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Fast *In-Vitro* Regeneration of an Important Medicinal Plant Karaveera (*Nerium odorum* L.)

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ABSTRACT

Nerium odorum, *Linn*. (Apocynaceae) an important evergreen shrub which is heat, salinity, and drought tolerant. Plants with milky sap has medicinal value, mainly cardenolides, flavonoids, and terpenes. It comprises of total 01 species. Explants of an alkaloid producing plant cultured *in vitro* and has been found to retain the capacity to synthesis alkaloids identical to that in the intact plant. It is used for wastewater purification and for restoration of riparian woodlands. In view of these facts the study was conducted for micro propagation of *Nerium odorum*. MS media supplemented with different concentrations (0. 5-10.0 mg/l) of NAA, 2, 4-D, BAP and KIN was used singly and in combination. Among all the growth hormones, in single combination 2, 4-D was the best for callus induction (75% in stem and 79% in leaf) and in combination 2, 4-D and BAP was found the most suitable for callus induction (78% in stem and 81% in leaf). Day of callus induction started from 19th to 37th day. This variation is due to the difference in culture conditions and the age of explants. In single combination BAP was the best for shooting (75%) and in double combinations BAP (1.5mg/l) and NAA (0.5mg/l) were the best for shooting (81%). Higher induction of root (85%) was observed at NAA (1.5 mg/l) and in double combinations BAP (1.5mg/l) and NAA (0.5mg/l) were the best for rooting (86%). Regenerated plants after hardenings were transferred to soil and they showed 77% survival. The protocol was optimized by manipulations of different plant hormones for enhanced multiplication. Protocol explained here provides a rapid plant regeneration system which could be used for the commercial purposes.

Key words: *Nerium odorum*, callus culture, micro propagation, plant growth regulators.

INTRODUCTION

N*erium odorum* (L.) is an important alkaloid-yielding medicinal and an ornamental plant belonging to family Apocynaceae is an evergreen shrub. It is heat, salinity and drought tolerant. It contains only one species of genus *Nerium* i.e. *N. odorum*. It goes by the name Karaveera in Sanskrit and Kanher in Marathi. In Sanskrit medical works of this plant is described as hot and poisonous.

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It is one of the most poisonous plants known to this family and it is effective in snakebite cure. The powdered leaves and bark are used as a rat poison and an insecticide [1]. Its root is recommended for external application in skin diseases and is a popular remedy for venereal diseases. The oil prepared from the root-bark is recommended for skin diseases of a scaly nature and for Leprosy. The leaves contain small amounts of latex that can be used to make rubber, though the amount is too small for commercial utilization. The plants have an extensive root system and are often used to stabilize soil. This species also produces secondary metabolites [2]. Among alkaloids some of which are of pharmacological interest, mainly cardenolides, flavonoids and terpenes [3,4]. For example, oleandrin has been identified as a potent antitumor compound [5]. *Nerium odorum* is also used for wastewater purification and for restoration of riparian woodlands [6].

This plant is conventionally propagated through stem, seed etc, the natural proliferation rate is very low due to drought in first summer season to young plantlets. *In vitro* culture of plants have gained importance during recent years because, this technique can be used for the rapid multiplication of these plants [7]. Tissue culture techniques have been reported on somatic embryogenesis from oleander leaf explants [8], whereas more recently, a protocol was referred for the medium-term conservation of *N. odorum* buds in synthetic seeds [9]. *In vitro* propagation of ornamental plants allows the continuous production of clones and can also be used to clean up plant material affected by some pathogens such as for oleander plants can be leaf scorch, caused by *Xylella fastidiosa* sub species *sandyi* and leaf blight, and caused by *Pseudomonas savastanoiipv, nerii* [10].

In view of these facts the study was conducted for the development of an efficient protocol for *in vitro* multiplication of this plant by optimizing the growth regulators such as auxins and cytokinins.

MATERIALS AND METHODS

Collection of explant

Explants stem (nodal) and leaf of *Nerium odorum* were collected from the medicinal plant Garden of Patna Science College under Patna University Patna, India (Fig. 1). The plants were identified, confirmed and authenticated by Dr. M. P. Trivedi, Associate Prof. in Botany of Patna Science College, Patna [11]. After authentication of this plant, *in vitro* culture studies were carried out.

