Table 1). Of the four concentration of cefotaxime tested, 250 mgl^{-1} had minimum phytotoxic effect on *W. coagulans* cultures and completely eliminated bacterial infection. Cefotaxime at concentration of 500 and 750 mgl^{-1} strongly inhibited shoot growth and however, eventually resulted in plant death (Table 1). Although most micro-shoots developed normal leaves, detectable morphological variations were scored. After cefotaxime treatment, some sectorial chlorophyll deficiency in leaf blades was sporadically observed.

Table 1. Effect of cefotaxime concentrations on explants growth and endogenous contamination in *W. coagulans.*

Concentrations (mgl-1)	Survival of explant (%)	Phytotoxic effect (%)	Explants growth	Contamination
0	25	-	+	++++
100	65	-	++	+
250	100	-	+++	-
500	33	++	-	-
750	-	+++	-	-

+ = Low (0–40); ++ = medium (40-80); +++ = high (>80).

Table 2. Influence of different cytokinins and auxin on bud break and shoot proliferation from nodal segments of *W. coagulans* after 8 weeks in MS medium.

Concentration (mgl^{-1})	Bud break (%)	No. of shoot/explant	Shoot length (cm)
Hormone free	0	0	-
BA			
0.5	52.0±1.3	1.2±0. 2	2.9±0. 2
1.0	88.0±2.3	1.4±0.5	3.6±0.1
2.0	92.0±2.5	5.4±1.4	1.2±0.1
IBA			
0.25	80.0±2.3	1.6±0.6	2.4±0.5
0.5	64.0 ±2.1	2.5±0. 2	1.2±0.2
2 BA+0.25 IBA	100±0.0	5.4±0.5	6.1±1.0
2 BA+0.5 IBA	100±0.0	7.2±1.0	7±1.4[a]
4 BA+0.25 IBA	100±0.0	6.8±0.5	3.5±0.8
4 BA+0.5 IBA	100±0.0	3.6±0.3	5.2±0.8

[a]Values represent means ±SD of four explants per treatment in five repeated experiments. Means followed by the same letters are not significantly different by the DMRT at 5% probability level.

Shoot multiplication

For initiation and proliferation of shoot buds, various concentrations of hormones (auxins and cytokinins) were tested. Multiple shoot buds were developed from nodal explant when cultured in MS medium fortified with BA (2 to 4 mgl^{-1}) and IBA (0.25 or 0.5 mgl^{-1}) either singly or in combination with different concentrations (Table 2). Plant growth regulators (PGRs) and their concentrations significantly influenced the *in vitro* bud break and shoot formation. Within few weeks of culture, multiple shoot buds developed from the dormant buds of the explants. Percentage of bud breaking and shoot multiplication was generally influenced by the concentrations and combinations of PGRs. Higher response of explants in terms of shoot bud production was observed in MS medium containing BA + IBA as compared to several other combinations. Multiple shoot buds were induced without intervening callus phase, that is, direct organogenesis occurred (Figure 1A) and resulted in about 1 to 7 micro shoots in 4 weeks (Figure 1B and Table 2). Profuse proliferation of shoot buds was also observed and MS medium supplemented with 2 mgl^{-1} BA and 0.5 mgl^{-1} IBA was proved to be more efficient (Figure 1B). In this treatment, cultured explants produced the highest number of shoots (7.2 ± 1.00 per explant) and showed the highest shoot length (7 ± 1.4 cm per shootbud) (Table 2).

Root initiation and acclimatization of plantlets

Root formation was induced in the *in vitro* proliferated shoots by culturing them on ½MS medium containing 2% (w/v) sucrose and IBA (1 to 6 mgl^{-1}) or IAA (0.25 to 1.0) or Kin (1 to 2 mgl^{-1}). Highest number of roots (35 ± 0.2 per shoot bud) was recorded in ½MS medium supplemented with 2.0 mgl^{-1} IBA, where 100% of the inoculated microshoots induced root (Table 3, Figure 1C). IBA was more competent than IAA in terms of number and length of roots induced per shoots. However, no rooting was observed in Kin supplemented media (Table 3).

Well-rooted plantlets were taken out gently from the test tubes and washed with sterile water to remove adhered agar and traces of the medium to avoid

