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Shoot regeneration from leaf explants of *Withania coagulans* (Stocks) Dunal and genetic stability evaluation of regenerates with RAPD and ISSR markers

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ABSTRACT

Withania coagulans (Stocks) Dunal (Solanaceae) is a critically endangered medicinal plant known for its multiple medicinal properties. We report here an efficient and high frequency plantlet regeneration system through direct organogenesis in *W. coagulans* using *in vitro*-derived leaves as explants. On Murashige and Skoog's (MS) medium containing 4.44 μM 6-benzylaminopurine (BAP) 73.7 ± 4.3% explants responded and produced 11.4 ± 0.9 shoot buds per explant. The regenerated shoot buds were elongated (6.7 ± 0.22 cm) on MS medium with 1.11 μM BAP and 0.57 μM indole-3-acetic acid (IAA). The elongated shoots were rooted both *in vitro* and *ex vitro*. The regenerates were acclimatized by slow and gradual exposure to different regimes of temperature and relative humidity. The leaf-regenerates were assessed for genetic stability using RAPD and ISSR molecular markers and confirmed true-to-type. The *in vitro* regeneration system developed would be useful for genetic restoration program as the overexploitation and reproductive failure forced *W. coagulans* towards the verge of complete extinction. The regeneration method would also be useful for genetic transformation for genetic improvement and improved phyto-pharming.

1. Introduction

Withania coagulans (Stocks) Dunal (Solanaceae) popularly known as Vegetable rennet, Paneer-Bandh or Indian cheese maker, is an important plant species of Indian and other traditional systems of medicine. The plant species is highly valued for its multiple medicinal properties (Maurya and Akanksha, 2010) *viz.* anti-tumor, anti-diabetic, anti-hyperglycemic, hepato-protective, cardiovascular, hypo-lipidemic, immunosuppressive and central nervous system anti-depressant activities (Maurya and Akanksha, 2010; Haq et al., 2013). Fruits are known as Paneer-dodhi and have ability to coagulate the milk. Different parts of plant are used to treat ulcers, rheumatism, dropsy, and senile debility (Haq et al., 2013). Its multiple medicinal properties are associated with one or more of its characteristic withanolides produced by the plant (Chen et al., 2011). Production of neuroactive metabolite withanolide A in aerial parts of *W. coagulans* compared to roots of *W. somnifera*, implies easy and economical harvest of the withanolides (Mishra et al., 2012). As there are no cultivation practices for *W. coagulans*; the plant species is harvested from wild, causing loss of natural diversity of the germplasm. Poly-gamo-dioecious nature of flowers and self-incompatibility (Gilani et al., 2009) limits the chances of seed setting and the plant species is found in vegetative state under arid conditions (Bhandari, 1990). The natural regeneration rate does not sustain to keep up with the exploitation rate and the plant species is threatened in its habitat and declared critically endangered (Jain et al., 2010; Rathore et al., 2012).

Overexploitation, hostile environmental factors, habitat disturbances and reproductive failure threaten the survival and render the plant species vulnerable to complete extinction (Rathore et al., 2012). Hence harvest of plant material from wild is not a viable option to meet the commercial demand and some other strategies must be devised to meet the requirements. *In vitro* multiplication strategies as a key component of biotechnological tools have several benefits with continuous supply of plant material from elite lines. These can make significant contributions to the exploitation of therapeutic properties of the plant species and eliminate the need for harvest from wild. *In vitro* propagation of rare or threatened plant genetic resources would help to prevent the erosion and encourage the amplification of germplasm. A number of tissue culture studies have been carried out on *W. somnifera*, but *W. coagulans* received little attention so far for biotechnological studies (Mirjalili et al., 2011; Mishra et al., 2012; Kushwaha et al., 2013; Rathore and Kheni, 2015). A few attempts were made on *in vitro* propagation of *W. coagulans* using different explants and mode of regeneration (Jain et al., 2009, 2010; Valizadeh and Valizadeh, 2009, 2011; Rathore et al., 2012). Plant regeneration

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techniques are attractive and potential ways for utilization of plant genetic resources as well as their conservation. Therefore, an efficient *in vitro* regeneration system for *W. coagulans* is a pre-requisite for its sustainable utilization to meet the pharmaceutical needs. This would also serve the primary requirement for genetic restoration program through true-to-type multiplication, and genetic transformation for genetic improvement and improved phyto-pharming.

The *in vitro* cultures can induce variations among cells, tissues and organs thereby generating variations among the somaclones (Bairu et al., 2011) and these are referred as somaclonal variations. These tissue culture derived variations limit the utility of *in vitro* regeneration systems and it is important to establish the genetic stability of regenerates to minimize the risk of introducing genetic variability. In recent molecular markers are reliable tools to assess the genetic stability of regenerates. Randomly amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) are the most commonly used markers systems (Gantait et al., 2010; Rathore et al., 2011, 2014) as these overcome most of the limitations associated with different molecular markers. Also these are simple, fast, and cost-effective methods of ascertaining the genetic integrity of plantlets. Cost-effectiveness does not add much to the cost of plant production; therefore, these were the methods of choice during present investigation. In present study, attempts were made to develop a simple, cost effective and efficient plantlet regeneration system using leaf as explant. Subsequently the regenerates were assessed to evaluate the reliability of *in vitro* regeneration system for genetic restoration program.

