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***In vitro* Regeneration of Hairy Root from *Brassica nigra* in Response to Different PGRs**

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Abstract: *Brassica* sp. is the most common oil yielding plant in India. Cotyledon, root, hypocotyl, etc. collected from *Brassica nigra* and *Brassica juncea* used as explants were incubated in MS medium containing different auxins and cytokinins either singly or in combination. Explants from *Brassica nigra* (except hypocotyl) when incubated in media containing 0.5 mg L⁻¹ NAA and 2.0 mg L⁻¹ BAP or 2.0 mg L⁻¹ Kin exhibited formation of profuse hairy root. Best response was found in 0.5 mg L⁻¹ NAA and 2.0 mg L⁻¹ Kin combination. Roots were cottony in appearance. 2, 4-D either singly or in combination with other cytokinins never produced hairy root. Explants incubated in medium containing 0.5 mg L⁻¹ IBA produced very few hairy roots. Explants of *Brassica juncea* transferred to medium containing similar hormone combination produced green colored compact callus but without any hairy root. Total proteins extracted from both normal and hairy roots were analyzed by 12% SDS-PAGE. A high molecular weight band was observed in hairy root but not found in normal root.

Key words: Anthraquinone, alizarin, callus, cotyledon, endogenous auxin, SDS-PAGE

INTRODUCTION

Brassicaceae includes plants of wide economic importance, most of which are used as spices, oil, vegetables etc. Black mustard or *Brassica nigra* and brown mustard or *Brassica juncea* are cultivated in different parts of India as well as in different countries of the world. Different parts of the plants have medicinal uses. Explants collected from different members of this family produced callus in hormone supplemented media (Toriyama *et al.*, 1987) or in media devoid of any PGRs (Frances *et al.*, 1995). Exogenous auxin induces formation and development of lateral and hairy roots (Lui *et al.*, 2002). Hairy roots are produced when plants are infected with different strains of *Agrobacterium rhizogenes* (Bhalla and Singh, 2008). Joshua *et al.* (2009) cited that Christey *et al.* (2005) reported that transformed T-DNA of the bacterium is integrated into the host DNA and it results in induction of hairy roots (Joshua *et al.*, 2009) from cut ends of the explants. The oncogenes hosted in the T-DNA promote formation of excessive hairy root. Hairy roots have been produced in *Brassica napus* and *Brassica juncea* by infecting the host plant with *Agrobacterium rhizogenes* (Bhalla and Singh, 2008). Hairy roots with *Agrobacterium rhizogenes* infection has also been reported in *Gossypium herbaceum* (Triplett and Moss, 2008), *Rubia tinctorum* (Tercan and Taskan, 1999), *Aconitum heterophyllum* (Giri *et al.*, 1997) and many other plants. It has been reported that production of hairy root increases the production of

secondary metabolites in plants (Srivastava and Srivastava, 2007). Formation of hairy root promotes production of peroxidase (Agostini *et al.*, 1997; Rudrappa *et al.*, 2009). Hairy roots promote formation of peroxidase isoenzyme which can degrade phenolic xenobiotics emitted by industrial waste (Singh *et al.*, 2006). Hairy roots from *Brassica nigra* has also been reported to remove phenol from aqueous solution (Coniglio *et al.*, 2008). Induction of hairy roots with application of PGRs has been reported for the first time in *Brassica nigra*. The objective of this investigation was to study the nature of formation of hairy roots in response to different PGRs. Differences in protein profile of both normal and treated individual had also been investigated.

MATERIALS AND METHODS

Explant source and development of callus: *Brassica nigra* (B-54) and *Brassica juncea* (B-9) seeds were purchased from local market. Seeds were sterilized by rinsing thoroughly in 70% ethanol and 0.01% Tween 20, sterilized with 0.1% HgCl₂ and followed by repeated washing with sterilized distilled water. Sterilized seeds were placed in MS media (Murashige and Skoog, 1962). Different parts (cotyledons, hypocotyls, roots etc.) from ten days old seedlings were used as explant source. Explants were transferred to MS media containing different concentrations of PGRs (2, 4-D, IBA, NAA, BAP, Kin). Callus derived from explants was subcultured at regular intervals.

Determination of anthraquinone content: Anthraquinone content in the normal and hairy roots were determined by using 1 mg/100 mL Alizarin as a standard by measuring the absorption at 450 nm.

