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PROPAGATION OF *Portulaca oleracea* L. IN LIQUID MEDIUM: IMPLICATIONS OF PLANT GROWTH REGULATORS IN CULTURE

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ABSTRACT

Portulaca oleracea L. is a medicinal plant, growing in warm and moist regions of north hemisphere of the world. A protocol for *in vitro* propagation using nodal shoot segments as explants has been outlined. The percent shoot response with shoot induction rate, 6.4 ± 0.7 shoots per explant, was achieved when cultured on agar-gelled Murashige and Skoog (MS) medium containing 2.0 mg/L of BAP (6-benzylaminopurine). The cultures were amplified by passages on MS medium with 1.0 mg/L each BAP and kinetin (Kn). The best shoot amplification (37.5±0.9 shoots per vessel) was achieved by subculturing of *in vitro* regenerated shoot clumps on liquid MS medium. Shoots regenerated *in vitro* by both the processes were rooted on ½ strength of MS medium + 2.0 mg/L of indole-3 butyric acid (IBA). Ninety six percent of the shoots rooted *in vitro.* The *in vitro* rooted plantlets were hardened under different regimes of temperature and humidity in the greenhouse. The hardened plantlets were transferred to mixture of soil and manure in polybags.

Keywords: Micropropagation, *in vitro*; Murashige and Skoog medium; *Portulaca oleracea* L.

INTRODUCTION

Portulaca oleracea (Linn.), belongs to the family Portulacaceae, is an annual succulent, commonly called as pigweed, little hogweed or common purslane. It is native to the India and the Middle East and grows in warm and moist regions of north hemisphere, especially temperate and tropical regions of the world (**Radhakrishnan *et al.*, 2001**). It is a pharmaceutically important plant, used for a variety of conditions like, headache, stomach ache, painful urination, enteritis, mastitis, lack of milk flow in nursing mothers and in postpartum bleeding and also eaten as a salad and vegetable all around the world (**Wyk and Gericke, 2000**). Externally it is used to treat burns, earache, insect stings, inflammations, skin sores, ulcers, itching skin, eczema and abscesses (**Leung and Foster, 1996**). *P. oleracea* has antibacterial, antifungal, wound healing, anti-inflammatory, uterine stimulant, muscle-relaxant and diuretic properties (**Okwuasaba *et al.*, 1986; Rashed *et al.*, 2003**). The whole plant is considered antiphlogistic bactericide in bacillary dysentery, diarrhea, haemorrhoids, enterorrhaghia, etc. (**World Health Organization, 1990**). It is used as a cataplasm of fresh leaves for maturing of abscesses and prescribed as antidiabetic medicine. The whole plant is said to be an emollient, calmative, diuretic, a refreshing agent, antiscorbutic and vermifuge (**Boulos, 1983**). The herb is used generally for treatment of heart trouble. A similar preparation is used as a sedative in fits of insanity. A decoction is used in lotions as an anodyne on the forehead for headache (**Burkill, 1997**).
Extensive studies have been done on *P. oleracea* regarding its medicinal properties (**Liu *et al.*, 2000**). The information of tissue culture of this plant is largely deficit. **Safdari and Kazemitabar (2009)** and **Sharma *et al.*, (2011)** reported some tissue culture work on *P. grandiflora* and *P. oleracea* respectively. Development of tissue culture protocol and optimization of *in vitro* conditions can be useful for further studies for this plant. Moreover plantlet regeneration protocol is prerequisite for regeneration of genetically transformed cells, tissues and plants for any plant system. Therefore, we report an efficient *in vitro* regeneration method for this valuable medicinal plant using liquid medium.

MATERIALS AND METHODS

Healthy plants were identified and collected during January - April, 2012 from the coastal areas of Pondicherry, India. Fresh sprouts with axillary buds were collected from six-week-old plant. Some plants were maintained in the green house to harvest better shoots as explants.

Collection and surface sterilization of explants

Two-to three-cm long fresh shoots each with 1-2 nodes were harvested and used as explants. The explants collected were surface sterilized with 0.1% Bavistin® (systemic fungicide, BASF, India Ltd.) and $HgCl_2$ solution for 4-6 minutes. These were washed thoroughly with autoclaved distilled water 6-8 times. Explants were finally dipped in ethanol and inoculated on the medium.