2. Material and methods

2.1. Plant material and explant preparation

Both *in vivo* and *in vitro*-derived leaves were evaluated as suitable explant for leaf regeneration. The seeds of *W. coagulans* were germinated and seedlings were maintained under nursery conditions. Leaves were harvested from 4-weeks-old seedlings and used as *in vivo*-derived explants. These were surface sterilized with 1.0% sodium hypochlorite solution for 5 minutes (min), followed by several washes with sterile water. Alternatively multiple shoot cultures of *W. coagulans* were established by culturing nodal explant from greenhouse-maintained mature plant on 0.8% agar-gelled Murashige and Skoog's (MS) basal medium (Murashige and Skoog, 1962) supplemented with 8.88 μM 6-benzylaminopurine (BAP), 0.57 μM indole-3-acetic acid (IAA), and additives (100 mgl^{-1} L-ascorbic acid, 25 mgl^{-1} each citric acid, adenine sulfate, and L-arginine) (Rathore et al., 2012). Further the shoot cultures were multiplied by repeated transfer and subsequently maintained on MS medium supplemented with 1.11 μM BAP and 0.57 μM IAA. These cultures were regularly transferred on fresh medium after a regular interval of 4 weeks. Leaves were harvested from 4 weeks old *in vitro* cultures and used as *in vitro*-derived leaf explants.

2.2. Shoot bud proliferation

The *in vivo* and *in vitro*-derived leaf explants were cultured on 0.8% agar-gelled Murashige and Skoog's (MS) basal medium supplemented with different concentrations of BAP (0.0–13.32 μM)/Kinetin (Kin; 0.0–13.92 μM)/thidiazuron (TDZ; 0.0–1.362 μM) to optimize the type and concentration of plant growth regulator (PGR) for direct shoot bud regeneration. The cultures were incubated in a culture room at 26 ± 2 °C, 55–60% relative humidity (RH), under 12 h per day (hd^{-1}) photoperiod with a light intensity of 35–40 μmol m^{-2} s^{-1} spectral flux photon (SFP) of photo-synthetically active (460–700 nm) radiations (provided by cool white florescent tubes Philips, Mumbai, India).

The *in vitro* regenerated shoot buds were cultured on fresh shoot bud induction medium for 28 d for further proliferation. The regenerated shoots were separated and cultured for elongation on MS medium supplemented with 0.0–4.44 μM BAP or 0.0–4.65 μM Kin for further proliferation. Effects of 0.57–1.14 μM indole-3-acetic acid (IAA) along with 0.55–2.22 μM BAP/0.58–2.32 μM Kin was tested for further improvement in shoot bud elongation. The cultures were incubated in a culture room.

2.3. Root regeneration and acclimatization

The healthy and elongated shoots were cultured on 0.8% agar-gelled ¼, ½, ¾ and full-strength MS salts supplemented with 0.2% activated charcoal (AC) for *in vitro* rooting. Indole-3-butyric acid (IBA) and β-naphthoxyacetic acid (NOA) with half and full strength of MS medium was also evaluated for *in vitro* root regeneration. The rooting response was recorded after 4 weeks. The *in vitro* rooted plantlets were taken out of the culture vessels and washed thoroughly with sterile water to remove adhered nutrient agar to avoid microbial contaminations. These plantlets were transplanted into plastic bags containing sterile sand and soil in the ratio of 1:1 (v/v) for subsequent hardening. Alternatively, the healthy and elongated shoots were pulse-treated with different concentrations of IBA (0.0–4.92 mM) and NOA (0.0–4.95 mM) for 3–5 min and transplanted into plastic bags containing sterile sand and soil for *ex vitro* rooting and subsequent acclimatization. Both *in vitro* rooted plantlets and pulse treated shoots for *ex vitro* rooting were covered with transparent plastic bags to maintain high RH. These plantlets were nutrified with ¼ strength of MS macro-salt solution and incubated in a culture room.

After certain level of acclimatization of plantlets on soil, the plantlets were gradually exposed to culture room conditions. After acclimatization under culture room conditions, the established plantlets were transferred under greenhouse conditions for further acclimatization. After 8 weeks the acclimatized plantlets were transplanted into large polythene bags or earthen pots containing garden soil and farmyard manure; and transferred under natural conditions.

2.4. Genomic DNA extraction and PCR amplification

Fifteen regenerates (1–15) were selected randomly for genetic stability assessment. Genomic DNA from leaves of regenerates and mother plant (P) was extracted using CTAB protocol (Doyle and Doyle, 1990) with slight modifications (Rathore et al., 2014). The total genomic DNA was quantified spectrophotometrically (BioTek Instruments Inc. USA) and aliquots were diluted to the final concentration of 10–15 ng μl^{-1}.

PCR reactions for RAPD and ISSR were performed in a programmable thermal cycler (Master cycle epgradient S, Eppendroff, Germany). PCR-RAPD amplifications were performed (Williams et al., 1990) using decamer arbitrary primers (Operon Technologies Inc, and IDT, USA). Initially for each RAPD and ISSR fingerprinting, 25 primers were screened. The PCR reactions were carried out in a volume of 15 μl of reaction mixture containing 25 ng template DNA, 1X PCR buffer (Fermentas, USA), 0.2 mM each dNTP's, 3.0 mM $MgCl_2$, 0.4 μM primer, and 1 U *Taq* DNA polymerase (Fermentas, USA). The PCR-RAPD amplification reactions were carried out using the program of initial denaturation at 94 °C for 3 min followed by 42 cycles of denaturation at 94 °C for 30 sec, primer annealing at 32 °C for 1 min, extension at 72 °C for 2.5 min and final extension at 72 °C for 4 min. The conditions for PCR-ISSR cycles consisted of an initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at optimum annealing temperature for particular ISSR primer for 30 sec, extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min. The amplified PCR-RAPD and ISSR products were electrophoresed in 1.5% agarose in 1X TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.0) buffer. The gels were stained with ethidium bromide and documented using gel documentation system (Syngene, UK).

2.5. Data analysis

For plantlet regeneration, all the experiments were set up in a Randomized Block Design (RBD). Each experiment was repeated thrice