Rapid Micropropagation and Conservation Method, in the Face of Changing Climate for *Vinca rosea* L.—A Potential Plant of High Medicinal Value

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ABSTRACT

Direct regeneration of shoots and roots through field grown juvenile explants has been achieved in shoot tips and nodes cultured on MS medium containing various concentrations of auxin and cytokinin separately and in combination. Most effective sterilization was achieved using 5% Sodium Hypocholrite for 7 minutes. A combination of 1–5 µM of NAA and 1–5 µM of BAP induced high frequency of axillary shoots and root differentiation in 4 weeks. BAP alone did not induced multiple shoot formation in the explants. Morphogenetic response such as percent shoot and root differentiation was recorded at regular intervals of time.

Keywords: Micropropagation, shoot tips, Shoot multiplication, nodal segments

Abbreviations: BAP-6-Benzyl Amino Purine, NAA-Napthelene Acetic Acid, MS-Murashige and Skoog medium

INTRODUCTION

Plant derived drugs are being widely used not only in developing countries but also in the most advanced countries. It has been reported by WHO that about 80% of the world's population rely on medicinal plants for their primary health care (Veeresham and Chitti, 2013). Until recently plants are being the important source of novel pharmacologically active compounds, with many blockbuster drugs derived directly or indirectly from plants. Despite the current occupation with synthetic chemistry as a vehicle to discover and manufacture drugs, the contribution of plants to disease treatment and prevention is still enormous (Raskin *et al*., 2002; Newman *et al*., 2000).

Different strategies, using an *in vitro* system, have been extensively studied to improve the production of plant chemicals. Application of tissue culture technology is important for the production of some important plant pharmaceuticals. *In vitro* regeneration or micro-propagation has tremendous potential for rapid multiplication and production of high quality medicinal plants (Murch *et al*., 2000).

Vinca rosea (Syn *Catharanthus roseus*) belonging to family Apocynaceae is one of the very few medicinal plants which have a long history of uses as diuretic, antidysenteric, hemorrhagic and antiseptic (Pahwa, 2009). It is known for use in the treatment of diabetes in Jamaica and India. Prevention of cancer, cancer

treatment, anti-diabetic, stomachic, reduces high blood pressure, externally against nose bleeding, sore throat and mouth ulcers (Mehta *et al*., 2013).

The chemotherapy agent vincristine is extracted from this plant and is used to treat some leukemias, lymphomas, and childhood cancers, as well as several other types of cancer and some non-cancerous conditions (Gaines, 2004; Negi, 2011; Mehta *et al*., 2013). Vinblastine is a chemical analogue of vincristine and is also used to treat various forms of cancer. These vinca alkaloid bind to tubulin dimers and inhibiting microtubule structures of the cells, thus inhibiting the metaphase of cellular mitosis (Johson *et al*., 1963; Patil and Ghosh, 2010). Dimeric alkaloids such as vincristine and vinblastine are produced by the coupling of smaller indole alkaloids such as vindoline and catharanthine (Heihden, 2004;Verma *et al*., 2012).

Keeping in view the importance of plant tissue culture in producing large number of plantlets which can be supplied to pharmaceutical companies for extraction of metabolites, present study of *in vitro* propagation of this valuable medicinal plant from shoot tip and nodal explants was undertaken.

MATERIAL AND METHODS

SOURCE OF EXPLANT

Shoot tips and nodes were used as explants. The explants were collected from the plants of *Catharanthus roseus* growing in Naseem Bagh Campus of University of Kashmir, Srinagar.

SURFACE STERILIZATION

Shoot tips and nodal explants were washed in running tap water and then washed again thoroughly by adding a few drops of Tween-20 to remove the superficial dust particles as well as fungal and bacterial spores. They were surface sterilized with 5% Sodium Hypocholrite for 5 min followed by rinsing them five times with double distilled water inside the Laminar Air flow chamber.

