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Original Article

Development of Shoot Cultures from Leaf Explant of *Portulaca quadrifida* L.

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Abstract

Portulaca quadrifida (Portulacaceae) is an annual succulent herb having medicinal value and is consumed as a vegetable or salads in India. In the present study, leaf explants were inoculated on Murashige and Skoog's (MS) medium fortified with sucrose (3%) and combinations of N^6-benzyladenine (6-BA) and kinetin (KIN) individually and in combination with 1-naphtalene acetic acid (NAA). Rapid regeneration was observed in medium fortified with combinations of 6-BA (8 µM) and NAA (1 µM) which formed 19.40 ± 0.64 shoots with 100% response. Variation in sucrose concentrations (4-6%) was tried but it failed to increase the shoot number. When the optimized medium was fortified with different carbon sources viz. dextrose, glucose and maltose, they could not evoked better response and sucrose proved to be more effective for regeneration. Rooting of *in vitro* shoots was achieved in ½MS + sucrose (1%) + indole-3-butyric acid (IBA, 2 µM).

Keywords: carbon source; indirect organogenesis; leaf explant; Murashige and Skoog; shoot cultures

Introduction

Portulaca quadrifida L. commonly called 'looni' which belongs to the family Portulacaceae is a small diffused, succulent, annual herb found throughout the tropical parts of India. It emits a mild pleasant smell and is used in salads or consumed as a cooked vegetable. In India, boiled leaves of the plant are mixed with sorghum or pearl millet flour in preparing a kind of bread. The plant has medicinal properties and is used to cure asthma, cough, urinary discharge, inflammation, ulcers, abdominal complaints, erysipelas and hemorrhoids (Kirthikar and Basu, 2001; Mulla and Swamy, 2010). It also has neuropharmacological effects and antifungal activities against *Aspergillus fumigates* and *Candida albicans* (Hoffman *et al*., 2004; Syed *et al*., 2010). Due to its nutritional and medicinal importance it was taken up for regeneration studies.

Leaves are commonly used explant for efficient shoot regeneration in many important medicinal plants (Joshi and Padhya, 2010; Joshi *et al*., 2010; Pathak and Joshi, 2017) and is also suitable for genetic transformation and protoplast fusion (Kumar *et al*., 2010; Xu *et al*., 2013). However the regeneration of plant under *in vitro* conditions depends on many factors, like genotype, explant, medium composition, growth regulators, gelling agent, light intensity and quality, photoperiod, temperature and vessels (Reed, 1999). The cultures developed under *in vitro* conditions need external carbon sources, which help in proliferation and differentiation of shoots (Kozai, 1991; Gurel and Gulsen, 1998; Nowak *et al*., 2004).

The aim of the present study was to optimize the medium for shoot regeneration fortified with a suitable carbon source using leaf explants of *P. quadrifida*.

Materials and Methods

Plant material and surface sterilization

Young healthy twigs of *Portulaca quadrifida* L. were collected from the Botanical Garden of the M.S. University of Baroda. Pieces of stem containing two to three nodes were kept in running tap water for 1 h, and then washed with labolene (Fisher Scientific, India) for 5 min. Surface sterilization was done with bavistin solution (0.01%) followed by $HgCl_2$ (Merck, India) solution (0.1%) for 3 min each. Entire leaves were excised and placed abaxially on medium.

Culture medium for shoot regeneration

Murashige and Skoog (MS, 1962), medium fortified with sucrose (SRL, Mumbai, India) (3%) served as a control for the study. This medium was supplemented with 6-BA and KIN (SRL, Mumbai, India) individually (5 and 10 µM) and in the range of 0.5-10 µM with NAA (0.5 and 1 µM) (SRL, Mumbai, India). The pH of the medium was adjusted at 5.80 using NaOH/HCl (1 N) and agar (SRL, Mumbai, India) (0.8%) was used as the solidifying agent. Media sterilization was done in autoclave at 121 °C (15 psi)

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for 30 min. All the cultures were maintained at 26 ± 2 °C under 16/8 hr (dark/light) photoperiod at 40 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lights (Philips India Ltd., India).

