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Full Length Research

Effect of plant growth regulators, explants type and efficient plantlet regener protocol through callus induction in *Naringi crenulata* (Roxb.) Nicolson and biochemical investigation

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Abbreviations: BAP, 6-Benzylaminopurine; **NAA**, α-naphthalene acetic acid; **Kn,** kinetin, **IBA**, indole-3-butyric acid.

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Abstract

Naringi crenulata (Roxb.) Nicolson, is a rare medicinal plant belonging to the family Rutaceae. It is a spinous tree and has great medicinal value. *N. crenu* has in recent years suffered over-exploitation and has therefore been listed in the Red Data list of *International Union for Conservation of Nature* as a vulnera induction and *in vitro* plantlet regeneration system for *N. crenulata* (Roxb.) Nicolson was optimized by studying the influence of explants type (leaf, nodal s and different concentrations of plant growth regulators. Callus formation and shoot differentiation was initiated on Murashige and Skoog's (MS) mediu concentrations of auxin and cytokinin. The best result was obtained using leaf explants and callus production was maximum at 0.5 mg/L BAP (6-benzy mg/L NAA (α-naphthaleneacetic acid) and for nodal and shoot tip explants, callus production was maximum at 2.0 mg/L BAP and 0.5 mg/L NAA. Highly maximum number of shoots (25±0.3) were obtained on MS medium supplemented with BAP (2.0 mg/L) and NAA (0.5 mg/L) from leaf explants. E development of shoot buds into shoots was achieved on MS medium fortified with 0.5 mg/L BAP and 0.5 mg/L Kn. However, the result reflected the existenc variability in response to growth regulators. *In vitro* rooting of shoots was achieved on ½ strength MS medium supplemented with IBA. Best rooting was a MS medium supplemented with 1.0 mg/l IBA (indole-3-butyric acid). The highest total soluble protein contents and peroxidase activity was observed in the cultures derived from leaf explants and this changing pattern can be used as biochemical marker for differentiation. So, this protocol can be used for the *Naringi crenulata* through indirect organogenesis using a wide range of explants.

Key words: *Naringi crenulata*, callus, regeneration, leaf explants, peroxidase, total soluble protein.

Introduction

Herbal medicine is one of the most remarkable uses of plant based biodiversity. As many as 75 to 90% of the world's rural people rely on herbal medicine f care (Mousumi et al., 2007). The success of any health care system depends on the availability of suitable drugs on a sustainable basis. Natural medici strength of the body.

Naringi crenulata (Roxb.) belonging to the family Rutaceae is a spinous glabrous shrub or small tree distributed throughout India. Its synonym is *He* (Roxb.) M. Roem or *Limonia crenulata* Roxb.

The plant is known by a wide range of common names in almost four languages across India – Beli (Hindi), naibela (Kannada), bilvaparni (Sanskrit), and na the therapeutic applications of *N. crenulata* are: The leaves are supposed to be a remedy for epilepsy (Kirtikar and Basu, 2005), the root is purgative, sudo the cure of colic and cardialgia (Nadkarni, 2002). The dried fruit is tonic, diminishes intestinal fermentation and has the power of resisting the infection of sm pestilent fevers and is also considered an excellent antidote to various poisons. The bark is aromatic and cooling and is useful in vitiated conditions Chennaiah, 1997). The plant shows anti- inflammatory activity. Its powered stem wood is used traditionally as a natural skin conditioner especially a Myanmar and some parts of Northern Thailand. Intensive and unabated collection and exploitation of this plant has declined its natural population numb extent that it has been categorized as vulnerable in Rajasthan (Shetty and Singh, 1987).