MEDIUM AND CULTURE CONDITIONS

Murashige and Skoog's (1962) basal medium containing 0.8% agar and sucrose [30.0 g l-1] was used during the entire experiment. The pH of the medium was adjusted to 5.6. Growth regulators were supplemented at various concentrations [Table 1]. Routinely 30ml medium was dispensed in each flask [100ml capacity], and sterilized by autoclaving at 121 °C. The explants were then placed horizontally over the semisolid MS medium. During the experiments, a light regime of 16 hours light intensity of 2000–2500 lux provided by cool-white fluorescent tubes at 25 ± 2ºC followed by 8 hr dark period was provided to the cultures. Phenotypic changes taking place among the cultured explants were noted down every week.

Subculturing

Once culture conditions for shoot induction from explants were established, the shoots produced *in vitro* were sub cultured on fresh medium every 4 wk.

Conservation

For short term conservation various concentrations of growth retardants including Maleic hydrazide and Absissic acid were added to MS media. Sucrose concentration was also reduced.

ACCLIMATIZATION

The *in vitro*-rooted plantlets were transferred to small pots containing soil: sand: peat moss in the ratio of 4:2:1 and irrigated with 1/4 MS salt solution. These pots were kept in controlled environmental conditions of culture room. After 3 wk of growth, the plantlets were transferred to mist house for further growth.

RESULTS

Shoot Proliferation

Shoot tips and nodal segments cultured on basal medium failed to show any response and resulted in the browning and wilting of explants. On supplementing the medium with various concentrations of BAP (1, 5, 7, 10 µM the explants showed elongation followed by browning (Fig. 1). Shoot proliferation was induced in both the explants on augmenting the various concentrations of BAP with NAA concentrations. Axillary shoot formation was initiated at BAP 5 µM + NAA 1 µM with shoot number of 2.4 ± 0.5 (Fig. 2). The highest shoot number of 11.6 ± 1.6 shoots was obtained on BAP (5µM) + NAA (2.5 µM) combination (Fig. 3). The shoot number further decreased on increasing the hormonal concentration.

Root Induction

Rooting of the explants was also initiated on the combination of BAP (5µM) + NAA (1 µM) but the longest and healthy roots were obtained on medium supplemented with BAP (5 µM) + NAA (2.5 µM) (Fig. 4).

Acclimatization

The plantlets obtained were successfully acclimatized with a success rate of 70%.

Conservation

Out of the growth retardants used absissic acid showed best result of 2 months subculturing period. The best conservation of six months was observed on MS (1/2) + BAP (5µM) + NAA (2.5µM) + ABA (2µM) (Fig 5). The explants were successfully recovered after six months.

Table 1: Effect of Auxins and Cytokinins and Their Combinations on Field Collected Shoot Tip and Nodal Explants of *Vinca rosea* on MS Medium

Treatment	Response	Number of Cultures Forming Shoots/ Roots (% Response)	No. of Shoots Formed ± SD*	Shoot Length ± SD	No. of Roots Formed ± SD*	Root Length ± SD
Basal	Browning and wilting of explants	-	-	-	-	-
BAP (1, 5, 7, 10 µM)	Elongation of explants followed by browning of explants	-	-	-	-	-
MS+BAP(5µM) + NAA(1µM)	Aillary shoots formation. The cultures also formed thin and long adventitious roots.	90	2.4 ± 0.5	2.5 ± 0.5	2.5 ± 0.4	3.8 ± 0.8
BAP(5µM)+NAA(2.5µM)	-do-	90	11.6 ± 1.6	14.8 ± 0.8	14.5 ± 1.9	3.6 ± 0.5
BAP(5µM)+NAA(5µM)	-do-	90	4.4 ± 0.8	2.1 ± 0.8	7.2 ± 0.8	2.6 ± 0.4
BAP(7µM)+NAA(3µM)	-do-	80	2.1 ± 0.5	1.6 ± 0.1	2.4 ± 0.8	1.8 ± 0.3
BAP(7µM)+NAA(5µM)	Mild callus formation at base	70	-	-	-	-
BAP(10µM)+NAA(3µM)	Browning of explants	70	-	-	-	-
BAP(10µM)+NAA(5µM)	Browning of explants	80	-	-	-	-

*Data scored after every six weeks and representing mean of ten cultures.