Effect of sucrose concentration on shoot regeneration

Optimum shoot regeneration was achieved in MS medium supplemented with sucrose (3%) and 6-BA (8 µM) along with NAA (0.5 µM). The effect of higher sucrose concentrations (4-6%) on regeneration was also evaluated.

Effect of carbon source variation on shoot regeneration

As sucrose at 3% concentration was considered as control, it was replaced by other carbon sources like dextose, maltose and glucose at 3% concentration and their effect on shoot regeneration was evaluated.

Rooting stage

Eight weeks old shoots (> 3 cm) were cultured into ½MS liquid medium supplemented with sucrose (1%) and IBA (2 µM). Leaves from lower nodes of the shoots were excised and they were transferred to tubes containing a filter paper bridge, with the arms of bridge dipping in liquid medium (20 ml). Single shoot was inserted in the center of the bridge and the last 2-3 nodes were dipped in the medium. The tubes were kept in the culture room and lower portion of the tube was covered with aluminium foil till the root induction started.

Statistical analysis

All experiments were carried out in ten replicates, mean and standard error for each combination was calculated. The means were analyzed using ANOVA ($\alpha = 0.05$) and significant means were further analyzed by Dunnett's test using Graph Pad Prism 6.01.

Results and Discussion

Establishment of shoot cultures from leaf explant

Entire leaves of *P. quadrifida* when inoculated on basal MS medium as well as on medium with 6-BA and KIN individually, resulted in only swelling and failed to induce any morphogenic response. Synergistic combinations of 6-BA and NAA when tried, the explants swelled and friable callus differentiated in all the combinations within a week. Lower concentrations of 6-BA (0.5, 1, 2 and 4 µM) with NAA (0.5-1 µM) failed to regenerate shoots, whereas indirect organogenesis was observed in higher concentrations i.e. 8 and 10 µM of 6-BA (Table 1). In medium fortified with 6-BA (8 µM) and NAA (0.5 µM) callus formation was observed at cut end which proliferated, turned nodular with simultaneous appearance of pink protuberances at the end of second week (Fig. 1A). They formed shoot buds by the end of fourth week (Fig. 1B) and after subculture they proliferated along with elongation into shoots during sixth week (Fig. 1C). This proliferation and elongation of shoots continued till eight week and at the end healthy shoots were observed (Fig. 1D). Optimum regeneration of 19.40 ± 0.64 shoots was observed in medium fortified with 6-BA (8 µM) + NAA (0.5 µM) with 100% response within eight weeks (Table 1). Further increase in 6-BA concentration to 10 µM with NAA (0.5 µM) induced same morphogenic response, but number of shoots decreased to 16.70 ± 0.53. Increasing the NAA concentration to 1 µM with same 6-BA concentrations (0.5-10 µM) resulted in similar response and higher concentration of 6-BA (8 and 10 µM) formed nodular callus which further differentiated 15.10 ± 0.81 and 13.30 ± 0.53 shoots respectively (Table 1). Three week old nodular callus was fixed in FAA and the anatomy showed large number of meristemoids. These were round structures with isodimetric cells containing dense cytoplasm and prominent nucleus which were darkly stained and the differentiating shoot buds as leaf primordia (Fig. 1F). When 6-BA was replaced by KIN in combination with NAA, it induced callus which differentiated roots. This callus ceased to proliferate and even after transferring to a fresh new medium, it only increased the length and number of roots till eight week.

