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Full Length Research Paper

In vitro callus induction protocol for *Leptadenia pyrotechnica* using various explants

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An efficient protocol has been developed for a rapid callus induction in *Leptadenia pyrotechnica.* Nodal, internodal and pod explants from mature plant of *L. pyrotechnica* were cultured after surface sterilization on Murashige and Skoog medium supplemented with plant growth regulators (PGRs) cytokinins and auxins individually and with various combinations. Nodal segments proved the best explants (90% callus induction) compared with internodal and pod explants (5% and no callus induction respectively). Different treatments were employed for surface sterilization of explants revealing that combination of sodium hypochlorite ($NaOCl_2$) and mercuric chloride (MC) were found significant. Minimum contamination (7%) occurred at 30% $NaOCl_2$ + 2 g/L MC, while 80% occurred at 30% $NaOCl_2$ + 1 g/L MC. The nodal segments cultured on MS medium supplemented with 0.5 mg/L NAA that produced maximum callus (90%) within four weeks. It was followed by 60% callus induction at 5 mg/L NAA + 2.5 mg/L benzyl-adenine (BA) and 30% at 10 mg/L 2,4-D + 3 mg/L 2ip + 3 g/L activated charcoal (AC), while only 20% appeared at control (without hormone application). This is the first successful attempt to establish consistent callus formation protocols from nodal stem segments of *L. pyrotechnica*. This study may contribute in conservation management of this native plant species found especially in the deserts of Pakistan.

Key words: Callus induction, *Leptadenia pyrotechnica,* plant growth regulators, cytokinins, auxins.

INTRODUCTION

Leptadenia pyrotechnica (Forssk.) Decne is an erect, generally leafless, much branched, ever green shrub that belongs to the family Asclepiadaceae. The plant is found distributed in Pakistan, India, Iran, Arabia, Egypt, Sudan, Somalia, Chad, Libya and Algeria (Ali, 1983). It is also found growing from northern dry sandy Sahel region from Mauritania to Nigeria, and in the semi-desert areas across Africa to western India (Burkill, 1985). This species is found in the sandy deserts of Sindh, Baluchistan and Punjab, Pakistan. The plant is known as Khimp in India and Khipp/Barda in Pakistan (Qureshi, 2004; Qureshi and Shaheen, 2011). *L. pyrotechnica* is a strong soil binder and one of the pioneer shrubs that fix sand dunes due to prolonged and extensive root system. The pods of this shrub are used as vegetables/ potherb. Its branches are dried and soaked in water and woven for rope making. Besides, the same are being used in thatching huts (Qureshi, 2002). The plant is browsed by cattle especially by camels, cows and goat. Medicinally, the whole plant is boiled in water and given to cattle after delivery for the expulsion of placenta (Bhatti et al., 2001).

Various desert plants have been used as major sources of natural products like food and food additives, medicine, flavors, fragrance ingredients, agricultural tools, forage, etc. *L. pyrotechnica*, a very commonly distributed shrub of desert habitat has been reported for being used as antispasmodic, anti-inflammatory,

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antihistaminic, antibacterial diuretic, urolith expulsion, expectorant, anti-rheumatic and purgative (Moustafa et al., 2009). Since the plant is heavily used in deserts of Pakistan to cover different human and animal needs, therefore this species is under huge pressure, which appeals to take measures for its conservation.

Due to overexploitation and misuse of medicinal plants, we are faced with the problem of losing our precious plant resource in the future. This situation calls for effective and in time conservation measures to enrich our lives with the services of plants. In this regard, Vinod et al. (2003) stressed the need of conservation and sustainable utilization of biodiversity. Various research groups across the world have attempted conservation of plants to protect biodiversity (Parabia et al., 2007; Nadeem et al., 2000; Ray and Bhattacharya, 2008; Malik et al., 2005). Different techniques for conservation of plants have been practiced worldwide, the most important being tissue culture (Parabia et al., 2007). Tissue culture is advantageous in producing multiple copies of a plant species within minimum time and space.

Various studies have been carried out on the biochemical (Moustafa et al., 2009), ethnobotanical (Qureshi, 2004; Bhatti et al., 2001) and ecological (Mousa and Ksiksi, 2006; Ksiksi et al., 2006; Qureshi and Bhatti, 2008) aspects of *L. pyrotechnica*, but it is worthwhile to mention that no research has ever been done on its tissue culture aspect before. However, earlier reports are available on micropropagation of *Leptadenia reticulata* from nodal explants (Parabia et al., 2007; Sudipta et al., 2011).

Throughout the world, there is reawakening the importance of wild plants for the benefit of human being especially ethnomedicinally important species and for which globally various studies have been carried out to conserve these germplasm for the protection of biodiversity from the extinction. This has been done by various research groups using different approaches in this regard. However, from Pakistan, conservation of economically important plants has been neglected so far and needs to be addressed in near future to maintain profits of plants and their monitory benefits for our lives. The objectives of this study were to investigate the best surface sterilization method and effects of explant type and plant growth regulators on callogenesis in *L. pyrotechnica*.