Culture medium and establishment of cultures

For the present study MS basal medium (**Murashige and Skoog, 1962**) was used as a nutrient medium. Sucrose or sugar cubes were added as a source of carbon. The sterilized explants were inoculated vertically on agar-gelled MS medium for culture initiation. Different concentration and combination of cytokinins (BAP and Kinetin ranging from 1.0 to 5.0 mg/L) and auxins (IAA and IBA ranging from 0.1 to 2.0 mg/L) were incorporated in the medium for bud breaking. These cultures were incubated at 25±2°C in the dark for 2-3 days. Subsequently these were kept under diffused light (22 µ $molm^{-2}$ s^{-1} Spectral Flux Photons, SFP) for 8-10 days. The light was provided by fluorescent tubes and incandescent bulbs.

Multiple shoots regeneration in liquid MS medium

The explants with regenerated shoots were taken out from the culture vessels after four weeks and transferred to the multiplication medium. Agar-gelled as well as liquid MS medium was tested for multiple shoot regeneration *in vitro*. The medium was supplemented with a range of 0.1 mg/L to 2.0 mg/L of BAP and Kn alone or in combination with auxins (IAA or IBA) ranging from 0.1 mg/L to 1.0 mg/L. Cultures in the liquid medium were put on the gyratory shaker for proper aeration and availability of nutrients at 100 rpm.

Rooting of *in vitro* regenerated shoots

Experiments were conducted to induce roots from the *in vitro* regenerated shoots on agar gelled MS medium. Healthy and lengthy shoots were isolated from the liquid cultures and transferred to the agar-gelled half-strength and one-fourth strength MS medium. The rooting medium was augmented with auxins (IAA or IBA). The cultures were kept in the dark for one week for root induction from the cut ends of the shoots.

Hardening of plantlets

The rooted shoots were removed from the glassware and washed with autoclaved distilled water to remove adhered nutrient agar. These plantlets were further transferred to the bottles containing sterilized soilrite® (Keltch Ltd.) and moistened with one-forth strength of MS basic salts. Bottles were kept in the green house conditions for two weeks. After four weeks, acclimatized plantlets were transferred to pots containing a mixture of soil and manure.

Observation and Data Analysis

The cultures were regularly subcultured on fresh medium after 4 week interval. The observations were taken after every 4-weeks of inoculation. The experiments were repeated thrice with ten replicates per treatment. The rate of multiplication represents the number of shoots produce per explant on a specific medium after a number of days of its inoculation as mentioned in the results. The data were subjected to statistical analysis.

RESULTS AND DISCUSSION

Induction of shoots from the nodal meristem of explants

Fresh shoot segments taken from the green house grown plants were responded quickly as compared to the plants grown in nature. About a week after culture, 93% explants initiated to form shoots in the treatment containing 2.0 mg/L BAP (Fig. 1A). In another one week these shoots proliferated rapidly and formed a cluster of shoots (Fig. 1B). Shoot regeneration from nodal segments in the MS medium containing 2.0 mg/L Kn occurred lately with less percent of response (89%) (Fig. 2). The combined effect of BAP and Kn on shoot regeneration percentage was low as compared to the used alone. **Bohidar *et al.*, (2008)** used different concentrations of Kn and BAP for shoot induction from nodal explants of *Ruta graveolens*. They achieved the maximum shoot regeneration (96.6%) with 1.0 mg/L BAP, whereas, **Safdari and Kazemitabar (2009)** reported maximum shoot regeneration percentage (78%) in purslane belonging to 8.88 µM BAP and it was low with 4.44 µM of BAP (39%). Researchers reported that in equivalent concentrations, BAP is better than Kn for shoot regeneration from hypocotyl explants of *Portulaca* species (**Bhuiyan and Adachi, 2002; Safdari and Kazemitabar, 2010; Shekhawat *et al.*, 2014a**), which corresponds with our results of shoot induction from nodal segment culture of *P. oleracea*.

Safdari and Kazemitabar (2009) reported *in vitro* culture studies on two purslane varieties and observed that the treatments containing10 µM IBA in combination with 10 or 5 µM BAP are suitable for callus induction from the leaves of wild purslane. Callus formation was also observed in our results from the cut ends when IAA and IBA was incorporated in the medium supplemented with BAP or Kn, again low rate of response was observed when the concentration of BAP or Kn was used higher or less than 2.0 mg/L in *P. oleracea*. **Sharma *et al.*, (2011)** achieved five shoots per explant when the explants were inoculated on MS medium supplemented with 0.5 mg/L Kn with the highest percentage of shoot response (86%).