Figure-1. Whole plant of *Nerium odorum*

Surface Sterilization and Culture Media

Explants – leaf and stem, washed thoroughly with running tap water for 30 minutes and then dipped for 15 sec. in 70% ethanol after that submerged in a disinfectant calcium hypochlorite (0.5%) for 25 minutes. Tween 80 added to the above solution to improve contact between tissue and disinfectant. Explants removed from disinfectant and washed 5 times in sterile distilled water. Explants blotted on filter paper in 5 replicates in Laminar Air Flow before placing it on Murashige and Skoog (MS) media.

Standard procedure was followed for the preparation of media with slight variations [12]. The pH of the media was adjusted to 5.8 and heat resistant growth regulators (NAA, 2, 4-D, BAP and KIN) were added to the media prior to sterilization done at 15 lbs/in for 15 min at 121^{o}C. All media were solidified with 8g/l agar. After autoclaving further work done under Laminar Air Flow.

Callusing

For callus induction juvenile stem (nodal) and leaf about 5 mm in length were aseptically prepared and were implanted vertically on MS medium prepared with specific concentrations of hormones. Culture of stem and leaf explants were initially incubated under darkness in a culture chamber at 25°C for callus induction.

Shoot Regeneration on Callus

The callus was cut into small pieces when it was observed in entire explants. Each piece of callus was transferred to MS media having same growth hormones in similar composition and concentration as for callus induction. Subsequently, calli were incubated under a 16/8 h (light/dark) photoperiod with light fluorescent lighting at an intensity of 60 µE m^{-5} s^{1} on a constant temperature as of callusing (25^{o}C) for shooting. Sub culturing was done after every 15th day.

Root Regeneration and Acclimatization

For initiation of roots the 5-7 weeks old shoots 3.5 cm in length were cultured on MS media having same growth hormones in similar composition, concentration and incubation as for shooting.

The complete rooted plantlets 75 days old were washed to free them of agar and dipped in 0.2% bavistin fungicide for 10 minutes to protect from fungal attack in near future. These plants were potted in small plastic pots containing sterilized soil. The plantlets were covered with polythene bags to maintain high humidity. These were acclimatized at 28^{o}C less than 16 hours, photoperiod and watered regularly. After 3-4 weeks the polythene bags were removed and established plantlets were transplanted in earthen pots in a greenhouse with watering at 2-3 days intervals.

RESULTS

All the experiments were carried in triplicates and the mean value was recorded (Fig. 2).

Callusing:

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 1-naphthaleneacetic acid (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 0.5mg/l the callusing response was recorded less and it increased up to 2mg/l. At 2.5mg/l on word callusing response was reduced and found minimum at 5mg/l. At 10mg/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.

Figure-2. Explants in triplicates on MS media with growth hormones for callus induction

2, 4-Dichlorophenoxyacetic acid (2, 4-D) with different concentration (0.5-10mg/l) showed stimulatory effects on callus induction (Table-2 and Fig-3). Maximum callusing response (75% in stem and 79% in leaf) was noted at 2.5mg/l.

No callus formation was observed on stem and leaf explants inoculated on MS media supplemented with 0.5mg/l to 10mg/l of Kin (Table-2).

With 6 benzylaminopurine (BAP) maximum callusing response (50% in stem and 55% in leaf) was noted at 2.5mg/l (Table-3). Lower concentration of BAP (0.5mg/l to 1.5mg/l) was unable to induce callusing and higher concentration of BAP (10 mg/l) in media had an inhibitory effect on callus induction.

Effects of different concentration and combination of growth hormones on leaf and stem callus induction:-

2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 6 benzylaminopurine (BAP) with different concentration (0.5-10mg/l) showed stimulatory effects on callus induction (Table-4 and Fig.-4). Maximum callusing response (78% in stem and 81% in leaf) was noted at 1mg/l and 1.5mg/l for BAP and 2, 4-D respectively. At 3mg/l of BAP and 2, 4-D 1 mg/l swelling of callus was observed. At 5mg/l to 10mg/l of BAP and 2, 4-D no callusing or growth was observed.

Figure-3. Callus induction of MS fortified with 2, 4-D (2.5 mg/l).

