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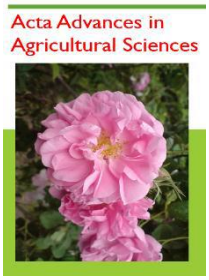

Research Article

Callus Induction Studies in Different Explants of *Podophyllum hexandrum* (Royle)

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ABSTRACT

A protocol for callus induction and *in-vitro* regeneration through organogenesis was established for *Pophyllum hexandrum* Royle (Berberidaceae). Callus was induced on Murashige and Skoog (MS) basal medium supplemented with cytokinin (Kn or BA) and auxin (2,4-D/IBA/NAA/IAA) from leaf, stem and rhizome explants of this economically valuable medicinal plants. Our study also persented both the approaches i.e shoot and root multiplication. No significant callusing was observed when MS medium is fortified with 1.0 mg l^{-1} of both 2,4-D and Kn in leaf, stem as well as rhizome explants. However, higher concentration of Kn and 2,4-D (2.5 – 3.0 mgl^{-1}) exhibited best callusing in leaf and better in stem explants. Also, higher concentration of another cytokinin BA (1.5 – 2.0 mgl^{-1}) produced excellent calli when combined with 2.0 mg l^{-1} of NAA in both leaf and stem explants. BA and NAA in the range of 2.0 – 2.5 mg l^{-1} performed better proliferation in callus mass in both explants i.e. stem and leaf respectively whereas lower concentration of BA and NAA (0.5 mgl^{-1}) produced poor calli in these explants. Though rhizome explants showed best callus mass when 1.5 - 2.0 mgl^{-1} of BA was incorporated with 1.5 - 2.0 mg l^{-1} of both the auxins IBA and NAA. Remarkable callus induction was achieved at higher concentration of BA with IAA and NAA. Along with this as the concentration of auxin and cytokinin was increased the induction frequency was also increased.

Introduction

Podophyllum hexandrum Royle (Berberidaceae) also known as the Indian podophyllum is a perennial herb, growing on the lower slopes of the Himalayas abundantly obtained from the forest region of Afghanistan eastwards to Central China (Chatterji 1952; Fu L-G 1992). Moreover, its rhizomes contain vital lignans which are the dimerisation product of phenylpropanoid compound which has carbons as it,s side chain and act a antitumor compound (Kamil and Dewick 1986b; Arumugam and Bhojwani 1994). Predominating, it also contain

podophyllotoxin, belonging to diarylnaphthelene group which is used as the starting compound for the chemical synthesis of etoposide (VP-16-213), and teniposide (VM-26) and ethophos that are used for the treatment of lung and testicular cancer **(**Stahelin and Warburg 1991**),** leukemia and rheumatoid arthritis (Lerndal and Svensson 2000). However, *P. hexandrum* has been noted as a critically endangered species of Indian Himalayan region (Nadeem et al. 2000). Previously also, Arumugam and Bhijwani (1989) reported the in *vitro* multiplication of Podophyllum plantlets via somatic embryogenesis. Limited availability of *Podophyllum* sp. plant is due to its long juvenile phase and poor fruit setting ability as well as the time consuming collection of the plants also results in shortage of it,s vital resins. Therefore, production of *Podophyllum* sp. using tissue culture techniques considered as attractive technology for mass cultivation (Yousefzadi et al. 2010). Keeping these facts in mind present investigation envisaged in *vitro* mass propagation of *P. hexandrum* (Royle) by using various explants such as leaf, stem and rhizome with optimization of phytohormones.

Materials and Methods

For proper cultivation of *P. hexandrum,* basal medium (BM) comprised the mineral salts and organic nutrients of the Murashige and Skoog (MS) medium (Murashige and Skoog 1962) was prepared, containing 3% sucrose and 8% agar. In our experiment MS medium were also supplemented with different type of growth regulators like auxin [2,4 dichlorophenoxy acetic acid (2,4-D), indole -3-butyric acid (IBA) and α-napthalene acetic acid (NAA)], cytokinin [kinetin (Kn) and 6-benzyladenine (BA)] onto the basal medium with varying concentration ranges from 0.5 to 3.0 mgl^{-1} and combinations as clearly described in result section. The pH of all the media combinations was adjusted to 5.8±0.1 by using 0.1 N NaOH or 0.1 N HCL. Autoclaving was done at 1.06 kg cm^{-2} pressure at 120^0 C and 60% relative humidity was also maintained with 16: 8 hrs in light: dark photoperiod. With respect to variable concentration of cytokinin and auxin the treatments were designated as T_1, T_2, T_3 and T_4. All treatments were conducted in triplicates. Each culture flask contained 40 ml of MS culture medium and up to minimum 1 to 4 explants was inoculated per flask. The data were analyzed statistically using Karl Pearson method as described by Kumar et al (2002).