There are mainly two type of organogenesis i.e. direct and indirect, and the ratio of cytokinin to auxin in the medium is a key factor behind this (Skoog and Miller, 1957). In the present study, leaf explants of *P. quadrifida* differentiated callus with different morphology in presence of 6-BA and NAA. Similar results are reported for *Suaeda nudiflora* (Singh *et al*., 2004), *Gomphrena globosa* (Jack *et al*., 2005) and *Hemidesmus indicus* (Pathak and Joshi, 2017) where BAP and NAA influenced callus development along with variation in its morphology. In *P. quadrifida* lower concentrations of 6-BA was able to induce only callus whereas in higher concentration differentiated shoots. Similarly high cytokinin level was responsible for organogenesis from leaf explant whereas lower level formed only callus in leaf explant of *Hemidesmus indicus* (Pathak and Joshi, 2017). Optimum regeneration was achieved in MS medium fortified with 6-BA and NAA, and in leaf explants of *Astragalus adsurgens* (Luo and Jia, 1998), *Beta vulgaris* (Yildiz *et al*., 2007) and *Astragalus nezaketae* (Erisen *et al*., 2010) maximum shoots regenerated in medium having 6-BA + NAA. When KIN was supplemented in the medium, it suppressed callus formation, as well as shoots buds, and instead regenerated roots in all the combinations. Similarly in *Hemidesmus indicus* (Pathak and Joshi, 2017) the combination of KIN with NAA evoked less callus formation as compared to combinations of 6-BA with NAA. However contrary result was observed in leaf explants of *Suaeda nudiflora* (Singh *et al*., 2004) where medium fortified with Kn and NAA produced multiple shoots. Bhojwani and Razdan (1996) reported that the ratio between both the types of PGRs regulates the type of organ differentiation *in vitro*. In *P. quadrifida* leaf explants adopted an indirect mode of organogenesis which can become a source of somaclonal variants which enrich the genetic diversity (Patel *et al*., 2014).

Table 1. Effect of BA (0.5-10 µM) with NAA (0.5-1 µM) on shoot regeneration from leaf explants of *P. quadrifida* (eight weeks)

BA (µM)	NAA (µM)	Callus Proliferation	No. of shoots/explant (Mean ± S.E.)[a]	% Response
0	0	-	-	-
0.5	0.5	+	-	-
1	0.5	++	-	-
2	0.5	++	-	-
4	0.5	+++	-	-
8	0.5	++++	19.40 ± 0.64****	100
10	0.5	++++	16.70 ± 0.53****	100
0.5	1	+	-	-
1	1	++	-	-
2	1	++	-	-
4	1	+++	-	-
8	1	++++	15.10±0.81****	100
10	1	++++	13.30±0.53****	100

[a]Means (n = 10) were subjected to ANOVA and significant means (*) were analyzed using Dunnett's test in comparison to control i.e. basal medium ($p < 0.05$).
-: No callus induction, +: less proliferation, ++: medium proliferation, +++: high proliferation, ++++: profuse proliferation.

