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Full Length Research Paper

Callus and azadirachtin related limonoids production through *in vitro* culture of neem (*Azadirachta indica* A. Juss)

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A protocol was established for the induction of callus and suspension cultures for azadirachtin production from neem explants. Different concentrations and combinations of plant growth regulators (2,4-D, NAA, IAA and BAP) were supplemented in MS medium. Immature flowers, nodular stem sections, leaves immature embryos and mature seeds were used as explants. The highest callus development (78%) was observed when immature flowers were inoculated on MS basal medium with addition of 1.0 mg/l 2,4-D, 1.0 mg/l BAP, 0.2 mg/l NAA and 3% sucrose. The azadirachtin containing liminoids were determined from calli obtained from different explants. Effect of sucrose, glucose, NH_4NO_3, KNO_3 and urea on cell suspension cultures and azadirachtin contents were also investigated. The dry cell weight and azadirachtin contents increased to 373.1 and 359.2 µg/ 50 ml when 0.25 and 0.5 g/l NH_4NO_3 was added in MS liquid media and supplemented with 1.0 mg/l 2,4-D, 0.2 mg/l BAP and 3% sucrose.

Key words: Neem (*Azadirachta indica* A. Juss), *in vitro,* indoleacetic acid (IAA), naphthylacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), benzylaminopurine (BAP).

INTRODUCTION

Neem (*Azadirachta indica* A. Juss) is an evergreen tropical forest tree that is a renewable source of various useful products. The seed, flowers, leaves, bark and branches of neem tree have multiple uses and several compounds of medical importance have been reported (Biswas et al., 2002). The neem holds the promise of providing highly effective, non-toxic and environmentally friendly means of controlling or eliminating insects and pests that cause losses in agricultural production (Govindachari et al., 1991). Among these, azadirachtin-A, nimbin and salannin are the major triterpenoids having several biological activities from neem (Subapriya and Nagini, 2005). The azadirachtin was first isolated from the neem seeds (Butterworth and Morgan, 1968).

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Abbreviations: IAA, indoleacetic acid; **NAA**, naphthylacetic acid; **2,4–D**, 2,4-dichlorophenoxyacetic acid; **BAP**, benzylaminopurine; **DCW**, callus dry weight.

Currently, azadirachtin is extracted from seed kernels of naturally growing neem and its availability is dependent upon a reliable supply of quality seeds. The seeds are produced once a year in neem but due to operational and storage problems, only a fraction of these seeds are used for azadirachtin extraction. Moreover, large variations in azadirachtin content exist based on the diverse genotype and wider geographical distribution of neem trees (Sidhu and Behl, 1996). The current supplies of bioactive azadirachtin containing liminoid compounds from neem tree have not being able to meet increasing demand (Sexena, 1989). Biotechnological advancement using plant cell culture has received sufficient attention as an attractive alternative production system for this important bioactive molecule as reported by Prakash et al. (2002). This *in vitro* culture technique could provide an alternative supply of important compounds used in medicine as well as stimulate the production of novel compounds which are not found in intact plant (Furmanowa et al., 1997; Zhao et al., 2001). The tissue culture allows for the continuous production of azadirachtin-containing liminoids that is free of pathogens

and toxins, independent of environmental conditions. The possibility of continuous production of azadirachtin through alternative route of plant cell culture has been reported (Balaji et al., 2003; Prakash et al., 2002; Raval et al., 2003). However, increase of azadirachtin containing liminoids through use of biotechnological techniques is the main objective of future research. The present investigation is based on neem callus and suspension cultures for production of azadirachtin-related liminoids.