Extraction of protein and analysis in SDS-PAGE: Plant materials grown axenically were used as protein source. Tissues were ground thoroughly in extraction buffer containing 100 mM potassium phosphate buffer pH-7.8, 1 mM EDTA, 1% Tween 20, 1% glycerol and 0.1 mM PMSF as protease inhibitor. The homogenate was centrifuged at 10,000 rpm for 15 min. All steps were carried out at 4°C. The supernatant was used as source of protein. Protein content was estimated by using dye binding method of Bradford (1976). Protein profile was analyzed by using 12% SDS-PAGE following standard method described by Laemmli (1970). Gels were stained with 0.2% Coomassie blue R-250 followed by destaining.

RESULT

Induction of callus from different species of *Brassica* in response to different PGRs used: Different parts collected from both *Brassica nigra* and *Brassica juncea* were incubated in media containing 0.5 mg L⁻¹ NAA and 2.0 mg L⁻¹ BAP. Vigorous callus growth took place from all the explants of both the species in all the different PGRs concentration used. Callus induced from explants of *Brassica nigra* regenerated hairy root after 10 days in medium consisting of 0.5 mg L⁻¹ NAA and 2.0 mg L⁻¹ Kin combination. Except hypocotyl explants of *Brassica nigra* all other explants of this species gave rise to hairy root from callus (Table 1). Explants in *Brassica juncea* never gave rise to such hair. In this case, callus turned brown after 18-20 days. Explants were collected from 10 days old seedlings and incubated in media containing varying concentration of NAA either singly or in combination with BAP or Kin. All explants induced callus within 10-12 days. For callus growth 0.5 mg L⁻¹ NAA and

2.0 mg L⁻¹ BAP showed best response. After 15 days other explants, except hypocotyl gave rise to hairy root. Roots were cottony in appearance, superficial in nature. 0.5 mg L⁻¹ NAA and 2.0 mg L⁻¹ Kin combination supported the best growth condition for hairy roots (Fig. 2a-c). The NAA when used singly also gave rise to hairy root at a concentration range of 0.04 mg L⁻¹ -10.0 mg L⁻¹ (Fig. 1). Cotyledonary explants when transferred to media supplemented with different concentrations of 2, 4-D either used singly or in combination of BAP or Kin induced callus in 7-10 days. Callus was initially green but gradually turned colourless and soft in nature. Best response was found in 0.5 mg L⁻¹ NAA and 2.0 mg L⁻¹ BAP combination. Callus grown in media containing 2, 4-D and BAP or Kin combination did not give rise to hairy root (Fig. 2). Cotyledonary explants were incubated in media containing IBA singly or in combination with 2.0 mg L⁻¹ BAP or Kin. Very few number of hairy root was obtained in medium containing IBA singly (Table 2). Induction of callus was also noted after 20 days in media supplemented with 0.5 mg L⁻¹ IBA singly and in combination with 2.0 mg L⁻¹ BAP. In IBA and Kin combination, the explants turned whitish in colour. Cotyledons were incubated in media containing different range of concentrations of Kin (0.5-2.0 mg L⁻¹). Induction of callus was noted only in 0.5 mg L⁻¹ Kin. In all other

Table 1: Response of different explants of *Brassica nigra* and *Brassica juncea*

Species	PGRs used	Explants	Response	Days for response
<i>Brassica nigra</i>	NAA- 0.5 mg L ⁻¹	Cotyledons	CH	15
		Hypocotyls	C	10
	BAP-2.0 mg L ⁻¹	Nodes	CH	12
		Roots	CH	12
<i>Brassica juncea</i>	NAA - 0.5 mg L ⁻¹	Cotyledons	C	7
		Hypocotyls	C	12
	BAP-2.0 mg L ⁻¹	Nodes	C	10
		Roots	C	8

Responses studied on the response of ten replicates. C: Callus, H: Hairy roots

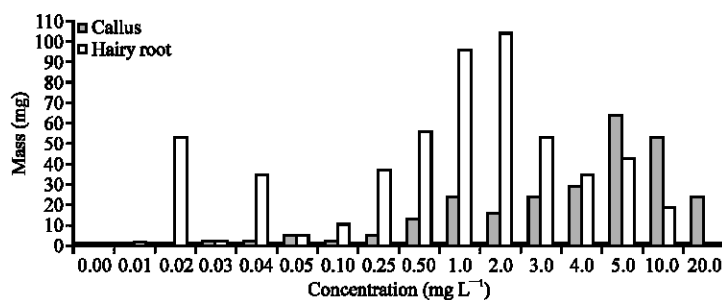


Fig. 1: Effect of different NAA concentration on hairy root induction. Mass of callus (mg) and hairy roots regenerating from cotyledons have increased with increasing concentration of NAA (from 0.01-20.0 mg L⁻¹). At 2.0 mg L⁻¹ NAA concentration highest hairy root has regenerated. In control (0.00 mg L⁻¹) no hairy root has been found