Figure 1A & 1B Induction of multiple shoots from nodal meristems of explants.
1C Multiple shoots in liquid MS medium on shaker.
1D & 1E *In vitro* rooted shoots in half strength MS medium with 2.0 mg/L IBA. **1F** Plantlet in hardening condition in green house.

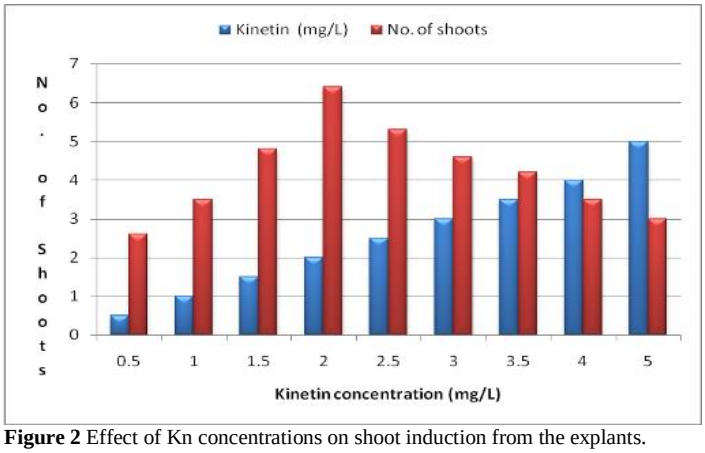

Figure 2 Effect of Kn concentrations on shoot induction from the explants.

The maximum number of shoots (6.4 ± 0.74 shoots per explant) was observed on MS medium supplemented with 2.0 mg/L BAP in the present study (Fig. 3). Direct shoot regeneration from shoot tips or petiole explants of wild purslane was observed only with 10 µM IBA alone (**Safdari and Kazemitabar, 2009**). They also reported that 8.88 µM BAP was found to be the best treatment to shoot regeneration from nodal segments of agronomic purslane. **Shekhawat *et al.*, (2011, 2014a, 2014b)** reported superiority of BAP over Kn in induction of shoots from nodal meristems of *Momordica dioica*.

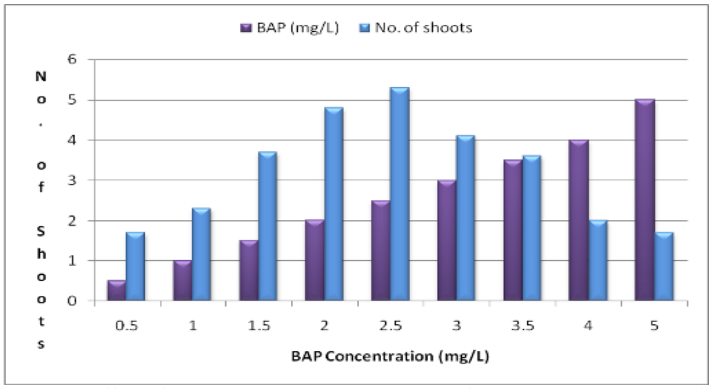

Figure 3 Effect of BAP on multiple shoot initiation from the nodal meristem of explants.

No shoot induction was observed from the explants when the medium was used without PGRs. Similar results were obtained with hypocotyl nodal shoots were used as explants (**Bhuiyan and Adachi, 2002; Safdari and Kazemitabar, 2010**). **Shekhawat and Shekhawat (2011)** used 2.0 mg/L BAP for regeneration of shoots from the stem nodes of *Arnebia hispidissima*. It seems that BAP in 2.0 mg/L and higher levels is suitable for shoot regeneration from nodal meristems in many plant species (**Shekhawat *et al.*, 2014a, 2014b**).

Multiplication of shoots in liquid medium

In the present study, liquid MS medium was tried in order to maximize the production of numbers of multiple shoots in a short period of time from each explant. Experiments were also conducted to multiply *in vitro* regenerated shoots on agar gelled MS medium for comparative studies. But there was a significant difference in the number and health of the shoots; less number of shoots was multiplied in agar-gelled MS medium as compared to the liquid medium (data not shown). So, further studies were confined to the liquid cultures for multiple shoots. The cultures with liquid medium were kept on gyratory shaker at 100 rpm. The MS medium supplemented with 1.0 mg/L each of BAP and Kn was found most suitable for multiplication in the present investigation. We used cytokinins as well as auxins for shoot multiplication; some researchers have also achieved suitable shoot regeneration in combinations of auxin and cytokinin (**Barwal *et al.*, 1986; Kosir *et al.*, 2004; Shekhawat *et al.*, 2014a**) in different species.