MATERIALS AND METHODS

Induction of callus cultures

The immature flowers, leaves, nodular stem sections, immature embryos and mature seeds were used as explants and were collected from a seven years old neem tree of Allama I. I. Kazi campus, University of Sindh, Jamshoro, Pakistan. The explants were disinfected for 15 - 20 min with 50 - 75% (v/v) commercial bleach (sodium hypochloride 5.25%, v/v) depending on explant type and then rinsed 4 times (5 min each wash) with sterile distilled water. The MS medium (Murashige and Skoog, 1962) with varying concentrations of 2,4-D, BAP and NAA were used for callus induction separately or in combinations. The pH of the media was adjusted to 5.8, solidified with 0.4% phytagel and sterilized at 121°C for 15 min. The proliferated calli were maintained on same medium. For carbohydrate effect, different concentrations of sucrose and glucose were used while for nitrogen effect, various concentrations of NH_4NO_3, KNO_3 and urea were used in the medium. All the cultures were incubated at light intensity of 2500 lux for photoperiod of 16/8 h light/dark at 25 ± 2°C. The proliferating calli were harvested after four weeks and six weeks for further analysis.

Establishment of cell suspension cultures

The suspension cultures were initiated in liquid MS medium supplemented with varying concentrations of sucrose, glucose, NH_4NO_3, KNO_3 and urea. The suspension cultures were maintained at light intensity of 2500 lux for photoperiod of 16/8 h light/dark at 100 rpm on shaking incubator at 25 ± 2°C.

Extraction and determination of azadirachtin related liminoids

The suspension cultures were centrifuged at 6000 rpm for fifteen minutes. The fresh weight of pellet was determined and dried at 50°C for 72 h. For extraction of azadirachtin related limonoids, 0.5 g of dried calli and suspension cultures were grounded in pestle mortar by adding 5 ml of dichloromethane, centrifuged at 7000 rpm for 15 min and supernatant of each sample was collected and procedure was repeated twice. The dichloromethane was then evaporated to sample dryness at 50°C in water bath. The dried samples were re-dissolved in 10 ml-distilled water and stored at -20°C.

Azadirachtin related liminoids were determined using colorimetric method described by Dai et al. (1999). 0.7 ml of test sample was added to 0.2 ml of methanol solution of vanillin (0.02 mg/ ml). After manually shaking, the sample was stand at room temperature for 2 min. Then 0.3 ml concentrated sulphuric acid was added into three portions (0.1 ml each) and the mixture was stirred for 10 s after each addition. Then 0.7 ml methanol was added to convert the obtained two-layer mixture into homogenous solution that developed into a blue-green color. The solution was left at room temperature for 5 min and then absorbance was measured at 577 nm using UV-visible spectrophotometer. The azadirachtin related limonoids were calculated from standard graph of azadirachtin.

RESULTS

The capacity of neem explants to produce calli on different media was investigated. The MS medium was supplemented with ten different concentrations of 2,4-D, BAP, NAA and IAA in different combinations. After five weeks of inoculation, immature flowers showed maximum callusing response on different media followed by nodular stem sections, immature embryos, leaves and mature seeds, respectively. The immature flowers showed 78% callusing response on M9 medium (MS supplemented with 1.0 mg/l 2,4-D, 1.0 mg/l BAP and 0.2 mg/l NAA) followed by 71, 62, 53 and 42% callusing response on M8, M10, M7 and M6 media respectively as shown in Table 1. Among nodular stem sections, 54, 52 and 42% of them proliferated on M7, M6 and M4 medium, respectively. The leaf explants showed only 48 and 32% proliferation on M1 and M2 medium while it failed to proliferate on M5 to M10 medium. On the other hand, matured seeds were found to show less proliferation efficiency on all ten media and only 32 and 31% response was observed on M3 and M6 medium as in Table 1.

The callus dry weight (DCW) and azadirachtin containing limonoids of callus cultures raised from different explants on different media were determined after eight weeks of inoculation. The highest dry weight DCW (0.381 g) was obtained from immature flower calli on M9 medium while minimum DCW (0.199 g) was obtained from leaf on M2 medium after eight weeks of inoculation as shown in Table 2. Among these callus cultures, highest azadirachtin containing limonoids 254.2 and 235.6 µg/g dry wt were found in immature flower based calli obtained from M9 and M8 media while the lowest azadirachtin contents 16.1 µg/g dry wt was determined in calli raised from leaf on M1 medium.