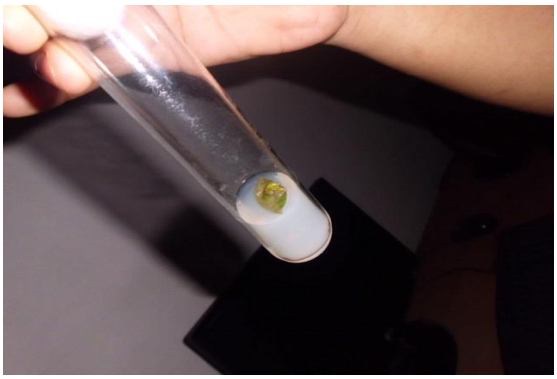

Table-1. Callus induction on stem (Nodal) and leaf explants on MS medium under the influence of different concentrations of NAA mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Composition of media 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 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) showed stimulatory effects on callus induction (Table 5). Maximum callusing response (75% in stem and 77% in leaf) was recorded at 0.5mg/l and 1 mg/l for BAP & NAA respectively.

Different concentrations (0.5–10.0 mg/l) of 1-naphthaleneacetic acid (NAA) and KIN showed stimulatory effects on callus induction (Table 6). Maximum callusing response (63% in stem and 67% in leaf) was recorded at 1mg/l and 1.5 mg/l for Kin & NAA respectively. At 2. 5mg/l to 10mg/l of kin and NAA no callusing or growth was observed.

In Vitro Shoot Regeneration:

Effects of different concentrations of auxins and cytokinins singly on shoot regeneration:

MS media supplemented with different concentrations (2–5.0 mg/l) of 6-benzylaminopurine (BAP) maximum shooting response in *Nerium odorum* 75% was noted at 2.5mg/l (Table-7*).* Lower concentrations of BAP (2mg/l) was having low value to induce shooting and higher concentrations of BAP (up to 5 mg/l) in media had lower effect on shooting. Days of shoot generation started from 21th to 37th days.

No shoot formation was observed on callus of *Nerium odorum* inoculated on MS media supplemented with 0.5 mg/l to 10 mg/l of 1-naphthaleneacetic acid (NAA), (Table-8) and 2, 4-Dichlorophenoxyacetic acid (2, 4-D), (Table-9).

Effects of different concentrations and combinations of growth hormones on shoot regeneration

2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 6-benzylaminopurine (BAP) with different concentration (0.1-2.5 mg/l) showed stimulatory effects on shoot regeneration (Table-10 and Fig.5). Maximum shooting response in *Nerium odorum* (61%) was recorded at 1.5mg/l and 1.5 mg/l for BAP and 2, 4-D respectively. Day of shoot generation started from 25th to 39th day.

MS media supplemented with different concentrations (0.1–3.0 mg/l) of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP*)* showed stimulatory effects on shoot regeneration (Table-11 and Fig. 6). Maximum shooting response in *Nerium odorum* (81%) was recorded at 1.5mg/l and 0.5 mg/l for BAP and NAA respectively. Days of shoot generation started from 19th to 39th day.

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

Composition of media 2,4-D 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	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.

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

Composition of media 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 mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Composition of media 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.

In Vitro Root Regeneration:

Effects of different concentrations of auxins and cytokinins singly on root regeneration:

MS media supplemented with different concentrations (2–5.0 mg/l) of 1-naphthaleneacetic acid (NAA) maximum rooting response in *Nerium odorum* (85%) at 1.5mg/l (Table-12). Lower concentrations of BAP (0.5- 1mg/l) was having low value to induce rooting and higher concentration of BAP (up to 5 mg/l) in media had lower effect on rooting. Day of root generation started from 09th to 17th day.

No root formation was observed on shoot of *Nerium odorum* inoculated on MS media supplemented with 0.5 mg/l to 10 mg/l of 6- benzylaminopurine (BAP), (Table-13) and 2, 4-Dichlorophenoxyacetic acid (2, 4-D), (Table-14)

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Effects of different concentrations and combinations of growth hormones on Root regeneration:

2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 6-benzylaminopurine (BAP) with different concentrations (0.1-2.5 mg/l) showed stimulatory effects on root

regeneration (Table-15). Maximum rooting response in *Nerium odorum* (59%) was recorded at 1.5mg/l and 1.5 mg/l for BAP and 2, 4-D respectively. Days of root generation started from 11th to 19th day.

