Industrial Crops and Products 76 (2015) 323–328

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Optimizing factors affecting adventitious shoot regeneration, in vitro flowering and fruiting of *Withania somnifera* (L.) Dunal

Iyyakkannu Sivanesan, Se Won Park*

Department of Molecular Biotechnology, Konkuk University, 1, Hwayang-dong, Gwangjin-gu, Seoul 143 701, Republic of Korea

ARTICLE INFO

Article history:
Received 2 March 2015
Received in revised form 24 April 2015
Accepted 8 May 2015

Keywords:
Cytokinins
In vitro propagation
Sucrose
Temperature
Winter cherry

ABSTRACT

The effects of plant growth regulators, sucrose and temperature on adventitious shoot regeneration, in vitro flowering and fruiting of *Withania somnifera* were investigated. The highest frequency of shoot regeneration (96%) with an average of 22.8 shoots per stem segment was obtained on Murashige and Skoog (MS) medium fortified with 2.0 mg l^{-1} kinetin, 0.5 mg l^{-1} α-naphthaleneacetic acid (NAA), 0.3 mg l^{-1} gibberellic acid (GA_3) and 40 g l^{-1} sucrose. The greatest frequency of rooting (100%) with an average of 14.1 roots per shoot was obtained when the regenerated shoots were cultured on the half-strength MS medium containing 2.0 mg l^{-1} indole-3-butyric acid (IBA) and 15 g l^{-1} sucrose. Regenerated plantlets were successfully established in the soil with 98% survival rate. The acclimatized plants grew well, developed flowers that were morphologically similar to the donor plants. The highest frequency of flowering (88%) with an average of 8.3 flowers per shoot and the greatest frequency of fruiting (74.9%) with an average of 5.1 fruit per shoot were obtained when shoot tips were cultured on MS medium fortified with 0.3 mg l^{-1} N^6-benzyladenine (BA) and 60 g l^{-1} sucrose, and maintained at 20 °C. Seeds were separated from the in vitro formed mature fruits and placed on MS medium containing 0.3 mg l^{-1} GA_3 to test their viability. The percentage of seed germination was 66%. The developed in vitro culture protocol can be useful for large scale propagation, genetic transformation and plant breeding studies.

1. Introduction

Withania somnifera (L.) Dunal (Solanaceae), is an important medicinal plant commonly known as ashwagandha, Indian ginseng or winter cherry. It is a perennial shrub distributed throughout the drier parts of Africa, Australia, East Asia, India and Middle East. It has been used in traditional medicine for the treatment of Alzheimer's disease, arthritis, cardiovascular disease, cold, dementia, dropsy, fever, female disorders, and hiccups (Kirtikar and Basu, 1975). Phytochemical studies revealed that *W. somnifera* contains a number of active constituents such as alkaloids, flavonoids and steroidal lactones (Pati et al., 2008; Mirjalili et al., 2009). In recent years, the demand for this plant species has sharply risen due to its biological activities. Pharmacological studies revealed that *W. somnifera* possess adaptogenic, anticancer, antibacterial, anti-inflammatory, antioxidant, antistress, cardioprotective and immunomodulatory activities (Tiwari et al., 2014). The plant is endangered in the wild due to large scale and indiscriminate collection (Siddique et al., 2005; Supe et al., 2006). Cultivation is a sustainable alternative to collection of *W. somnifera* from the wild. It is conventionally propagated through seeds. However, large scale propagation of this plant species is often restricted due to poor seed germination, viability, and low survival of seedlings (Kattimani et al., 1999; Vakeswaran and Krishnasamy, 2003). Further, it is prone to a number of diseases and pests (Nagraj and Reddy, 1985; Pati et al., 2008).

Plant tissue culture has become an important tool for medicinal plants conservation and improvement. There are several reports available on in vitro clonal propagation of *W. somnifera* using apical buds, nodal segments and shoot tips (Sivanesan, 2007; Sivanesan and Murugesan, 2008; Nayak et al., 2013). Adventitious shoot regeneration is a pre-requisite for genetic transformation studies. In vitro direct or indirect adventitious shoot regeneration of *W. somnifera* has been achieved using various explants such as embryo, cotyledons, epicotyls, hypocotyls, leaves, roots and stem segments (Kulkarni et al., 2000; Rani et al., 2003; Sivanesan and Murugesan, 2005; Dewir et al., 2010; Ghimire et al., 2010; Rout et al., 2011). Kulkarni et al. (2000) reported that high concentration of BA (5.0 mg l^{-1}) required for de novo shoot regeneration from internodes obtained from in vitro grown seedlings. On the other

Abbreviations: 2iP, N^6-(2-Isopentenyl)adenine; BA, N^6-benzyladenine; GA_3, gibberellic acid; IBA, Indole-3-butyric acid; MS, Murashige and Skoog medium; NAA, α-naphthaleneacetic acid.

