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Effect of Various Growth Hormone Concentration and Combination on Callus Induction, Nature of Callus and Callogenic Response of *Nerium odorum*

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Abstract *Nerium odorum*, *Linn*. (Apocynaceae) is an important evergreen shrub. It is heat, salinity and drought tolerant. Plants with milky sap have medicinal value, mainly cardenolides, flavonoids and terpenes. It is used for wastewater purification and for restoration of riparian woodlands. In view of these facts, the study was conducted for micropropagation of *N. odorum.* Murashige and Skoog (MS) media supplemented with different concentrations (0.5–10.0 mg/l) of 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and kinetin (Kin) were used singly and in combinations. Among all the growth hormones, 2,4-D was the best for callus induction (75 % in stem and 79 % in leaf) and in combination 2,4-D and BAP (78 % in stem and 81 % in leaf). The day of callus induction started from the 19th to the 37th day. This variation is due to the differences in culture conditions and the age of explants. The fresh and dry weight and moisture content showed good growth of callus, which is used in further studies of alkaloid production. Micropropagation of this plant allows the production of clones at a fast rate and in continuous manner. This work can lead to the development of an efficient protocol for callus induction and other issues.

Keywords Growth hormone · Callus induction · Callogenic response · *Nerium odorum*

Introduction

Nerium odorum, *Linn*. (Apocynaceae) is an important evergreen shrub. It is heat, salinity and drought tolerant. Plants with milky sap have medicinal value [1] and have mainly cardenolides, flavonoids and terpenes [2]. The species can be used for wastewater purification and for restoration of riparian woodlands [3]. Tissue culture techniques of *Nerium* have been described [4]. In vitro propagation is also used to clean up plant infections such as oleander plant-leaf scorch, caused by

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Xylella fastidiosa subsp. *sandyi*, and leaf blight, caused by *Pseudomonas savastanoii pv. nerii* [5]. This work can lead to the development of an efficient protocol for callus induction and other issues.

Materials and Methods

Collection of Explant

Explants {leaf and stem (nodal)} of *N. odorum,* were collected from the departmental garden of Botany, Patna University, Patna-5.

Surface Sterilization

Explant leaf and stem (nodal) were washed thoroughly with running tap water for 30 min and then dipped for 15 s in 70 % ethanol. Later on, they were submerged in calcium hypochlorite (0.5 %) for 25 min. Tween 80 was added to the above solution to improve contact between tissue and disinfectant. Explants were removed from the disinfectant and were washed five times in sterile distilled water. Explants were blotted on filter paper in five replicates in laminar air flow before being placed on Murashige and Skoog (MS) media.

Explant Implantation and Culture Conditions

Standard procedure was followed for the preparation of media [6]. The pH of the media was adjusted to 5.8, and heat-resistant growth regulators (1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and kinetin (Kin)) were added to the media prior to sterilization done at 15 lbs/in. for 15 min. All media were solidified with 8 g/l agar. After autoclaving, further work was done under laminar air flow. Stem and leaf about 5 mm in length were aseptically prepared and were implanted on MS medium prepared with specific concentrations of hormones. Stock culture, stem and leaf explants were incubated in the dark in a culture chamber at 25 °C.

Determination of Callus Fresh Weight

The callus was collected from tissue culture lab, and its media were washed with sterile distilled water. They were placed under a fan (on a blotting paper) to remove water and weighed.

Determination of Callus Dry weight

After fresh weight determination, the materials were placed on Petri dishes and kept in an oven for 48 h at 65 °C for drying. Dry weight was weighed with an electronic balance.

Determination of Callus Moisture Content

The moisture content was determined using the fresh and dry weight of callus by the following ways:

A=weight of empty Petri dish
B=weight of Petri dish with fresh cell material

C=weight of Petri dish with dried cell material

$$\text{Moisture content percentage} = (B-A)-(C-A)/(B-A) \times 100$$

Results

All the experiments were carried out in triplicates and the mean value was recorded (Fig. 1).