MATERIALS AND METHODS

This work was carried out in the Biotech. Laboratory of Date Palm Research Institute, Shah Abdul Latif University, Khairpur, Sindh, Pakistan in 2010 to 2011.

Plant materials

The plant materials were collected from Saleh Pat, a catmint area of Nara desert, Sindh. Young stem nodal segments, pods and internodal segments were selected as an explant source.

Media preparation

The potential of 2 different PGRs Auxin and Cytokinin were analyzed for the induction of callus. The Nutrient medium either alone or supplemented with different combinations of PGRs (Table 1) was used with MS (Murashige and Skoog, 1962) as a basal medium containing (in mg L^{-1}): 100.0 myo-inositol; 1.0 nicotinic acid; 1.0 pyridoxine-HCl; 1.0 thiamine-HCl; 2.0 glycine; 200.0 glutamine; 40.0 adenine sulfate; 2400.0 agar (Agar Technical, Oxoid, Inc.); 1400.0 Gel (Gellan Gum, Caisson Laboratories, Inc.) and 30000.0 sucrose. After preparation of the medium, pH was adjusted to 5.7 ± 0.1 before autoclaving. Media were dispensed into small culture tubes (25 × 150 mm) in aliquots of 15 ml per tube and were capped with aluminum foil. The same were then autoclaved for 20 min at 1.11 kg/cm^2 and 121°C and stored at 4°C before inoculation with explants.

Explant surface sterilization

Freshly harvested nodal, pods and internodal segments were cleaned and dead/decaying parts separated, that were washed with the running tap water for 10 min to remove the dust or sand particles. These were then surface sterilized by using two different methods. In the first method, all explants were treated with 70% ethanol for 2 to 3 min and then washed with 50% sodium hypochlorite (bleach) for 20 min and then dispensed thrice with autoclaved distilled water so as to remove bleach completely. In the second method, explants were treated with 30% of sodium hypochlorite for 15 min and then immersed in 1 and 2 g/L MC solution for 3 min. Few drops of Tween-20 were also added as a surfactant. After that, the plant material was washed three times with sterile distilled water with gentle shaking under sterile conditions.

Inoculation of explants

A cut was given on both basal as well as the top portion of the explants to remove undesirable/dead portions after surface sterilization and all explants were then transferred aseptically in the test tube containing medium while keeping them in an erect position (Plate 1A).

All of the explant cultures were maintained in controlled environment of growth room under illumination, provided by white fluorescent tube lights, with a photoperiod of 16 h in every 24 h. Cultures were visited regularly to observe their response to tissue culture. Data were recorded after every week, for four weeks and the values presented as scores as +, ++, +++, - represent poor, moderate, high and no response, respectively, according to the method described by Abul-Soad et al. (2002) and Mujib et al. (2005). Callus induction rate on each media formulation was calculated using the following equation:

$$\text{Frequency} = \frac{\text{No. of explants showing response}}{\text{Total No. of explants}} \times 100$$

RESULTS AND DISCUSSION

Effect of sterilization method

The surface sterilization was optimized that helped in preventing blackening of tissues and establishment of clean cultures. The sterilization with 30% $NaOCl_2$ + 2 g/L MC proved the most successful procedure as all of the

Table 1. Effect of different medium compositions on callus formation.

Conc. No.	Media composition (mg/L)	Replicates/ treatments	Percentage age of callus induction nodal explants	Percentage age of callus induction pod explants	Percentage age of callus induction internodal explants
1	MS	5	15	-	-
2	MS + 0.5 NAA	5	90*	-	-
3	MS + 1 IAA	5	-	-	-
4	MS + 2 IAA	5	-	-	-
5	MS + 0.1 IAA + 1 BAP	5	-	-	-
6	MS + 0.1 NAA + 1.5 BAP	5	-	-	-
7	MS + 0.1 NAA + 1 BAP	5	-	-	-
8	MS + 0.5 BA	5	20	-	-
9	MS + 0.5 NAA + 0.5 BA	5	30***	-	-
10	MS + 0.5 NAA + 2.5 BA	5	60**	-	-
11	MS + 2.5 NAA + 0.5 BA	5	20	-	-
12	MS + 10 2,4-D + 3 2ip + 3000 A.C	5	30***	-	-

*, ** and *** indicated 1st, 2nd and 3rd significant combination showed callus induction.