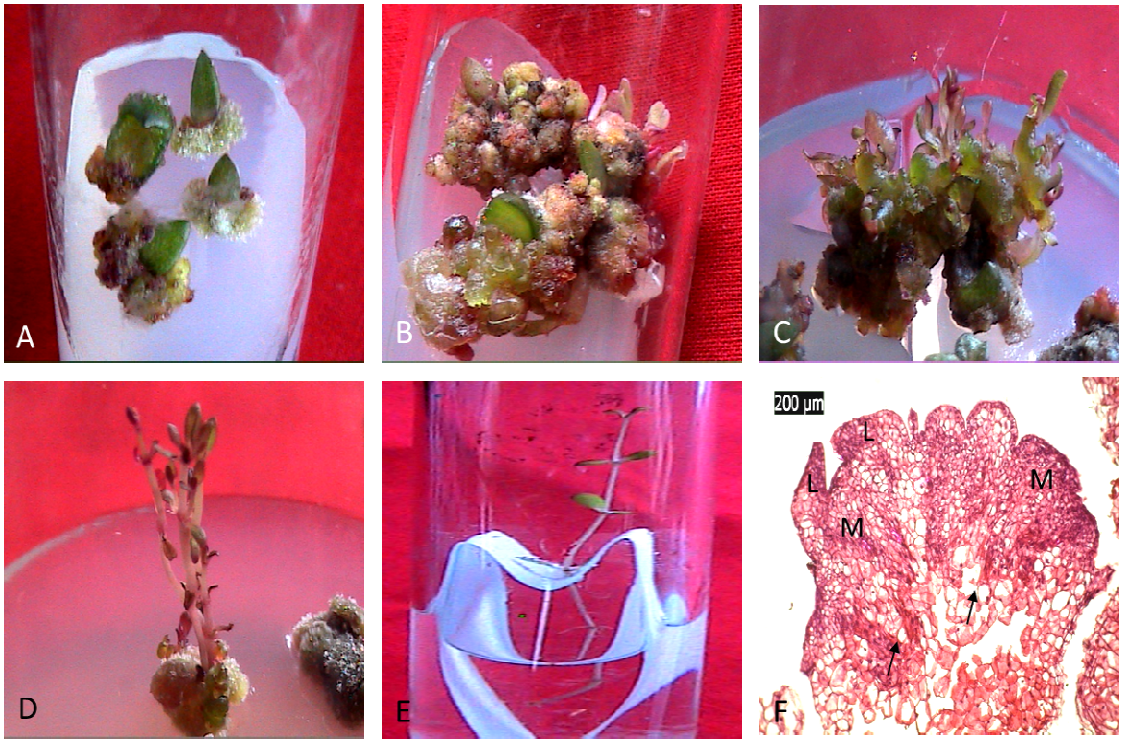

Fig. 1. Indirect organogenesis in *P. quadrifida* in MS + sucrose (3%) + BA (8 µM) + NAA (0.5 µM): (A) nodular callus with pink shoot tips after two weeks, (B) formation of shoot buds after four weeks, (C) proliferation and elongation of buds into shoots after sixth week, (D) healthy elongated shoots at the end of eight weeks, (E) rooting of shoots in ½MS + sucrose (1%) + IBA (2 µM) and (F) anatomy of nodular callus showing aadventitious shoot with leaf primordial (L), shoot meristems (M) and vascular bundles attaching to callus (arrow).

Effect of sucrose concentration on shoot regeneration

Sucrose is known to be a potent source of carbohydrate, which affects the regenerative potency of the explant. Hence optimized medium [6-BA (8 µM) and NAA (0.5 µM)] was fortified with different concentrations of sucrose (4-6%) and its effect on shoot regeneration was observed. Increasing the level of sucrose to 4% in medium evoked an early response and within three week callus started to differentiate shoot buds. An increase in shoot buds was observed however at the end of eight weeks 17.56 ± 0.58 shoots were observed in 100% cultures (Table 2). Increase in sucrose concentration to 5% and 6% induced the leaf explants to differentiate shoot buds, but number of shoots and % response was less in comparison to 3% sucrose. A total of 12.34 ± 0.84 number of shoots were formed in medium fortified with 5% sucrose (75% response) and 9.12

± 0.23 in presence of 6% sucrose (50 % response) (Table 2).

Generally sucrose has been used as a major carbon source which helps in maintaining the osmotic potential of culture and permits the absorption of mineral nutrients present in the medium. This is prerequisite for optimal proliferation, however it may vary from culture to culture (Nowak *et al*., 2004; Siwach *et al*., 2011). In shoot cultures of *Paederia foetida* (Amin *et al*., 2003) and *Elaecarpus robustus* (Rahman *et al*., 2004), sucrose at 3% concentration evoked optimum growth and development of shoots. Gubiš *et al*. (2005) also tried different concentrations of sucrose (1-3%) in tomato and observed that sucrose at 3% concentration regenerated maximum shoots. Naik *et al*. (2010) reported that lower sucrose (2% and 3%) were better for shoot regeneration whereas further increase in sucrose concentration adversely affected shoot regeneration in *Bacopa monnieri*. This may be due to the growth suppression by osmotic stress of high concentration of sucrose, as sugars are perceived by cells as chemical signals and their high concentrations *in vitro* acting as stressing agents (Steinitz, 1999; da Silva, 2004). Charriere and Hahne (1998) observed the direct influence of sucrose concentration on uptake of 6-BAP into the sunflower explants, and it modified the endogenous auxins and cytokinin level which in turn triggered organogenic or embryogenic response. Nowak *et al*. (2004) reported that the sucrose in the medium, not only acts as a carbon source but also as an osmotic, which was also supported by findings of Karim *et al.* (2007) in *Araria elata*.

Effect of carbon source variation on shoot regeneration

Sucrose (3%) regenerated optimum shoots and hence different carbon sources were used to evaluate their effect on shoot regeneration. When dextrose was supplemented in the medium, it evoked similar response as sucrose but resulted in inducing less number of shoots and was able to regenerate only 15.38 ± 0.82 shoots within eight weeks (Table 3). Presence of maltose in the medium also showed a poor response and only 11.70 ± 0.28 vitrified shoots were formed. Whereas the explants when inoculated on medium fortified with glucose as a carbon source it failed to induce any type of morphogenic response (Table 3).