MS media supplemented with different concentrations (0.1–3.0 mg/l) of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) showed stimulatory effects on root regeneration (Table -16 and Fig. 7). Maximum rooting response in *Nerium odorum*

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

Composition of media 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 & NAA mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Composition of media 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.

(86%) was recorded at 1.5mg/l and 0.5 mg/l for BAP and NAA respectively. Days of root generation started from 09th to 18th day.

Figure-4. Shoot regeneration of *Nerium odorum* on MS fortified with BAP (1.5 mg/l) and NAA (0.5 mg/l).

Figure-5. Root regeneration of *Nerium odorum* on MS fortified with BAP (1.5 mg/l) and NAA (0.5 mg/l).

Figure-6. Hardening- Plants covered with plastic.

Figure-7. Hardening- Plant ready to transfer into soil.

Table-7. Shoot regeneration from callus on MS medium under the influence of different concentrations of NAA mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Composition of media mg/l	*Nerium odorum*		
NAA	% of Shooting	degree of shooting	day of shoot induction
0.5	-	-	-
1	-	-	-
1.5	-	-	-
2	-	-	-
2.5	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
10	-	-	-

(-) indicates no regeneration.

Table -8. Shoot regeneration from callus on MS medium under the influence of different concentrations of 2, 4-D mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Composition of media mg/l	*Nerium odorum*		
2,4-D	% of Shooting	degree of shooting	day of shoot induction
0.5	-	-	-
1	-	-	-
1.5	-	-	-
2	-	-	-
2.5	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
10	-	-	-

(-) indicates no regeneration.

Table -9. Shoot regeneration on MS medium under the influence of different concentrations of BAP mg/l (2, 2.5, 3, 4 and 5).

Composition of media mg/l	*Nerium odorum*		
BAP	% of Shooting	degree of shooting	day of shoot induction
2	67	++	29
2.5	75	+++	27
3	60	++	31
4	33	+	32
5	17	+	37

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = poor, ++ = good, +++ = excellent.

Table -10. Shoot regeneration on MS medium supplemented with different concentrations and combinations of BAP and 2, 4-D mg/l (0.1 to 2.5).

Composition of media mg/l		*Nerium odorum*		
BAP	2,4-D	% of Shooting	degree of shooting	day of shoot induction
0.1	2	21	+	39
0.5	2	35	+	39
1	1.5	47	+	35
1	2	41	+	38
1.5	1.5	61	++	29
1.5	2	51	++	33
1.5	2.5	45	+	37
2.5	1	55	++	31
2.5	2	29	+	39

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = poor, ++ = good, +++ = excellent.

Table -11. Shoot regeneration on MS medium supplemented with different concentrations and combinations of BAP and NAA mg/l (0.1, 1, 1.5, 2, 2.5 and 3).

Composition of media mg/l		*Nerium odorum*		
BAP	NAA	% of Shooting	degree of shooting	day of shoot induction
0.5	0.5	-	-	-
1	0.1	66	++	25
1.5	0.5	81	++++	23
2	0.1	72	+++	27
0.1	1	33	+	31
0.5	1	19	+	38
1	1	33	+	31
1.5	1	65	++	27
2	1	55	++	35
1	2	23	+	37
3	2	19	+	39

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = poor, ++ = good, +++ = excellent.

Table-12. Root regeneration from shoot on MS medium under the influence of different concentrations of NAA mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Composition of media mg/l	*Nerium odorum*		
NAA	% of Rooting	degree of Rooting	day of Root induction
0.5	53	++	13
1	65	++	12
1.5	85	++++	10
2	83	++++	12
2.5	81	++++	14
3	71	+++	15
4	69	++	16
5	47	+	17

(-) indicates no regeneration and (+) indicates status of callus induction; + = poor, ++ = good, +++ = excellent.

Table-13. Root regeneration from shoot on MS medium under the influence of different concentrations of 2, 4-D mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Composition of media mg/l	*Nerium odorum*		
2,4-D	% of Rooting	degree of Rooting	day of Root induction
2	-	-	-
2.5	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-

(-) indicates no regeneration.