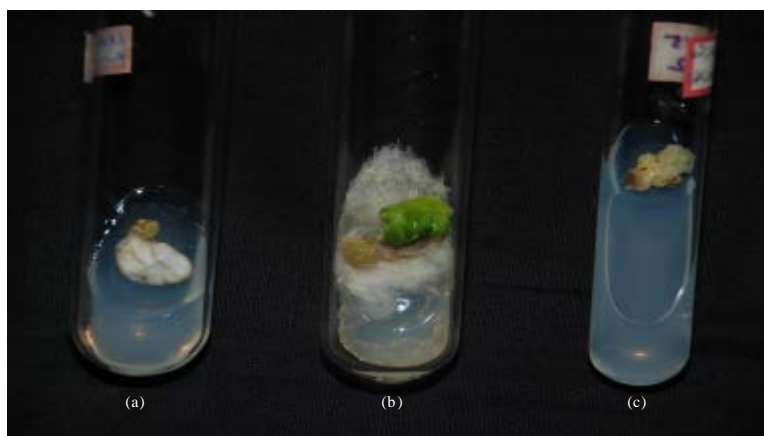


Fig. 2: Regeneration of callus and hairy roots from cotyledonary explants of *Brassica nigra* after one month in culture condition. (a) Explants incubated in medium containing 0.5 mg L⁻¹ IBA and 2.0 mg L⁻¹ Kin, explants turned white without any callus or hairy root formation. (b) Explants incubated in medium containing 0.5 mg L⁻¹ NAA and 2.0 mg L⁻¹ Kin Profuse hairy root is found with lesser amount of callus. (c) Explants incubated in medium containing 0.5 mg L⁻¹ 2, 4-D and 2.0 mg L⁻¹ Kin, callus without any hairy root regenerated from explant

Table 2: Response of *Brassica nigra* explants in response to different PGRs used.

Days for response	Explants Response	Explants responded* (%)	PGRs used (mg L ⁻¹)				
			Kin	BAP	NAA	IBA	2,4-D
7	C ⁺	40	-	-	-	-	0.5
7	C ⁺	40	-	-	-	-	1
10	C ⁺⁺⁺	30	-	2	-	-	0.5
7	C ⁺⁺	30	2	-	-	-	0.5
20	C ⁺ H ⁺	20	-	-	-	-	0.5
20	C ⁺ H ⁺	20	-	-	-	1	-
20	C ⁺	20	-	2	-	-	0.5
-	-	-	2	-	-	-	0.5
10	C ⁺⁺ H ⁺⁺	20	-	-	0.5	-	-
10	C ⁺⁺ H ⁺⁺	40	-	-	1	-	-
7	C ⁺⁺⁺ H ⁺	40	-	2	0.5	-	-
7	C ⁺ H ⁺⁺⁺	80	2	-	0.5	-	-
25	C ⁺	10	-	0.5	-	-	-
25	C ⁺	10	-	1	-	-	-
18	C ⁺⁺	20	-	2	-	-	-
-	-	-	0.5	-	-	-	-
-	-	-	1	-	-	-	-
-	-	-	2	-	-	-	-

C: Callus, H: Hairy roots, +: Callus or hairy roots present, ++: Moderate callus or hairy roots, +++: Profuse callus or hairy roots, -: No response found. *All data are taken on the basis of ten replicates in each concentration and repeated twice

concentrations explants turned brown. Cotyledons used as explants when transferred to media containing different concentrations of BAP (0.5-2.0 mg L⁻¹) gave rise to callus. In media supplemented with 2.0 mg L⁻¹ BAP shoot regeneration took place from callus after 18-20 days.

Alizarin test for *Agrobacterium rhizogenes*: Anthraquinone is a marker for *Agrobacterium rhizogenes*. Alizarin test was performed to detect the presence of any

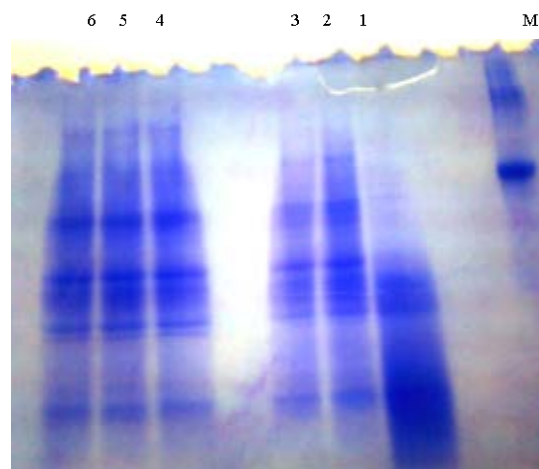


Fig. 3: SDS-PAGE analysis of proteins isolated from normal roots and hairy roots. M: BSA used as marker. Lane 1, 2, 3: Proteins from normal roots. Lane 4, 5, 6: Proteins from hairy roots. Proteins were analyzed in 12% SDS-PAGE. Lane 4, 5 and 6 showed expression of a high molecular weight protein in hairy root

anthraquinone in the hairy roots. No anthraquinone could be detected in hairy roots, ruling out the possibility of *Agrobacterium rhizogenes* infection.