To conserve the genetic stocks of this valuable plant, *in vitro* propagation can be utilized successfully. Perusal of literature shows only single report on (Francisco et al., 1992) which describes the non embryogenic callus induction of 28 citrus relatives and an attempt was made to isolate protoplast from Ramani et al. (2010) carried out the pharmacognostical, phytochemical and anthelmintic evaluation of leaves of *N. crenulata.* Plant tissue culture technique alone or in combination with genetic transformation can be useful as tools for crop improve-ment and for investigating the production of important seconda induction and subsequent green plant regeneration is genotype specific. It is mentionable that the success of plantlet regeneration under *in vitro* culture syst type of medium, type of tissue or explant. When plants are grown *in vitro,* they come under stress because of accumulation of ammonia in culture vessels. to increase tolerance to stress, the activity of antioxidant enzymes, such as guaiacol peroxidase, superoxide dismutase, catalase, ascorbate peroxi reductase, is generally increased in plants (Foyer et al., 1997). Therefore, it is very important to note the level of these enzymes at different level of *in vitro* p during different stages of callogenesis, where cultures are maintained for longer time under *in vitro* conditions. Therefore this study was undertake regeneration capacities of different explants types and to compare the responses of different explants to various plant growth regulator (PGR) describing a rapid regenerative and efficient protocol for regeneration of this threatened species. It also describes the changes in peroxidases an contents during different stages of callus growth and regeneration.

Materials and Methods

Field trial, plant material and experimental design

Plant material and explants preparation

Explants of *N. crenulata* were excised from mature plants growing in wild, in semi-arid regions of Jaipur, Rajasthan. First, fully expan-ded leaves of 1 cm^2 r nodal stem segment explants of 1 cm and shoot tip of 6 mm in size were used. The explants were first washed thoroughly in running tap water for about h then treated with 2% (v/v) Tween 20 (a commercial grade detergent) followed by several rinses in sterile distilled water. The disinfected explants were su aseptic conditions in a laminar flow chamber. The explants were treated with 70% ethanol for 30 s and washed thrice in sterile distilled water. The explants v 0.1% mercuric chloride solution and again rinsed thrice in sterile distilled water.

Callus induction and shoot initiation

For callus induction, leaf, nodal segment and shoot tip explants were placed on full strength Murashige and Skoog's, (1962) basal medium (MS medium) a with different concentrations and combinations of growth regulators such as BAP (0.5 to 5.0 mg/L), NAA (0.5 to 5.0 mg/L) and for shoot elongation from le 5.0 mg/L) containing 3% (w/v) sucrose and 0.8% (w/v) Agar. The pH of the medium was adjusted to 5.8 and it was autoclaved at 121ºC under 15 psi for incubated at 26±2°C under 16 h photoperiod illuminated by fluorescent light of 2000 to 3000 lux intensity and 55±5% relative humidity. Each experiment was replicates per treatment. Periodic observations were recorded.

Root initiation and hardening of regenerated shoots

For rooting, 2 to 3 cm long shoots were transferred to ½ MS medium alone or supplemented with IBA (0.5 to 2.5 mg/L). The *in vitro* rooted shoots were care culture vessel and they were gently washed with sterile distilled water to remove every trace of media. Thereafter plantlets were dipped in 0.05% Bavistin (s 10 seconds to minimize the microbial infection. Again a second wash was given with sterile distilled water. The treated plantlets were then transferred asept pots containing mixture of vermiculite and sterilized soil in growth chamber with controlled temperature, light and humidity to acclimatize with the outs

strength liquid medium was added periodically. The plantlets were covered with polythene bags to ensure a relative humidity of 70 to 80%. The acclimatiz were then transferred to the field.

Biochemical investigation

To determine the total soluble protein, 500 mg of *in vitro* tissues for each types were ground with 10.0 ml of 5% trichloro acetic acid (TCA), using a p homogenate was centrifuged at 2000 rpm for 20 min and the supernatant was discarded. The residue was dissolved in 5 ml of 0.1 N NaOH. 0.1 ml of this so 1.0 ml with distilled water, protein contents were estimated by Lowry's method (1951). For peroxidase enzyme, 500 mg sample was prepared by homog (fresh) in 5 ml of phosphate buffer (pH 7.0) and then centrifuged at 5000 rpm for 20 min. The supernatant thus obtained was assayed for peroxidase by Worthington enzyme manual (1972).