Leaf, stem and rhizome explants collected from the healthy plants of *P. hexandrum* obtained from the Division of Medicinal and Aromatic Plants (MAP), Uttarakhand University of Horticulture and Forestry, Bahrsar, Pauri, India. Before inoculating the explants they were washed with tap water followed by a surface sterilized by 1% (v/v) Labolene detergent then with 70-90% ethyl alcohol for 30 seconds and by 0.1 (w/v) $HgCl_2$ containing 0.2 ml Tween 80 per 100 ml solution for 1 minute. The explants were rinsed several times with sterile double distilled water (DDW) (Chakraborthy et al. 2010). The cut surfaces exhibiting mercuric chloride damage were aseptically trimmed with a sharp, sterile surgical blade.

Results and Discussion

The callusing response in leaf, stem and rhizome parts of *P. hexandrum* were observed and categorized them poor, moderate, excellent on the basis of callus size developed per explants during growth period. As the callus initiation time and its growth (proliferation) period were found to vary with different explants as well as with different combination as clearly shown in (Table 1, 2 and 3). Our results clearly revealed that at higher concentration of Kn and 2,4-D (2.5 – 3.0 mgl^{-1}) exhibited excellent callusing in leaf and stem explants.

Table 1: *Callusing response of leaf explants of Podophyllum hexandrum on MS media supplemented with combination of different concentration of cytokinin (Kn/BA) and auxins (2,4-D/IBA/NAA).*

Concentration of cytokinin (mg/l)	Auxin (mg/l)						Treatments
	2,4-D						
	0.5	1.0	1.5	2.0	2.5	3.0	
Kinetin							T_1
0.5	0	0	*	*	*	*	
1.0	*	*	**	**	**	**	
1.5	*	**	***	***	***	***	
2.0	*	**	***	****	****	***	
2.5	0	*	**	***	****	****	
3.0	0	**	***	***	****	****	
BA	IBA						
	0.5	1.0	1.5	2.0	2.5	3.0	T_2
0.5	0	0	*	*	*	0	
1.0	*	*	**	**	**	*	
1.5	*	*	**	***	***	**	
2.0	*	*	**	****	****	**	
BA	NAA						
	0.5	1.0	1.5	2.0	2.5	3.0	T_3
0.5	0	0	0	0	0	0	
1.0	*	*	*	*	*	0	
1.5	*	*	***	****	**	*	
2.0	*	**	***	****	****	**	
2.5	0	**	**	****	****	***	

*0 = no response, * = poor, ** = moderate, *** = better, **** = excellent.*

Table 2: *Callusing response of stem explants of Podophyllum hexandrum on MS media supplemented with combination of different concentration of cytokinin (Kn/BA) and auxins (2,4-D/IBA/NAA/IBA).*

Concentration of cytokinin (mg/l)	Auxin (mg/l)						Treatments
	2,4-D						
	0.5	1.0	1.5	2.0	2.5	**3.0**	
Kinetin							T_1
0.5	0	0	*	*	*	*	
1.0	0	*	**	**	**	**	
1.5	*	*	***	**	***	***	
2.0	*	**	***	**	***	***	
2.5	*	*	**	***	****	***	
3.0	0	**	***	***	****	****	
BA	IBA						
	0.5	1.0	1.5	2.0	2.5	**3.0**	T_2
0.5	0	0	*	*	*	0	
1.0	*	*	**	**	**	*	
1.5	*	*	**	****	***	**	
2.0	0	*	**	****	****	**	
BA	NAA						
	0.5	1.0	1.5	2.0	2.5	**3.0**	T_3
0.5	0	0	0	0	0	0	
1.0	*	*	*	*	*	0	
1.5	*	*	***	****	**	*	
2.0	0	**	***	***	****	***	
2.5	*	**	**	***	***	**	
BA	IBA						T_4
	0.5	1.0	1.5	2.0	2.5	3.0	
0.5	0	0	0	0	0	0	
1.0	*	*	**	**	*	0	
1.5	*	**	***	***	**	*	
2.0	*	**	**	****	****	***	
2.5	*	**	**	***	***	*	