Table-14. Root regeneration on MS medium under the influence of different concentrations of BAP mg/l (2, 2.5, 3, 4 and 5).

Composition of media mg/l	*Nerium odorum*		
BAP	% of Rooting	degree of Rooting	day of Root induction
2	-	-	-
2.5	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-

(-) indicates no regeneration.

Table -15. Root regeneration from shoot on MS medium supplemented with different concentrations and combinations of BAP and 2, 4-D mg/l (0.1 to 2.5).

Composition of media mg/l		*Nerium odorum*		
BAP	2,4-D	% of Rooting	degree of Rooting	day of Root induction
0.1	2	21	+	17
0.5	2	31	+	16
1	1.5	33	+	15
1	2	37	+	14
1.5	1.5	59	++	11
1.5	2	39	+	13
1.5	2.5	43	+	12
2.5	1	57	++	11
2.5	2	29	+	16

(-) indicates no regeneration and (+) indicates status of callus induction; + = poor, ++ = good, +++ = excellent.

Table -16. Root regeneration from shoot on MS medium supplemented with different concentrations and combinations of BAP and NAA mg/l (0.1, 1, 1.5, 2, 2.5 and 3).

Composition of media mg/l		*Nerium odorum*		
BAP	NAA	% of Rooting	degree of Rooting	day of Root induction
1	0.1	-	-	-
1.5	0.5	86	++++	10
2	0.1	51	++	16
0.1	1	71	+++	11
0.5	1	69	++	13
1	1	67	++	14
1.5	1	61	++	13
2	1	65	++	15
1	2	49	+	16
3	2	29	+	17

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = poor, ++ = good, +++ = excellent.

DISCUSSION

Callusing

Standard procedure was followed for the preparation of media [13]. In the present study, two explants leaf and nodal stem were used in which leaf explants were found the best for callus induction than stem, which is in accordance with the earlier findings [14]. MS media without any growth hormone was unable to induce callus as reported [15]. Among all the growth hormones, 2, 4-D was the best for callus induction which is similar to the earlier finding [16].

In the present work Kin alone could not induce callus as according to earlier findings [17]. In further experiments Kinetin (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 fortified with 2, 4-D and BAP was found the best for callus induction as reported earlier [18].

Day of callus induction started from 17th to 37th day [19]. This variation observed in the present investigation may be attributed due to the difference in culture conditions and the age of explants.

Shoot generation

The callus was sub cultured in all BAP containing media differentiated into multiple shoots giving out an average of 5 shoots per piece of callus in MS +BAP (2.5mg/l). The media with lower concentration of NAA further gave multiple shoots as well as roots [20]. Inductions of callus and plant regeneration are the most reliable tools to multiply the plants in a large scale [20]. In combination of NAA and BAP normally lower NAA and higher BAP concentration favoured the production of shoot [21]. MS media with BAP and NAA is most suitable for shooting in our case and it is also supported by [22].

In our findings Shoot cultured were initiated on MS medium containing BAP (0.5 mg/l) with NAA (0.5 mg/L). Maximum shoot proliferation was achieved in medium containing BAP (1.5 mg/L) with NAA (0.5 mg/L) in *Nerium odorum* (81%). Among all the growth hormones, in single combination BAP (2.5 mg/l) was the best for shoot induction in *Nerium odorum* (75% from callus). Day of shoot generation started from 19th to 39th day.

Root generation

Root generation was satisfactory with BAP and NAA. NAA was the best in all other hormones like 2,4-D and BAP for rooting in our result and it was favored by [23]. In our findings, root cultures were initiated on MS medium containing BAP (1 mg/l) with NAA (0.1 mg/L). Maximum root proliferation was achieved in media containing BAP (1.5 mg/L) with NAA (0.5 mg/L) in *Nerium odorum* (86% from shoot). Day of root generation started from 11th to 17th day.

Hardening

Plantlets after 81 days old in secondary hardening were ready for field transplantation. Regenerated plants after hardenings were transferred to soil and they showed 77% survival. The regenerated plants were morphologically similar to control plants.

Therefore, in the present investigation, a protocol for micro- propagation of locally adapted population is successfully established which may be used for commercial application as a substitute to natural propagation through conventional methods.

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Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

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