Incorporation of auxins in the medium causing callus formation from the base of shoots and sometimes roots were also observed in this combination in the liquid medium. Reduced numbers of shoots were found in case of increased concentration of BAP and Kn in the medium. Stunted growth of shoots was observed when 2.0 mg/L each of BAP and Kn was added in the medium, but the shoots were thick and healthy on this media combination.

Maximum numbers of shoots (37.5±0.9 shoots per culture vessel) were obtained on 1.0 mg/L each of BAP and Kn supplemented medium (Fig. 1C & 4). **Sharma *et al.*, (2011)** multiplied and elongated shoots on MS medium supplemented with 0.5 mg/L Kn and finally they succeeded to achieve *in vitro* flower bud formation in *P. oleracea*. Cultures must be subcultured after four weeks in the liquid

medium otherwise necrosis and tip burning starts and the leaves could turn yellow in color because of fast absorption of nutrients in liquid cultures.

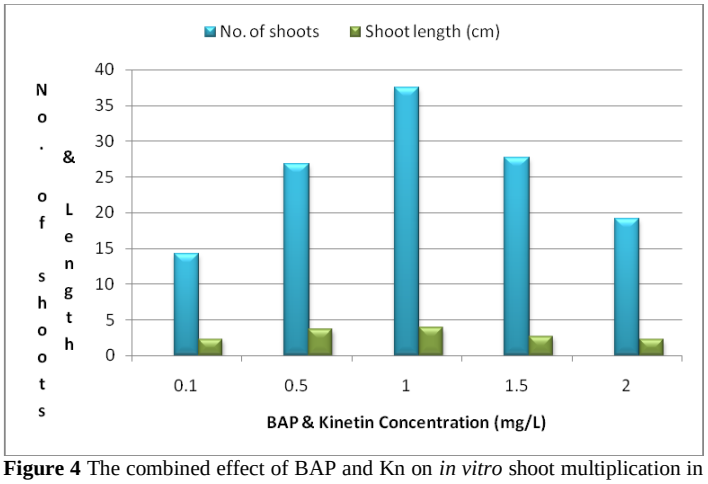

Figure 4 The combined effect of BAP and Kn on *in vitro* shoot multiplication in liquid MS medium.

Each shoot bunch was divided in 4-6 pieces and further subcultured in fresh medium to get the next crop of shoots within four weeks. These pieces are the best source to get multiple shoots *in vitro* in a short period of time because they could rapidly absorb liquid nutrient medium and grow fast at higher light intensity. **Safdari and Kazemitabar (2010)** reported *in vitro* multiplication of shoots in *P. grandiflora* and observed that the treatment containing 10 µM BAP was found to be the best one for shoot regeneration and 15 µM BAP alone or in combination with 5 µM NAA were found to be the best treatments for shoot regeneration from callus.

***In vitro* root induction and hardening of plantlets**

Two methods, *in vitro* and *ex vitro* were employed to induce roots from the shoots. Healthy and long shoots (3-5 cm) were isolated from the bunch of shoots during subculturing for multiplication, used to initiate roots. Half strength MS medium augmented with 2.5 mg/L IBA was found the most suitable combination for root initiation and elongation in *P. oleracea*. On this media combination 11.6±0.7 roots were initiated with 3.2±0.4 cm length (Fig. 1D, 1E & 5). Our results are similar to the findings of **Safdari and Kazemitabar (2009)**. They reported that IBA at level 2.5 µM was found to be the best treatment for rooting of regenerated shoots in both the races of purslane, but our results are better than those of the previous studies (**Safdari and Kazemitabar, 2009; Sharma *et al.*, 2011**). **Safdari and Kazemitabar (2010)** rooted *in vitro* raised shoots on MS medium supplemented with 0.25 mg/L IBA. Later on they achieved *in vitro* flowering in *P. oleracea.*