Statistical analysis

Five replicates were used per treatment and the entire experiment was repeated thrice to confirm the results. Data were recorded as the mean ± standard de

Results and Discussion

Influence of explants type

During the present set of experiments, different auxins NAA, IAA, IBA, 2,4-D alone were tried for callus induction and the auxin giving best response, th mg/L) was combined with cytokinin BAP (0.5 to 5.0 mg/L) or Kn (0.5 to 5.0 mg/L) on MS medium using three explants, including, leaf, nodal segments explants were inoculated on MS medium supplemented with various concentrations (0.5 to 5.0 mg/L) of auxins (2, 4-D, NAA, IAA and IBA). Generally, the c initiated in the form of swelling of the explants on the eighth day in case of leaf explants and after 2 to 3 weeks in case of nodal and shoots tip explants. H was markedly affected by the type of primary explants used in the following order LE >NS >ST. Leaves were the best source of explant both for ca regeneration. Over a period of 3 to 4 months, 80% of leaf derived callus produced shoots with the highest average of 25 ± 0.3 shoots per explant (Table 2).

Influence of plant growth regulators (PGRs)

Different cytokinin and auxin concentrations alone or in combination had a considerable effect on callus regeneration (Tables 1 and 2). Explants cultured on (control) did not produce any callus. In case of nodal segment and shoot tip explants, lower concentration of IAA (0.5 to 1.0 mg/L) resulted in moderate a was dark brown in colour (Table 1). IBA (1.0 mg/L) induced profuse callusing. Callus was initially white to green in colour, soft and fast growing but later response (Figure 1A and B; Table 1). 2,4-D at lower concentrations (0.5 mg/L) showed yellowish brown, slow growing and hard callus. NAA (0.5 to 2.0 mg/l and fast growing callus (Table 1). Similar results have been reported by Meena and Patni (2007).

In case of leaf explants, NAA (2.0 mg/L) was found to be the best auxin for producing creamish-green, fast growing and friable callus; the optimal concent Results are shown in Table 1 and Figure 1C. With increased concentrations of NAA (2.0 to 5.0 mg/L), the amount of callus also increased but it showed rhiz browning of callus. Lower concentration of IBA (1.0 mg/L) was also good for callus induction but after 4 weeks, the growth of callus stagnated.

The callus was soft and whitish green in colour. (Figure 1D). On higher concentration of IBA (2. to 5.0 mg/L) rhizogenic callus was produced with little g mg/L) moderate amount of brown, watery, slow growing callus was obtained (Figure 1E). At higher concentration of 2, 4-D (3.0 to 5.0 mg/L) only curling observed. On IAA (1.0 mg/L) moderate amount of callus was induced and the callus was whitish green in colour (Figure 1F). So, it could be concluded th explants (leaf/shoot tip/ nodal segment) tried, leaf explants proved to be the best and among the various auxins tried, NAA was the most suitab concentration.

Therefore, NAA (0.5 to 5.0 mg/l) was further combined with cytokinins BAP (0.5 to 5.0 mg/L) or Kn (0.5 to 5.0 mg/L). Callus induction was markedly enhar NAA (0.5 to 5.0 mg/L) and BAP. Result after the first subculture showed that both BAP and NAA were necessary for viable callus induction for all exp regenerating in the absence of NAA turned brown/ black and showed no further growth. From the combination of PGR's tested, callus production was n mg/L) and NAA (2.0 mg/L) for leaf explants (100%) and for nodal (65%) and shoot tip explants (50%), callus production was maximum at BAP (2.0 mg/L (Table 2).The callus so produced was green, compact, healthy and fast growing. Similar results were reported by Pathak and Heble (2002), Rajeshwa Mungole et al. (2009), Patel and Shah (2009), Safdari and Kazemitabar (2010) and Isikalan et al. (2010).