DISCUSSION

It is well known that the balance of cytokinin and auxin in plant tissues controls the direction of organogenesis (Skoog and Miller, 1957). To keep this balance ideal for development of new organs, exogenous cytokinin or auxin is usually added to the plant regeneration medium (Swanberg & Dai, 2008). In the present study MS basal medium without growth regulator did not show any promising results and showed only elongation of both explants. Similar results were observed by Cavallini and Lupi (1987); Paal *et al.* (1981) and Haq *et al.,* (2013). Shoot tips and nodal tissues of *Vinca rosea* were found to be sensitive to the presence and concentration of cytokinin and auxin. The ratio of cytokinin to auxin seemed critical for shoot regeneration. The plants contain endogenous hormones but the naturally occurring hormones in tissues were not able to induce shoot formation. Addition of exogenous PGRs to the medium was necessary for plant regeneration of V. *rosea* as was found in other plant species such as holy basil, lavender, chokecherry, black cherry, and gerbera (Dai *et al*., 2004; Espinosa *et al*., 2006; Kumar *et al*., 2004, Tyub *et al*., 2007; Tyub and Kamili, 2009). Plant regeneration is a challenge for *Catharanthus roseus*, especially through organogenesis using vegetative tissues. After testing numerous concentrations of BAP and NAA in isolation and in combination it was found that shoot induction was induced after a particular level of

hormones was achieved by both the explants. This study showed that both shoot tip and nodal explants showed the axillary shoot induction as is found in *V. rosea* plants in nature. This result correlates with previous studies that found shoot tip and nodal tissues to be best suited for regeneration of *Vinca* (Mollers and Sarkar, 1989; Swanberg & Dai, 2008; Negi, 2011, Haq *et al*., 2013, Mehta *et al*., 2013).

After obtaining the axillary multiple shoots successfully, short term conservation was also tried for the plantlets obtained. The *in vitro* conservation of plants for short to medium periods of time through a tissue culture approach can be achieved by using various procedures to reduce growth, such as by increasing the interval between subcultures (Rai *et al*., 2011). A well-known approach that is often used for slow-growth conservation is modification of the cultural and/or environmental conditions, such as the maintenance of cultures under reduced temperature and/or reduced light intensity or low oxygen concentration and the use of growth retardants, osmoticum or minimal growth medium (alteration of mineral content and/or sucrose in medium) (Engelmann *et al*., 2003; Gupta and Mandal 2003; Rai *et al*., 2008 a & b, 2009). The use of ABA for *in vitro* slow-growth conservation has been reported for many plant species (Watt *et al*., 2000; Gopal *et al*., 2004; Morata *et al*., 2006). In present also the MS half strength media augmented with BAP (5 µM) + NAA (2.5 µM) + ABA (2 µM) was found to be best suitable for *in vitro* conservation. The sucrose was found to be effective for extending subculturing time/duration and are in line with the findings of Javed and Ikram (2008). It was concluded that plant tissue culture is a successful technique for short term conservation when growth retardants like absissic acid and maleic hydrazide are used. These results are in accordance with some workers (Zimmermann, 1993). Rai *et al*., (2008a); Choi and Jeong (2002) who also achieved short term conservation of medicinal plants using the growth retardants in tissue culture medium.

Fig. 1: MS + BAP (5µM)

Fig. 2: MS + BAP (5µM) + NAA (1µM)

Fig. 3: MS + BAP (5µM) + NAA (2.5µM)

Fig. 4: MS + BAP (5µM) + NAA (5µM)

Fig. 5: Conservation Medium MS (1/2) + BAP (5µM) + NAA (2.5µM) + ABA (2µM)

So it is concluded that the present protocol is a cost effective and rapid method for production of multiple shoots and conservation of *V. rosea.*

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