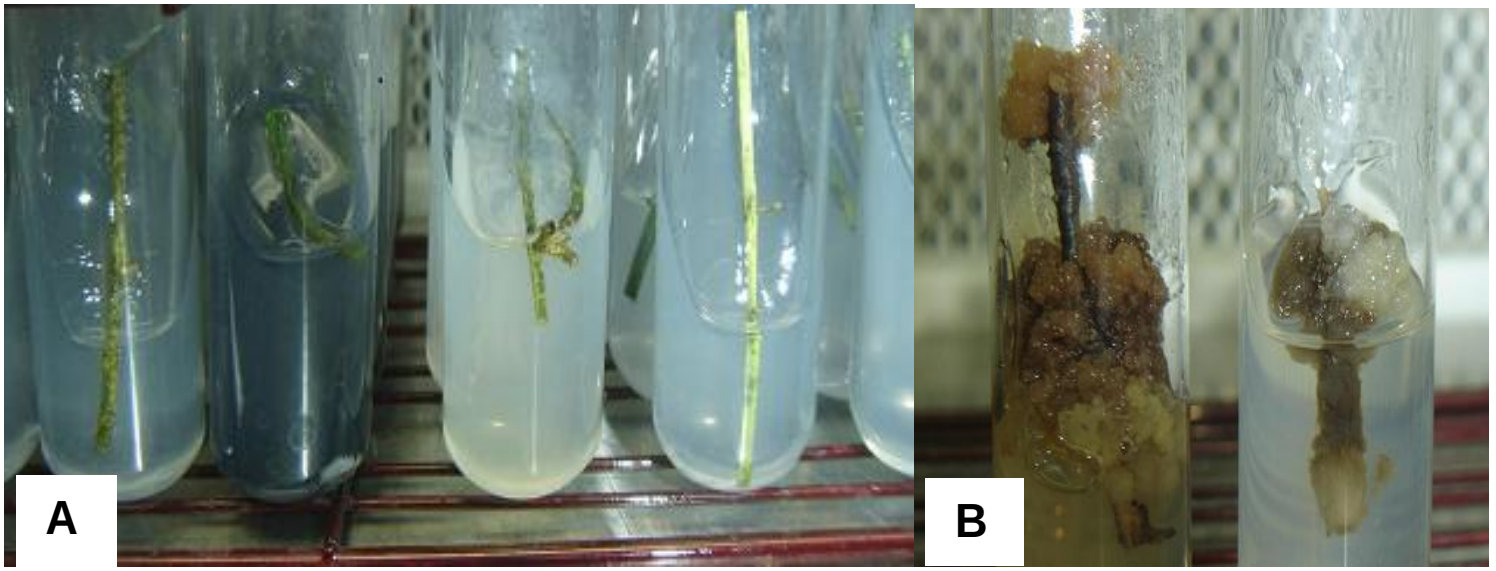

Plate 1. A. Initial explants at different concentrations, B. calli produced from cultured nodal explants.

cuttings were undamaged and responded to tissue culture medium which sprouted well and only 7% of them became infected. On the other hand, treatment with 70% ethanol + 50% $NaOCl_2$ and 30% $NaOCl_2$ + 1 g/L MC failed due to contamination of all explants.

Effect of plant material

Nodal segments found the best source of explants for callus induction that showed 90% response as compared with internodal and pod explants. Except nodal explants, rest of the materials expressed 5% with very poor or no response respectively in callus induction on any media formulation (Table 1) due to vigor loss which became gradually dead.

Callus induction

Auxins and cytokinins are major growth regulators that have profound influence on various phenomena of cell division, callus induction and regeneration (Munazir et al., 2000; Tang et al., 2000). In the present study, maximum callus induction (90%) was observed when 0.5 mg/L of NAA was used, followed by 2.5 mg/L of BA (60% callus induction) and 0.5 mg/L of NAA + 0.5 mg/L BA and 10 mg/L of 2,4-D + 3 mg/L 2ip + 3000 AC which showed 30% callus induction respectively (Table 1). The rest of combinations of NAA + BA, NAA + BAP, IAA + BAP and 2, 4-D + 2ip + A. C were capable of producing more or less poor results. The control treatment (that is, MS medium without any hormone) was capable to induce callus only in a trace amount. Rapid callus induction and its proliferation are vital to tissue culture as those calli are of no use which fails to proliferate (Munazir et al., 2000). It was observed that when MS was supplemented with 0.5 mg/L of NAA, it did not only result in maximum callus induction but also calli proliferated well at this concentration (Plate 1B). This shows that a suitable concentration of growth regulating substance is fruitful in tissue culture for further propagation.

Conclusions

In vitro culture of plants has gained importance during recent years because this technique can be used for the rapid multiplication and *ex situ* conservation of some plants. To the best of my knowledge, no report is available about micro-propagation of *L. pyrotechnica* and the aim of the present work was to determine the culture conditions for micro-propagation of this plant. The present study discovers nodal segment as an effective explant which expressed maximum callus at 0.5 mg/L NAA.

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