As MS liquid medium was supplemented with different concentrations of sucrose and glucose, variable DCW and azadirachtin contents were obtained after four weeks of inoculation. According to results shown in Table 3, the highest DCW 0.359 g/ 50 ml was obtained in 40 g/l sucrose containing medium followed by 0.352 and 0.348 g/ 50 ml obtained in 30 g/l of medium containing glucose and sucrose separately. Similarly, higher azadirachtin related limonoids 351.2 µg/ 50 ml was found in calli obtained from 30 g/l sucrose containing medium followed by 348.6 and 317.7 µg/ 50 ml from 40 g/l sucrose and 30 g/l glucose containing liquid media, respectively.

The addition of different concentrations of different nitrogen source as NH_4NO_3, KNO_3 and urea in MS liquid media supplemented with 30 g/l sucrose increased the DCW and azadirachtin contents. The additional supplement of 0.25g/L KNO_3 showed the highest DCW 0.412 g/ 50 ml followed by 0.398 and 0.382 g/ 50 ml on 1.0 g/l

Table 1. Percentage callusing response of different explants of neem on MS medium supplemented with 3.0% sucrose and different concentrations of 2,4-D, BAP, NAA and IAA after four weeks of inoculation.

Medium	Plant growth regulators (mg/l)				% Callusing response of neem explants				
	2,4-D	BAP	NAA	IAA	Nodular stem section	Immature Flower	Immature Embryo	Mature Seed	Young Leaf
M1	-	0.25	-	0.1	11	NR	NR	NR	48
M2	-	0.25	-	0.25	8	12	NR	NR	32
M3	0.5	0.5	-	-	18	16	21	32	6
M4	1.0	0	-	-	42	12	52	7	2
M5	0.5	1.0	-	-	33	19	17	26	NR
M6	1.0	0.5	-	-	52	42	39	31	NR
M7	1.0	1.0	-	-	54	53	51	NR	NR
M8	1.0	1.0	0.1	-	12	71	26	NR	NR
M9	1.0	1.0	0.2	-	NR	78	NR	NR	NR
M10	1.0	1.0	0.3	-	NR	62	NR	NR	NR

NR = No response.

Table 2. Gram dry weight and azadirachtin contents in callus cultures obtained from different explants on different media.

Explant	Callus harvested from medium	Callus growth (g dry wt)	Azadirachtin related limonoids (µg/g dry wt)
Immature flower	M9	0.381	254.1
Immature flower	M8	0.364	235.2
Nodular stem section	M7	0.21	162.6
Nodular stem section	M6	0.231	168.2
Immature embryo	M4	0.321	157.6
Immature embryo	M7	0.311	142.3
Mature seed	M3	0.279	266.5
Mature seed	M6	0.232	121.6
Leaf	M1	0.231	16.1
Leaf	M2	0.199	26.4

Table 3. Effect of sucrose and glucose on dry cell weight (DCW) and azadirachtin related limonoids in cell suspension cultures of neem.

Carbohydrate source (g/l)		DCW (g/50 ml)	Azadirachtin related limonoids (µg/50 ml)
Sucrose	10	0.298	162.2
	20	0.321	246.7
	30	0.348	351.2
	40	0.359	348.6
Glucose	10	0.120	248.8
	20	0.217	256.3
	30	0.352	317.7
	40	0.343	201.3

KNO_3 and 0.25 g/l NH_4NO_3 containing medium after four weeks of inoculation, while azadirachtin contents were increased by 373.1 and 359.2 µg/ 50 ml in MS liquid media supplemented with 0.25 and 0.5 g/l NH_4NO_3 as compared to different concentrations of KNO_3 and urea supplemented media as shown in Table 4.

Table 4. Effect of different nitrogen sources on dry cell weight (DCW) and azadirachtin related limonoids in cell suspension cultures of neem in MS liquid medium with 3% sucrose.