SDS-PAGE analysis: Destained gels showed almost same banding pattern in both normal and hairy roots. A high molecular weight band was observed in case of hairy root which was not found in normal roots (Fig. 3).

DISCUSSION

Callus can be obtained from different explants incubated in different hormone combinations and concentrations (Toriyama *et al.*, 1987). Explants from *Brassica nigra* and *Brassica juncea* induces callus in all hormone combinations. *Pongamia pinnata* a biodiesel yielding plant gives rise to adventitious roots when treated with IAA, IBA or NAA (Kesari *et al.*, 2010). Explants except hypocotyls, from *Brassica nigra* when incubated in media containing NAA and BAP or Kin combinations give rise to callus with hairy roots (Table 1). Hairy roots are also found from explants growing in medium supplemented with NAA in different concentrations and also from medium supplemented with 0.5 mg L⁻¹ IBA devoid of any BAP or Kin combination. Highest root induction is found in medium containing 0.5 mg L⁻¹ NAA and 2.0 mg L⁻¹ BAP or 2.0 mg L⁻¹ Kin combination (Fig. 2). NAA if used singly can also give rise to callus with profused hairy roots (Fig. 1). At lower concentration, very few hairy roots are produced and root length is comparatively longer than the higher concentrations. Slight callus formation with induction of hairy roots is observed at 0.04 mg L⁻¹ NAA concentration. Induction of hairy root is observed when dicotyledonous plants are infected with *Agrobacterium rhizogenes* (Giri *et al.*, 1997). Endogenous auxin level is increased in the infected plants and it induces formation of hairy roots. The absence of *A. rhizogenes* in the hairy roots of *Brassica nigra* was demonstrated by Alizarin test. In the experiments performed here, application of exogenous auxin is switching on the expression of some genes, which are supposed to synthesize *de novo* proteins. These proteins may trigger induction of hairy roots by increasing endogenous auxin level. Higher concentration of either NAA or IBA acts as inhibitor of adventitious rooting in plants (Kesari *et al.*, 2010). After increasing auxin concentration up to certain level, the auxin is acting as an antagonistic factor in auxin induced gene expression. At 20.0 mg L⁻¹ NAA callus is found without any hairy roots (Fig. 1). When Kin is coupled with NAA, the number of hairy root is maximum. Kin is acting as a stimulus for auxin activity i.e., increasing the number of hairy root. IBA if used singly can also give rise to very few hairy roots. 2, 4-D either used singly or in combination with BAP or Kin is unable to induce any hairy root, comparing with NAA. When any other cytokinin (BAP or Kin) is used, IBA action is suppressed. 2, 4-D at any situation is unable to induce any hairy root. It may be due to its failure in increasing the endogenous auxin level. Other cytokinin when combined with IBA is unable to promote auxin level. Explants of *Brassica juncea* incubated in similar NAA and BAP concentrations as

mentioned above showed only callus growth without any hairy root. In axenic culture, members of Brassicaceae (*Brassica nigra*, *Brassica juncea*, *Brassica oleracea*, *Raphanus sativus* etc.) exhibits presence of tuft of root hairs. But in tissue culture condition only explants from *Brassica nigra* showed hairy root growth. Explant once producing hairy root never gives rise to any shoot bud. Regeneration of shoot bud is only observed in medium supplemented with 2.0 mg L⁻¹ BAP. Probably, the endogenous auxin level is increased so much that it suppressed shoot regeneration in spite of presence of cytokinins. Subcultured callus after a certain number of passages stop producing any hairy root.

In SDS-PAGE analysis an extra high molecular weight band is observed in hairy root (Fig. 3). Due to applications of exogenous hormones some *de novo* proteins may have been synthesized, which has been observed as an extra band in hairy root. It signifies the presence of new protein with high molecular weight that has been expressed here.

Thus it can be concluded that the formation of hairy roots without *Agrobacterium rhizogenes* infection is not reported earlier. Different explants (except hypocotyl) of *Brassica nigra* show this unique feature.

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