Shoot bud organogenesis

Shoot bud organogenesis and subsequent plantlet regeneration involved transferring callus to shoot bud induction medium containing different concentratio mg/L) or Kn (0.5 to 5.0 mg/L) alone or in combination with NAA (0.5 to 5.0 mg/L). In case of leaf explants, maximum number of shoots (25±0.3) was obt supplemented with BAP (2.0 mg/L) and NAA (0.5 mg/L) (Figure 1F) while in case of nodal explants maximum number of shoots were 16±0.8 and in shoo Addition of lower quantities of NAA along with higher concentration of BAP seemed to have a positive response on the organogenic callus from lea concentration of BAP was increased above 2.0 mg/L, the number of shoot buds decreased. The shoots produced on this medium were very small, comp

separated easily.

Kinetin did not prove to be beneficial for producing green and healthy callus. The callus produced on kinetin sup-plemented medium was brown, watery an callus obtained on MS medium augmented with NAA (0.5 mg/L) and BAP (2.0 mg/L) grew profusely and exhibited high regeneration potential. Thus, neit proved beneficial in callus organogenesis/ differentiation of shoots from callus. Similar result was observed by Arya et al. (2008).

The number of shoot buds induced increased considerably when the callus was sub cultured on MS medium containing both BAP and NAA, the optimal c mg/L of BAP and 0.5 mg/L of NAA (Figure 1G and H).The entire callus turned into a globular mass and later differentiated into shoot buds within 3 t maximum number of shoot buds (25 ± 0.3) was initiated in the presence of BAP (2.0 mg/L) and NAA (0.5 mg/L), this medium was designated as 'shoot b This synergistic effect of BAP and auxin has been demonstrated in many plants by Swamy et al. (1992), Purohit et al. (1994), Casado et al. (2002), Fratern et al. (2002), Dode et al. (2003) and Ahmad et al. (2010).

Table 1. Effect of different concentration of auxins on callus induction from different explants of *N. crenulata*.

Auxin concentration (mg/L)	Callus response			Type of callus
	L.E.	N.S.	S.T	
Control: MS basal medium	Nil	Nil	Nil	Nil
NAA				
0.5	C+	C+	C+	N.S. and S.T- green hard callus. L.E.- Creamish green, fast growing, friable callus.
1.0	C++	C++	C++	
2.0	C+++	C++	C++	
3.0	C++ R+	C+	C+	
4.0	C+ R++	C+	C+	
5.0	C+ R++	C+	C+	
IBA				
0.5	C+	C+	C+	N.S. and S.T- white to green in colour, fast growing, L.E.- whitish green and rhizogenic.
1.0	C++	C+++	C+++	
2.0	C+R+	C+	C+	
3.0	C+R++	C+R+	C+	
4.0	C+R+	C+R++	C+	
5.0	C+R+	C+	C+	
IAA				
0.5	C+	C++	C+	N.S. and S.T-dark brown, L.E. - whitish green in colour
1.0	C++	C++	C++	
2.0	C+	C+	C+	
3.0	C+	C+	C+	
4.0	C+	C+	C-	
5.0	C-	C-	C-	
2,4-D				
0.5	C+	C+	C+	N.S. and S.T- yellowish brown, slow growing and hard. L.E.- brown, watery, slow growing callus
1.0	C+	C+	C+	
2.0	C++	C+	C+	
3.0	Nil	Nil	Nil	
4.0	Nil	Nil	Nil	
5.0	Nil	Nil	Nil	

L.E, Leaf explants; N.S, nodal segment; S.T, shoot tip; C, callusing response, +, slight callusing, ++, moderate callusing; +++, profuse callusing; -, no callus.