*0 = no response, * = poor, ** = moderate, *** = better, **** = excellent.*

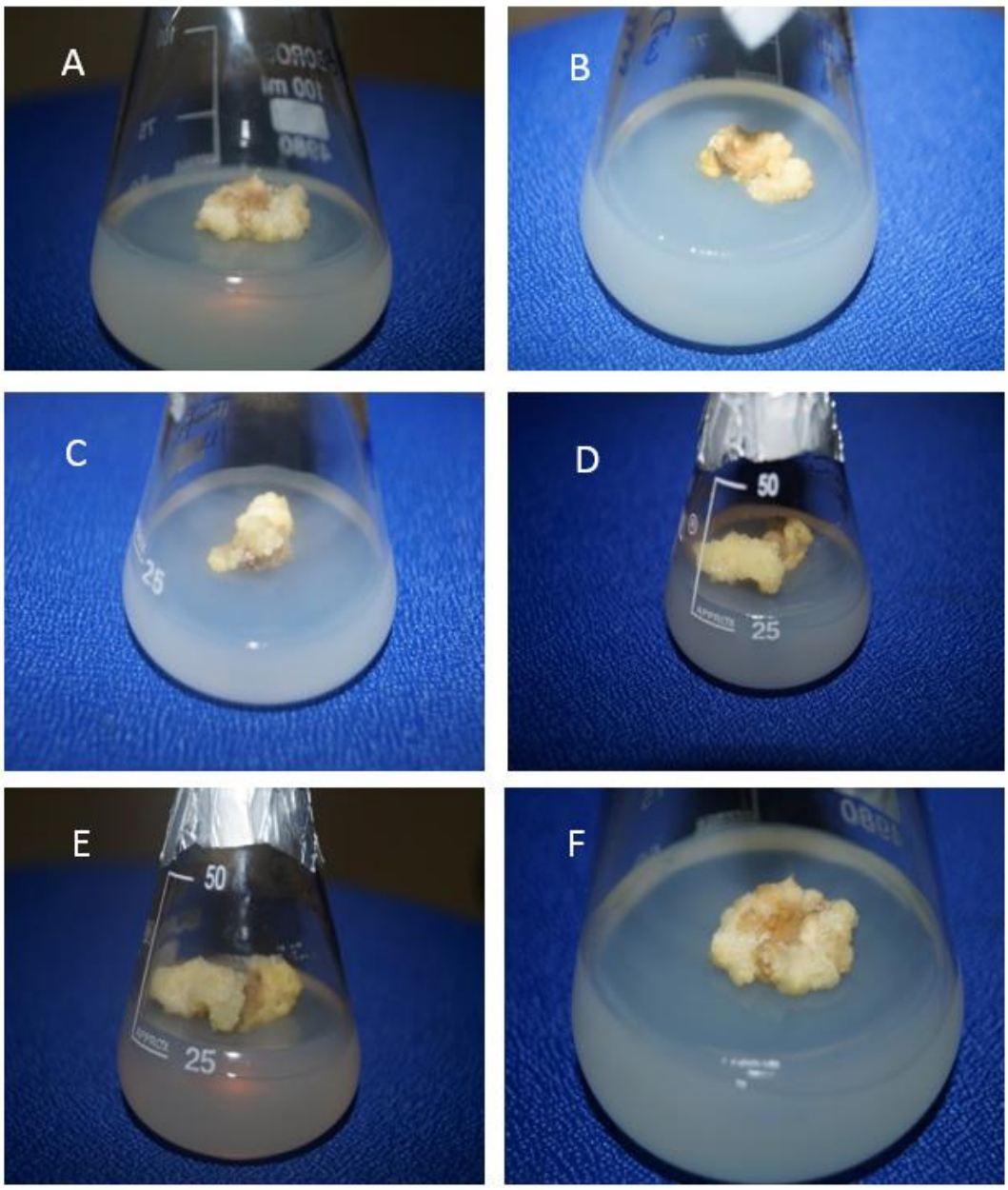

Figure 1: Formation of callus in leaf, stem and rhizome explants of *P. hexandrum* at different concentration with combination of cytokinin and auxin (A) leaf explants with 2.0, 2.5 mgl^{-1} of Kn and 2,4-D. (B) leaf explants with 1.5, 2.0 mgl^{-1} of BA and NAA (C) Stem explants with 1.5, 2.0 mgl^{-1} of Kn and IBA (D) Stem explants with 1.5, 2.0 mgl^{-1} of BA and NAA (E) Rhizome explants with 1.5, 2.0 mgl^{-1} of BA and IBA (F) Rhizome explants with 1.0, 1.5 mgl^{-1} of BA and NAA.