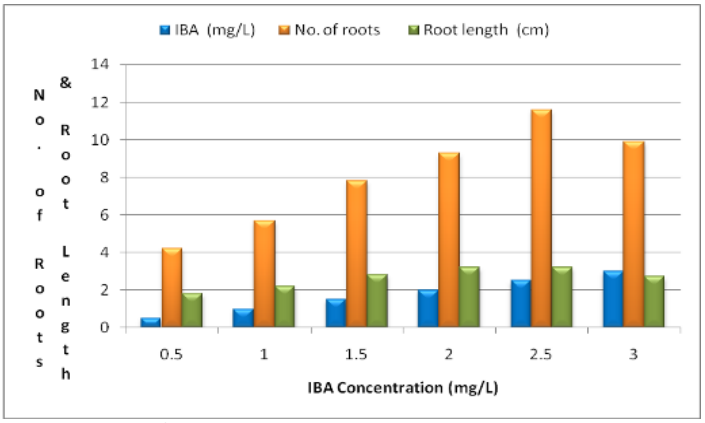

Figure 5 Impact of IBA on *in vitro* root initiation and root length.

Cultures responded better when these were put in the dark for one week. In dark conditions, roots were initiated within one week but in light conditions it took two weeks for initiation. More numbers of roots could be initiated on higher concentration of IBA but the length of the roots was short. The response of IAA was also observed but it was not significant as compared to IBA in root induction in this species (8.7±0.7 roots per shoot) (Fig. 6).

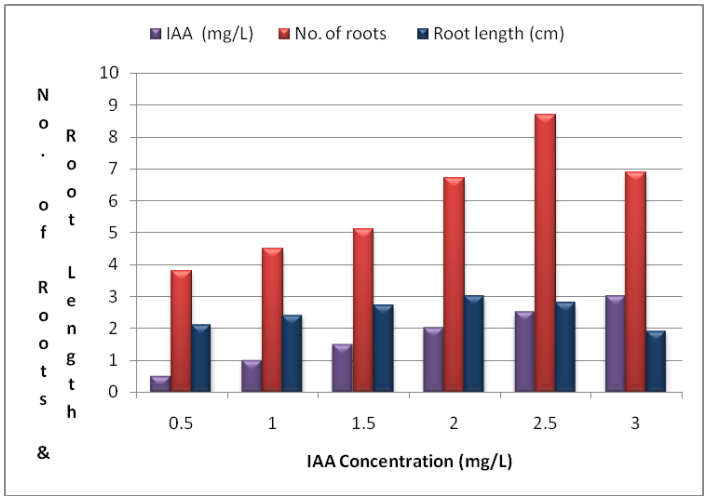

Figure 6 Effect of IAA concentrations on root induction from the cut ends of *in vitro* regenerated shoots.

The present study clearly indicates the supremacy of IBA in root induction. **Bohidar *et al.*, (2008)** used different levels of IBA or NAA to root induction from regenerated shoots of *R. graveolens*. They found that IBA is generally better than NAA for root induction and development. Also, they reported that the cultured shoots on media without auxins failed in root formation. Similarly we found that IBA is better than IAA for root induction in this study. In purslane, IBA especially at low concentrations (5 µM and lower) proved to be a suitable auxin to root inducer as in many other plants, for example, in bamboo **(Das and Pal, 2005)**, *Arnebia hispidissima*, *Turnera ulmifolia* **(Shekhawat, 2012; Shekhawat *et al.*, 2014a)**, *Salvadora persica* (**Phulwaria *et al.*, 2011**). The response of shoots in root induction in *ex vitro* rooting was not encouraging. Even a single shoot was not rooted in soilrite® containing bottles in the green house. It requires further studies to get roots from the shoots *ex vitro*. Well-rooted plantlets were transferred to soilrite® containing bottles (Fig. 1F) and kept in the green house for hardening for four weeks. The plantlets were ready for pot transfer after another one week in the green house.

CONCLUSION

Micropropagation could be an effective technique for the mass scale propagation of elite germplasm in a short period of time to overcome difference in demand and supply in the market, especially in case of medicinal plants. An efficient *in vitro* propagation protocol has been developed for *P. oleracea* in the present investigation. Liquid medium could provide better alternative method for shoot multiplication and root initiation in some plant systems. It was shown in this study that plants can better use nutrients from the liquid medium. This protocol can be used for further studies, like regeneration of transgenic *P. oleracea*.

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