Effects of Different Concentrations of Auxins and Cytokinins Singly on Callus Induction

MS media supplemented with different concentrations (0.5–10.0 mg/l) of NAA showed stimulatory effects on callus induction (Table 1). Maximum callusing response (69 % in stem and 74 % in leaf) was recorded at 1.5.0 mg/l of NAA. At 0.5 mg/l, the callusing response was recorded less, and it increased up to 2 mg/l. At 2.5 mg/l, onward callusing response was reduced and found minimum at 5 mg/l. At 10 mg/l, no callusing or growth was observed. It was observed that the higher concentration of NAA in media had an inhibitory effect on callus proliferation.

2,4-D with different concentrations (0.5–10 mg/l) showed stimulatory effects on callus induction (Table 2 and Fig. 2). Maximum callusing response (75 % in stem and 79 % in leaf) was observed at 2.5 mg/l. No callus formation was observed on stem and leaf explants inoculated on MS media supplemented with 0.5 to 10 mg/l of Kin (Table 2).

With BAP, maximum callusing response (50 % in stem and 55 % in leaf) was noted at 2.5 mg/l (Table 3). A lower concentration of BAP (0.5 to 1.5 mg/l) was unable to induce callusing, and a higher concentration of BAP (10 mg/l) in media had an inhibitory effect on callus induction.

Effects of Different Concentrations and Combinations of Growth Hormones on Leaf and Stem Callus Induction

2,4-D and BAP with different concentrations (0.5–10 mg/l) showed stimulatory effects on callus induction (Table 4 and Fig. 3). Maximum callusing response (78 % in stem and 81 % in leaf) was noted at 1 and 1.5 mg/l for BAP and 2,4-D, respectively. At 3 mg/l of BAP and 2,4-D 1 mg/l, swelling of callus was observed. At 5 to 10 mg/l of BAP and 2,4-D, no callusing or growth was observed.

Fig. 1 Explants in triplicates on MS media with growth hormones for callus induction

Table 1 Callus induction on stem (nodal) and leaf explants on MS medium under the influence of different concentrations of NAA (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10 mg/l)

Medium composition NAA mg/l	*Nerium odorum*					
	Stem			Leaf		
	% of callus induction	Degree of callusing	Day of callus induction	% of callus induction	Degree of callusing	Day of callus induction
0.5	17	+	31	21	+	29
1	55	++	27	63	++	25
1.5	69	+++	23	74	+++	21
2	65	+++	25	67	+++	19
2.5	55	++	27	59	++	26
3	31	+	28	35	+	28
4	23	+	29	27	+	29
5	15	+	31	19	+	30
10	no callusing	−	−	Swelling	−	−

(−) indicates no regeneration and (+) indicates status of callus induction

+ = poor, ++ = good, +++ = excellent

MS media supplemented with different concentrations (0.5–10.0 mg/l) of NAA and BAP showed stimulatory effects on callus induction (Table 5). Maximum callusing response (75 % in stem and 77 % in leaf) was recorded at 0.5 and 1 mg/l for BAP and NAA, respectively.

Different concentrations (0.5–10.0 mg/l) of NAA and Kin showed stimulatory effects on callus induction (Table 6). Maximum callusing response (63 % in stem and 67 % in leaf) was recorded at 1 and 1.5 mg/l for Kin and NAA, respectively. At 2.5 to 10 mg/l of kin and NAA, no callusing or growth was observed.

Effects of Different Concentrations of Growth Hormones Singly and in Combination on Nature and Moisture Content of Callus

The moisture content varied in the callus derived from different explants (leaf and stem-nodal) under the influence of various growth hormones. It was observed that moisture content varied from 69 to 80 % which supports good growth of callus (Table 7).

Leaf callus was loose in texture and friable. It was white, light green and green in colour (Table 7). Stem callus was compact and nonfriable, light yellow to green in colour (Table 7).

Discussion

In the present study, two explants, leaf and stem (nodal), were used in which leaf explants appear the best for callus induction which is in accordance with the earlier findings [7]. MS medium, without any growth hormone, was unable to induce callus [8]. Among all the growth hormones, 2,4-D was the best for callus induction which is similar to the earlier finding [9].