* Corresponding author.
E-mail address: sewpark@konkuk.ac.kr (S.W. Park).

http://dx.doi.org/10.1016/j.indcrop.2015.05.014
0926-6690/

hand, the internodes derived from in vitro grown seedlings failed to develop callus or shoot on the medium containing above 1.5 mg l^{-1} BA (Rout et al., 2011). The authors also reported that stem callus did not differentiate into shoot buds. Manickam et al. (2000) achieved shoot regeneration (6.2 shoots) from the stem callus cultures of *W. somnifera*. However, direct shoot regeneration from stem explants has not been reported previously.

The inclusion of cytokinin alone or in combination with auxin to the culture medium is required for adventitious shoot formation in *W. somnifera* (Ghimire et al., 2010; Rout et al., 2011). Moreover, the effect of cytokinin on shoot formation potential of in vitro plant cultures depends on the source of plant tissue, type and concentration of cytokinin. Thus, it is necessary to find out the optimal concentration of cytokinin in promoting adventitious shoot regeneration from stem explants of *W. somnifera*. The in vitro-regenerated shoots often fail to elongate in the shoot induction medium; therefore, elongation of shoot is necessary prior to rooting of shoots in *W. somnifera* (Waman and Sathyanarayana, 2014). It has been reported that gibberellic acid (GA_3) promote shoot elongation in *W. somnifera* (Sivanesan and Murugesan, 2005; Kumar et al., 2011; Waman and Sathyanarayana, 2014), but there are no reports on its effect on shoot regeneration in this plant species. In this study, GA_3 was added to the shoot induction medium, to evaluate its effect on shoot regenerations and to avoid the shoot elongation phase.

In vitro flowering and fruiting has a great potential in breeding programs for crop improvement. There are several reports available on in vitro flowering and fruiting in many plants such as *Capsicum annuum* (Bodhipadma and Leung, 2003), *Cleome viscosa* (Rathore et al., 2013), and *Cucumis sativus* (Sangeetha and Venkatachalam, 2014). In vitro flowering and fruiting has also been reported in *W. somnifera* (Saritha and Naidu, 2007), but the frequency of fruiting was low (7.5% with 1.0 fruit per plantlet), and attempts have not been made to studying the viability of seeds obtained from in vitro. Adventitious shoot regeneration, flowering and fruiting in vitro depends on the culture medium composition, culture conditions and plant growth regulators (PGRs) (Bodhipadma and Leung, 2003; Dewir et al., 2006). The objective of the present study was to evaluate the effect of PGRs, sucrose and temperature on adventitious shoot regeneration, flowering and fruiting of *W. somnifera*.