Higher concentration of another cytokinin BA (1.5 – 2.0 mgl^{-l}) produced significant calli when combined with 2.0 mg l^{-l} of NAA in both leaf and stem explants. Moreover, BA and NAA in the range of 2.0 – 2.5 mg l^{-l} exhibited fast proliferation in callus mass in both explants whereas lower concentration of BA and NAA (0.5 mgl^{-l}) produced poor calli in these explants. In rhizome explants higher callus mass was observed when 1.5 - 2.0 mgl^{-l} of BA in combination with 1.5 - 2.0 mg l^{-l} of IBA and NAA respectively (Table 3). Earlier, Chakraborthy et al (2010) demonstrated that no callusing was observed in rhizome explant and moreover they performed the in *vitro* regeneration of this endangered medicinal plant through somatic embryogenesis at different concentration of NAA, BA and 2,4-D only. Also, our results were also found that no morphogenetic responses on MS media alone were observed.

Table 3: *Callusing response of rhizome explants of Podophyllum hexandrum on MS media supplemented with combination of different concentration of cytokinin (Kn/BA) and auxins (2,4-D/IBA/NAA/IAA).*

Concentration of cytokinin (mg/l)	Auxin (mg/l)						Treatments
	2,4-D						
	0.5	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	
Kinetin							T_1
0.5	0	0	0	0	0	0	
1.0	*	*	***	****	**	**	
1.5	*	**	***	***	***	***	
2.0	*	**	****	****	****	***	
2.5	*	***	****	***	****	****	
3.0	*	**	***	***	****	***	
BA	**IBA**						
	0.5	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	T_2
0.5	0	0	0	0	0	0	
1.0	*	*	**	***	****	*******	
1.5	*	**	***	****	***	*******	
2.0	*	**	****	****	****	*******	
2.5	*	**	***	****	****	********	
3.0	*	*	***	***	****	*******	
BA	**NAA**						
	0.5	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	T_3
0.5	0	*	*	*	*	*	
1.0	*	****	****	**	**	*	
1.5	**	**	***	****	***	***	
2.0	*	**	***	***	***	***	
2.5	*	**	***	****	***	****	
BA	**IAA**						T_4
	0.5	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	
0.5	0	0	0	0	0	0	
1.0	*	**	****	**	**	*****	
1.5	*	***	***	***	**	******	
2.0	*	**	****	***	****	*******	
2.5	*	**	***	****	***	****	

*0 = no response, * = poor, ** = moderate, *** = better, **** = excellent.*

Earlier also, no morphogenetic response on MS media alone were observed (Kukreja 1996; Esshwara et al. 1998; Chakraborty et al. 2010). Previously, Saxena et al (1997) also experimented the callus formation in *Psoralea corylefolia* by using individual concentration of auxin and cytokinin, no callus were achieved. Present investigation also showed that lower concentration of Kn (0.5 mgl^{-1}) produced low callus mass with 1.5 – 3.0 mgl^{-1} of 2,4-D in leaf and stem explants (Table 1 and 2). Induction frequency was increased at higher concentration of cytokinin and auxin (2.5 – 3.0 mgl^{-1}) exhibited remarkable callusing in leaf, stem and rhizome (Plate 1). Previously other economic cultivated crops were also studied with optimum concentration of 2,4-D (2.0 mgl^{-1}) and BA (0.5 mgl^{-l}) for callus growth (Fian and Jian 1993). Callus induction studies conducted in cotyledon explants of apple cultivars also revealed that the combination of 2,4-D and Kn increases callus induction. While in stem explants mixed callusing response was observed using 1.5 mgl^{-1} of Kn with different concentration of IBA being poor (1.0mgl^{-1}), good (1.5mgl^{-1}) and best at 2.0mgl^{-1} of IBA. In another set of experiment on same explants (1.5 mgl^{-1}) of BA produced best calli with 2.0 mgl^{-1} of NAA. Present study also conducted both the approaches to achieve in *vitro* plant multiplication i.e shoot and root multiplication under variety of light and dark regime.

Conclusion

Conclusively, our investigation clearly indicated that significant callus induction was achieved at higher concentration of BA with IAA and NAA. Along with this as the concentration of auxin and cytokinin was increased the induction frequency was also increased. The Himalayan endangered plant *P. hexandrum* is slow growing so, micro propagation through tissue culture technique provide the opportunity for mass multiplication of this economically valuable plants. It is hoped that through this regeneration application the metabolic bioactive valuable compound contained in *P. hexandrum* can be efficiently produced.

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