In the present work, Kin alone could not induce callus [10, 11]. In further experiments, Kin was supplemented to the MS media in combination with auxins (2,4-D and NAA). It was observed that Kin had enhanced callus growth in the presence of auxins. MS media

Table 2 Callus induction on stem (nodal) and leaf explants on MS medium under the influence of different concentrations of 2,4-D and Kin separately (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10 mg/l)

Medium composition	*Nerium odorum*					
	Stem			Leaf		
	% of callus induction	Degree of callusing	Day of callus induction	% of callus induction	Degree of callusing	Day of callus induction
2,4-D mg/l						
0.5	–	–	–	–	–	–
1	–	–	–	–	–	–
1.5	–	–	–	–	–	–
2	64	++	23	67	++	21
2.5	75	+++	21	79	+++	21
3	45	+	27	47	+	25
4	33	+	30	41	+	27
5	21	+	33	25	+	29
10	No callusing	_	–	Swelling	_	–
Kin mg/l						
0.5	–	–	–	–	–	–
1	–		–	–	–	–
1.5	–	–	–	–	–	–
2	–	–	–	–	–	–
2.5	–	–	–	–	–	–
3	–	–	–	–	–	–
4	–	–	–	–	–	–
5	–	–	–	–	–	–
10	–	–	–	–	–	–

(−) indicates no regeneration and (+) indicates status of callus induction

+ = poor, ++ = good, +++ = excellent

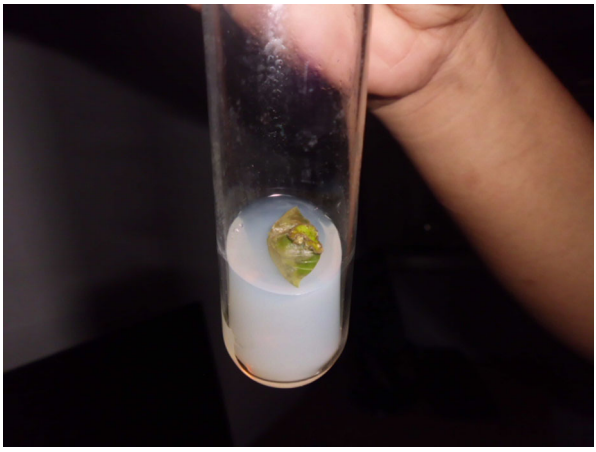

Fig. 2 Callus induction of MS fortified with 2,4-D (2.5 mg/l)

Table 3 Callus induction on stem (nodal) and leaf explants on MS medium under the influence of different concentrations of BAP (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10 mg/l)

Medium composition BAP mg/l	*Nerium odorum*					
	Stem			Leaf		
	% of callus induction	Degree of callusing	Day of callus induction	% of callus induction	Degree of callusing	Day of callus induction
0.5	–	–	–	–	–	–
1	–	–	–	–	–	–
1.5	–	–	–	–	–	–
2	49	+	29	53	++	27
2.5	50	++	27	55	++	25
3	41	+	29	42	+	29
4	37	+	31	39	+	30
5	21	+	33	25	+	32
10	no callusing	_	–	Swelling	_	–

(−) indicates no regeneration and (+) indicates status of callus induction

+ = poor, ++ = good, +++ = excellent

Table 4 Callus induction on stem (nodal) and leaf explants on MS medium supplemented with different concentrations and combinations of BAP and 2,4-D (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10 mg/l)

Medium composition mg/l		*Nerium odorum*					
		Leaf			Stem		
		% of callus induction	Degree of callusing	Day of callus induction	% of callus induction	Degree of callusing	Day of callus induction
MS		–	–	–	–	–	–
BAP	2,4-D						
0.1	2	21	+	33	17	+	34
0.5	2	61	++	25	55	++	27
1	1.5	81	+++	21	78	+++	22
1	2	65	++	20	57	++	27
1.5	1.5	25	+	32	21	+	33
1.5	2	39	+	29	50	++	27
1.5	2.5	35	+	30	29	+	31
2.5	1	30	+	32	25	+	33
2.5	2	23	+	33	19	+	34
3	1	no callusing	–	–	swelling	–	–

(−) indicates no regeneration and (+) indicates status of callus induction

+ = poor, ++ = good, +++ = excellent

Fig. 3 Callus on MS supplemented with 2,4-D (1.5 mg/l) and BAP (1 mg/l)

fortified with 2,4-D and BAP was found to be the best for callus induction as reported earlier [12–14].

Day of callus induction was indigenous which started from the 17th to the 37th day [15]. This variation, observed in the present investigation, may be attributed to the difference in culture conditions and the age of explants. The fresh and dry weight and moisture content showed good growth of callus.