2. Materials and methods

2.1. Adventitious shoot regeneration

Seeds of *W. somnifera* were obtained from CAS in Botany, University of Madras, India. Seeds were sown in plug trays containing soil + sand + vermiculite (1:1:1) and were placed in the germination bed with the fogging system in the greenhouse. After 35 days, seedlings were transplanted into the experimental field at University. Actively growing shoots obtained from three-month-old field-grown plants were used as explant sources. The shoots were thoroughly washed under running tap water and soaked in Teepol solution (0.1%, v/v) for 5 min, and then washed three times with distilled water. The remaining procedures were done in a sterile laminar airflow chamber. The explants were first disinfected in a 70% (v/v) ethanol solution for 60 s, and 0.1% (w/v) mercuric chloride for 10 min. Finally, they were thoroughly rinsed four times with sterile distilled water so as to remove the mercuric chloride completely. The explants were blotted in a sterile filter paper to remove excess water and then cut into 0.5–1.0 cm stem segments. To study the effect of cytokinins on shoot regeneration stem explants were cultured on Murashige and Skoog (1962) medium (MS) fortified with 0.0, 0.1, 0.3, 0.5, 1.0, 1.5, 2.0 or 3.0 mg l^{-1} N^6-(2-isopentenyl) adenine (2iP), N^6-benzyladenine (BA) or N^6-furfuryladenine (Kinetin). To evaluate the effect of combination of plant growth regulators on shoot regeneration, stem explants were cultured on MS medium fortified with 2.0 mg l^{-1} kinetin in combination with 0.1 or 0.5 mg l^{-1} α-naphthaleneacetic acid (NAA) and 0.3 mg l^{-1} GA_3. To study the effect of various concentrations of sucrose on shoot regeneration stem explants were cultured on MS medium fortified with 0, 20, 30, 40 or 50 g l^{-1} sucrose, 2.0 mg l^{-1} kinetin, 0.5 mg l^{-1} NAA and 0.3 mg l^{-1} GA_3. The culture medium consisted of MS salts and vitamins with 30 g l^{-1} sucrose and solidified with 0.8% (w/v) agar. The pH of the medium was adjusted to 5.70 ± 0.02 before autoclaving at 121 °C for 20 min. The cultures were maintained at 25 ± 2 °C under a 16 h photoperiod with 45 μmol m^{-2} s^{-1}. The number of explants developing shoots and number of shoots per explant were recorded after 45 days of incubation period.

2.2. Rooting and acclimatization

For rooting in vitro-regenerated shoots (2–3 cm) were excised and transferred onto half-strength MS medium containing 0, 15, 30, 45 or 60 g l^{-1} sucrose and 2.0 mg l^{-1} indole-3-butyric acid (IBA). After 30 days, the percentage of root induction, number of roots per shoot and roots length were recorded. Rooted plantlets were removed from the culture medium and washed with sterile distilled water to remove traces of culture medium. Plantlets were transplanted into plastic cups (200 ml) containing autoclaved soil and sand in the ratio of 1:1 or soil and vermiculite (1:1) or soil, sand and vermiculite (1:1:1) and irrigated two days interval with quarter-strength MS salt solution. The pots were covered with perforated polythene bags to maintain humidity for a week and kept under culture room conditions. Plantlet survival was recorded after 4 weeks. Acclimatized plants were transferred to polythene bags containing garden soil and maintained in the greenhouse (22 ± 5 °C, 60–70% relative humidity). After 2 weeks, plants were transferred to the field.

2.3. In vitro flowering and fruiting

To study the effect of sucrose on in vitro flowering and fruiting shoot tips were excised from 45 days old shoots regenerated on MS medium fortified with 2.0 mg l^{-1} kinetin, 0.5 mg l^{-1} NAA and 0.3 mg l^{-1} GA_3, and cultured on MS medium containing 0, 15, 30, 45 or 60 g l^{-1} sucrose and 0.3 mg l^{-1} BA. After 30 days of culture, the shoots were transferred to fresh medium containing the same composition of PGRs and ingredients. After 60 days, the results were recorded. To study the effect of temperature on in vitro flowering and fruiting shoot tips were excised from 45 days old shoots regenerated on MS medium fortified with 2.0 mg l^{-1} kinetin 0.5 mg l^{-1} NAA and 0.3 mg l^{-1} GA_3, and cultured on MS medium containing 60 g l^{-1} sucrose and 0.3 mg l^{-1} BA. The cultures were placed in three separate identical environment controlled plant growth chamber (VS-4DM, Vision Scientific Co., Ltd., Daejeon, Korea) at 20, 25 or 30 ± 2 °C under a 16 h photoperiod with 45 μmol m^{-2} s^{-1}. After 30 days of culture, the shoots were transferred to fresh medium containing the same composition of PGRs and ingredients. After 60 days, the results were recorded. The in vitro formed seeds were placed into flasks containing MS medium fortified with 30 g l^{-1} sucrose and 0.3 mg l^{-1} GA_3. After 30 days, the results were recorded.

2.4. Statistical analysis

All experiments were conducted in three replications and each of them contained 10 stem segments, shoot tips, plantlets or seeds. The experiments were set up in a completely randomized design and repeated thrice. Data obtained were subjected to analysis of variance (ANOVA) by using SAS program (Release 9.1, SAS Institute, NC, USA), and Duncan's multiple range test at $P < 0.05$ was used