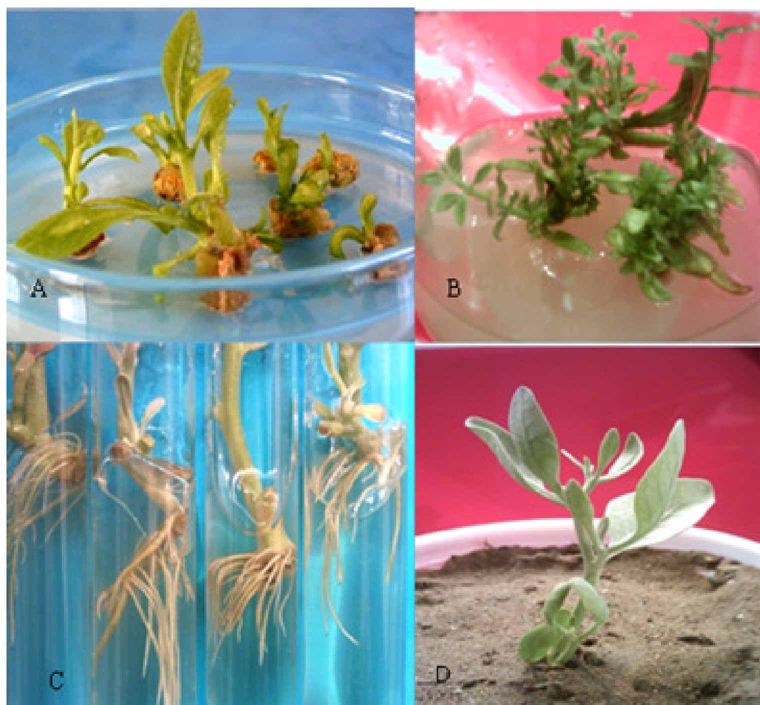

Figure 1. Clonal propagation of *W. coagulans* from nodal segments with axillary bud. A: Nodal explant after 2 weeks of culture; (B) multiple shoot induction on MS medium with 2 mg L^{-1} BA in association with 0.5 mg L^{-1} IBA; (C) rooting of shoot of *W. coagulans* by treatment with 2 mg L^{-1} IBA; (D) *in vitro* developed plantlets transferred to pot.

Table 3. Effect of various concentrations of IBA and Kin on rooting after 6 weeks in ½ MS medium.

Growth regulators (mgl^{-1})	Days of rooting (day)	Response	Shoots rooted (%)	No. of roots/shoot	Root length (cm)
Control	**-**	**S**	**N**	**N**	**N**
IBA					
1	16	R	100[a]	22±0.1	3±0.2
2	15	R	100	35±0.2	3.9±0.4
4	15	R	84	6±0.0	2.2±0.3
6	-	C	-	-	-
IAA					
0.25	18	R	100	8±0.6	1.5±0.6
0.5	25	R	76	3±0.4	1.8±0.9
1.0	25	R	64	4±0.7	2.5±0.4
Kin					
0.5	-	C	N	N	N
1	-	C	N	N	N
2	-	C	N	N	N

[a]Values represent means ±SD. S: swelling, R: rooted, C: callusing, N: no response.

microbial infection in the outside environment. These were then transferred to pots containing potting mixture of sterile soil and sand (2:1) and kept in a growth chamber. The hardened plants were then transferred to a field nursery (Figure 1D) where 75% of the plantlets survived.

DISCUSSION

W. coagulans is becoming rare because it has weak reproductive system, that is, poor seed setting and seed germination. Indiscriminate and ruthless collection for medicinal purposes is another cause of its rapid

disappearance in nature. It is possible to save local flora if proper conservation measures are taken in time (Gilani et al., 2009).

Shoot bud differentiation, elongation of microshoots and induction of roots of *W. coagulans* varied in different PGRs combinations and concentrations. Plant hormones are necessary for shooting, elongation and rooting (Debnath, 2008). The effects of auxins and cytokinins on shoot multiplication of various medicinal plants were reported (Rout et al., 2000). Shoot proliferation studies have been performed by Siddiqu et al. (2004) and Anjali et al. (2000) on *W. somnifera* (L.) Dunal. Sivansean and Murugesan (2008) reported that maximum number of multiple shoots was obtained on MS medium containing BAP + IAA each at 1.5 mgl^{-1} in *W. somnifera*. Saritha and Naidu (2007) showed that MS medium supplemented with BA (2.0 mgl^{-1}) and α-naphthalene acetic acid (0.1 mgl^{-1}) was found to be optimum for the production of multiple shoots from axillary buds in *W. somnifera*. Anjali et al. (2000) reported that inter nodal explants formed shoot buds on MS medium with 1.0 and 5.0 mgl^{-1} BA. Adevntitious shoot regeneration of *W. coagulans* was reported earlier by Jain et al. (2010). They showed that multiple adven-titious shoot bud differentiation occurred on medium fortified with 2.3 µM kinetin (Kn) and higher levels of BA (22.2 µM). Our investigation showed that the highest number of shoots was observed in MS medium supplemented with 2.0 mgl^{-1} BA and 0.5 mgl^{-1} IBA.

Stout root formation was observed in *in vitro* prolife-rated shoots of *W. coagulans* when grown in IBA supplemented with half strength MS medium but no rooting was observed in Kin supplemented media. *In vitro* induction of roots on growing shoots has been achieved in media. Moderate to high concentrations of all cyto-kinins inhibit rooting (Rout et al., 2000). The effectiveness of IBA in rooting has been reported for medicinal plants like *W. somnifera* (Sivansean and Murugesan, 2008; Sivansean, 2007). In *W. somnifera*, regenerated shoots produced highest number of roots when transferred to half strength MS medium supplemented with 2.0 mgl^{-1} IBA (Sivansean and Murugesan, 2008). Rani and Grover (1999) reported that regenerated shoots in *W. somnifera*, rooted best on MS medium containing IBA (2.0 mgl^{-1}) alone, and IBA (2.0 mgl^{-1}) with IAA (2.0 mgl^{-1}). Similar results were obtained in the present study. According to Nickell (1982), the slow movement and slow degradation of IBA facilitates its localization near the site of application and thus, its better function in inducing roots.

Conclusions

Natural stand of *W. coagulans* are fast disappearing in Iran because of indiscriminate collection and over exploitation of natural resources for commercial purposes and the protocol outlined here offers a potential system for its mass multiplication and conservation. This study highlights a complete micropropagation protocol for *W. coagulans* through adventitious shoot multiplication. It will also be of use in conservation and genetic transformation studies aimed at improving the plant. This is an important issue for future research.

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