Nitrogen source (g/l)		DCW (g/50 ml)	Azadirachtin related limonoids (µg/50 ml)
NH_4NO_3	0.25	0.382	373.1
	0.5	0.367	359.2
	1	0.362	345.6
KNO_3	0.25	0.412	343.3
	0.5	0.377	346.1
	1	0.398	328.8
Urea	0.25	0.378	332.1
	0.5	0.342	326.5
	1	0.321	267.8

DISCUSSION

It has been postulated that secondary metabolism is closely connected with differentiation processes (Fowler, 1981; Rhodes et al., 1986), which depend strongly on growth regulators. These processes must be triggered also in a callus. The potential for differentiation is limited, due to a given auxin-cytokinin ratio in the nutrient medium. The cytokinine and auxin are known to promote callus formation in tissue culture (Akiyoshi et al., 1983). The cultural conditions for neem callus and growth were established. It was observed that the callusing percentage and degree of callusing from different explants of neem were dependent on presence of BAP in combination with 2,4-D, NAA or IAA in the medium. The best callusing response (78%) was observed when immature flower was used as explant on M9 medium (MS medium supplemented with 3.0% sucrose, 1.0 mg/l 2,4-D, 1.0 mg/l BAP and 0.2 mg/l NAA). However, the degree of callusing was enhanced when immature flowers were first inoculated on MS medium supplemented with 1.0 mg/l 2,4-D, 1.0 mg/l BAP and 0.2 mg/l NAA and 10% sucrose for fifteen days and then sub cultured on same medium with 3.0% sucrose only (data not shown).

The variability in azadirachtin related liminoids was found in harvested calli raised from different explants on different media as shown in Table 2. However, these contents were significantly lower when compared to azadirachtin contents obtained from suspension cells in liquid media having different concentrations of carbohydrates or nitrogen source as shown in Tables 3 and 4. Babu et al. (2006) reported azadirachtin production in callus and suspension cultures and found the highest azadirachtin-A in nodal segment derived callus tissues on MS medium supplemented with 5 mg/l benzyladenine (BA), 10 mg/l each of IBA and NAA. The azadirachtin contents of callus cultures varied depending on the cell line, the nutrient medium and the carbohydrate source employed. The callus dry weight and azadirachtin related liminoids were also affected significantly by sucrose and glucose concentrations in the medium as shown in Table 3. Carbohydrate, phosphorus and nitrogen were the most essential ingredients of the nutrient medium that affected the growth and metabolism of cultured cells and have been studied in different species in relation to the biosynthesis of secondary metabolites. The possibility of production of azadirachtin through alternative route of plant cell culture has been reported (Balaji et al., 2003; Prakash et al., 2002; Raval et al., 2003). Sujanya et al. (2008) reported that reduction in sucrose to 15 g/l resulted in a proportionate decrease in biomass in standard MS medium, the growth being reduced from 43.4 to 40.52 g/l with a correspondingly lower growth rate, while Wewetzer (1998) observed significant increase in azadirachtin production with change of sucrose concentration. With additional supplement of NH_4NO_3 and KNO_3 in MS liquid media, increased in the overall production of azadirachtin related liminoids are as presented in Table 4. Variations in cell suspension cultures dry weight and azadirachtin related contents in MS liquid media with various concentrations of sucrose and glucose was also observed. Furthermore, the present results are in agreement with another study by Wewetzer (1998). It was found that low sucrose concentration in media favours more callus induction compared to high concentration but increase in sucrose concentration is obviously not a limiting factor in azadirachtin production. Similar findings were reported by Raval et al. (2003) that the increased production of azadirachtin in quantities well above the limit of detection in cell suspension cultures of neem depends on the cell line and components of the nutrient medium such as total nitrate, phosphate and sucrose levels. An increase in product formation is seen with increased nitrate and decreased phosphate levels (Sujanya et al., 2008).

Conclusion

This is the first report in Pakistan that shows the induction

of callus, degree of callus formation and azadirachtin containing liminoids production from different explants of neem. These results provide a unique opportunity towards further studies on different aspects on the biosynthesis of azadirachtin and other neem based compounds like salannin and nimbin for commercial production.

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