Table 5 Callus induction on stem (nodal) and leaf explants on MS medium supplemented with different concentrations and combinations of BAP and NAA (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10 mg/l)

Medium composition mg/l		*Nerium odorum*					
		Leaf			Stem		
		% of callus induction	Degree of callusing	Day of callus induction	% of callus induction	Degree of callusing	Day of callus induction
MS		–	–	–	–	–	–
BAP	NAA						
0.1	0.1	–	–	–	–	–	–
0.5	0.5	–	–	–	–	–	–
1	0.1	17	+	33	13	+	35
1.5	0.5	21	+	31	19	+	33
2	0.1	27	+	27	25	+	29
0.1	1	60	++	25	51	++	27
0.5	1	77	+++	21	75	+++	21
1	1	55	++	25	50	++	27
1.5	1	70	+++	19	67	++	23
2	1	61	+++	24	57	++	25
1	2	65	++	23	61	++	23
3	2	65	++	23	60	++	23
5	4	35	+	27	27	+	29
10	5	Swelling	–	–	Swelling	–	–

(−) indicates no regeneration and (+) indicates status of callus induction

+ = poor, ++ = good, +++ = excellent

Table 6 Callus induction on stem (nodal) and leaf explants on MS medium supplemented with different concentrations and combinations of Kin and NAA (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10 mg/l)

Medium composition mg/l		*Nerium odorum*					
		Leaf			Stem		
		% of callus induction	Degree of callusing	Day of callus induction	% of callus induction	Degree of callusing	Day of callus induction
MS		–	–	–	–	–	–
Kin	NAA						
0.1	2	–	–	–	–	–	–
0.5	2	40	+	27	33	+	29
1	2.5	27	+	29	25	+	31
0.5	2.5	35	+	29	30	+	31
1	1.5	67	++	23	63	++	23
1	2	57	++	25	55	++	27
1	2.5	35	+	27	31	+	31
1.5	1.5	27	+	29	22	+	31
1.5	2	15	+	31	13	+	33
1.5	2.5	11	+	35	10	+	37
2.5	2	No callusing	–	–	Swelling	–	–

(−) indicates no regeneration and (+) indicates status of callus induction

+ = poor, ++ = good, +++ = excellent

Table 7 Callus growth observations by measuring callus fresh and dry weight and nature of callus of randomly selected samples from different concentrations (milligrams per litre) of growth hormones of *N. odorum*

Medium composition mg/l	*Nerium odorum*							
	Leaf				Stem			
	Fresh weight (mg)	Dry weight (mg)	Moisture content (%)	Nature of callus	Fresh weigh (mg)	Dry weight (mg)	Moisture content (%)	Nature of callus
MS	–	–	–	–	–	–	–	–
2,4-D (2.5)	1.743	0.436	76	Whitish, f	1.673	0.387	77	Whitish, g
2,4-D (5)	0.832	0.176	79	Green, f	0.713	0.171	76	Yellowish green, nf
NAA (1.5)	1.657	0.379	77	Light green, f	1.531	0.412	73	Light green, nf
NAA (5)	0.875	0.187	79	Light yellow, f	0.765	0.159	77	Yellow, nf
BAP (2.5)	1.231	0.297	76	Light green,	1.213	0.288	76	Light green, nf
BAP (5)	0.677	0.197	71	Green, s	0.597	0.189	69	Green, s
Kin (0.5)	–	–	–	–	–	–	–	–
BAP (1)+2,4-D (2)	1.275	0.287	78	Light green, f	1.269	0.269	79	Light green, nf
BAP (0.5)+2,4-D (2)	1.351	0.293	78	Light green, f	1.277	0.297	77	Light green, nf
BAP (0.5)+NAA (1)	1.701	0.521	69	Whitish green, f	1.673	0.508	70	Whitish, g
BAP (1)+NAA (2)	1.297	0.265	79	Light green, f	1.285	0.273	79	Light green, nf
Kin (1)+NAA (2)	1.175	0.257	78	Light green, f	1.159	0.159	78	Light green, nf
Kin (0.5)+NAA (2)	0.759	0.175	76	Green, f	0.735	0.181	75	Green, nf

f friable, *s* swelling, *g* globular, *nf* nonfriable

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