Organic nutrition mostly in the form of sugars is the source of carbon and energy for growth of explants in culture media and its type as well as quantity plays a significant role in organogenesis. Earlier studies have reported that the sucrose is one of the important factors which controls induction and growth of shoots *in vitro* (Gurel and Gulsen, 1998; Gibson, 2000). Although sucrose has been the carbohydrate of choice in the majority of work on regeneration in different species, sometimes it is ineffective as the response is genotype dependent (Thompson *et al*., 1987; Cuenca *et al*., 2000). Results of variation in carbon source have depicted that sucrose is superior in comparison to other carbon sources as it differentiates healthy shoots from leaf explants. Gubis *et al*. (2005) used sucrose, glucose and maltose in the medium for tomato regeneration and observed that sucrose regenerated optimum shoots. The shoots regenerated in maltose supplemented medium showed vitrification and similar report by Bahmani *et al*. (2009) for apple in which the concentration of carbon source affected regeneration frequency and resulted in hyperhydricity of shoots in all the carbon sources. Cuenca and Vieitez (2000) reported that medium fortified with fructose showed vitrification symptoms in shoot cultures of *Fagus* species. Pasqualetto *et al*. (1988) has also reported that concentrations of carbon source and gelling agent influenced the vitrification in apple cultivars *in vitro*. Regeneration frequency and number of shoots varied between the carbon sources and this may be due to the complex interplay of sugar and hormone signalling in plant growth and development (Leon and Sheen, 2003).

Rooting stage

Generally *in vitro* rooting is affected by salts of the medium and reduced strength of MS medium (half or quarter strength) has been proved to be beneficial for rooting (Murashige, 1979; Skirvin and Chu, 1979; Garland and Stoltz, 1981; Zimmerman and Broome, 1981). *In vitro* shoots were excised and transferred to ½MS liquid medium supplemented with sucrose (1%) and IBA (2 μM). Within a week of inoculation, there was increase in shoot length. During second week, root formation was observed from the nodes and its length increased till four weeks (Fig. 1F). IBA has been used for rooting of many medicinal plants like *Bacopa monnieri* (Joshi *et al*., 2010) and *Hemidesmus indicus* (Pathak and Joshi, 2017). Similar reports were documented for *Schinopsis balansae* (Pedro *et al*., 2003), *Bambusa balcooa* (Das and Pal, 2005) and *Pappea capensis* (Mng'omba *et al*., 2007) where rooting was achieved in half strength MS medium supplemented with IBA.

Table 2. Effect of sucrose variation on shoot regeneration from *P. quadrifida* leaf explant (eight weeks)

Sucrose concentration (%)	No. of shoots/explant (Mean ± S.E.)[a]	% Response
3	19.40 ± 0.64	100
4	17.60 ± 0.65	100
5	12.90 ± 2.07*	80
6	09.20 ± 2.44***	60

[a]Means (n = 10) were subjected to ANOVA and significant means (*) were analyzed using Dunnett's test in comparison to control i.e. medium with 3% sucrose concentration ($p < 0.05$).

Table 3. Effect of different carbon source on shoot regeneration from *P. quadrifida* leaf explant (eight weeks)

Carbon source	No. of shoots/explant (Mean ± S.E.)[a]	% Response
Sucrose	19.40 ± 0.64	100
Dextrose	15.30 ± 0.53****	100
Maltose	11.70 ± 0.28****	100
Glucose	0.00 ± 0.00***	0

[a]Means (n = 10) were subjected to ANOVA and significant means (*) were analyzed using Dunnett's test in comparison to control i.e. medium having sucrose ($p < 0.05$).

Conclusions

It is concluded that leaf explant of *P. quadrifida* has a high regenerative potency in medium fortified with sucrose (3%), 6-BA (8 μM) and NAA (0.5 μM). The shoots regenerated through this medium will be further screened for qualitative and quantitative analysis of secondary metabolites.

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