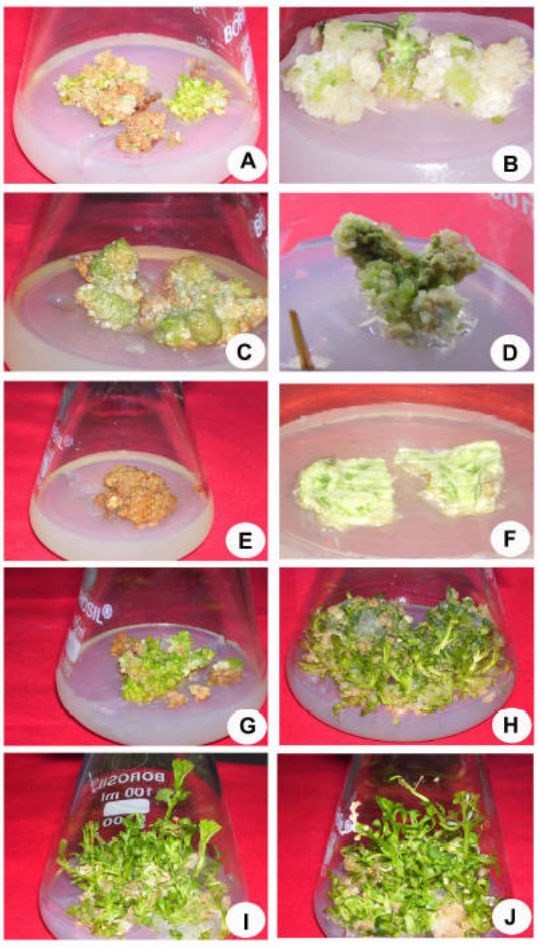


Figure 1. Callus induction, differentiation and elongation from nodal segment, shoot tip and leaf explants of *Naringi crenulata*. **A**, Callus

formation from nodal segment supplemented with IBA (1.0 mg/l); **B,** Callus formation from shoot tip explant with IBA (1.0 mg/l); **C,** Callus induction from leaf explant with NAA (2.0 mg/l); **D,** Callus induction from leaf explant with IBA (1.0 mg/l); **E,** Callus induction from leaf explant with 2,4-D (0.5mg/l); **F,** Callus induction from leaf explants with IAA (1.0 mg/l); **G,** Callus differentiation from leaf explant with BAP(2.0 mg/l) and NAA (0.5 mg/l); **H,** Further differentiation and proliferation of multiple shoots from callus with BAP (2.0mg/l) and NAA (0.5mg/l) after 6 weeks; **I** and **J,** Elongated and proliferated multiple shoots of *Naringi crenulata* with BAP (0.5mg/l) and Kn (0.5mg/l).

Table 2. Effect of different combination of NAA and BAP on callus induction % and shoot regeneration from different explants of

Growth regulator concentration (mg/L)		Callus induction (%)			Number of shoot/per explants ± S.D		
BAP (mg/L)	NAA (mg/L)	L.E	N.S	S.T	L.E	N.S	S.T
0.0	0.0	0	0	0	0	0	0
0.5	5.0	10	15	10	1.2±0.3	0.9±0.1	0.3±0.1
0.5	3.0	15	10	15	2.5±0.7	1.3±0.2	0.8±0.2
0.5	**2.0**	**100**	40	35	5.0±0.9	2.3±0.1	1.4±0.1
2.0	2.0	40	30	20	10.0±0.4	8.5±0.3	6.4±0.5
2.0	1.0	45	50	30	16.5±0.6	12±0.6	8.0±0.3
2.0	**0.5**	80	**65**	**50**	**25±0.3**	**16±0.8**	**10±0.3**
5.0	0.5	5	20	10	1.0±0.8	6.7±0.1	0.1±0.2

L.E., Leaf explants; N.S., nodal segment; S.T., shoot tip.

Table 3. Effect of cytokinins on shoot elongation from leaf explants of *N. crenulata cultured* on MS medium supplemented with BAP

Concentration of growth regulator		Number of shoot elongated ± S.D
BAP(mg/L)	Kinetin (mg/L)	
0.1	0.1	6.5 ± 0.2855
0.25	0.25	12.2 ± 0.179
0.5	**0.5**	22.6 ± 0.335
1.5	1.5	10.2 ± 0.1155
2.0	2.0	5.5 ± 0.557

Shoot elongation

The shoot buds produced on this induction medium did not develop further and remained stunted structures which failed to elongate on the same m elongation and development into healthy shoots, shoot buds were sub cultured on MS medium containing both BAP (0.5 mg/L) and Kn (0.5 mg/L) (Figures BAP and Kn in combination, though not ideal for shoot bud induction, were effective in converting shoot buds into sturdy and healthy shoots. Therefo designated as 'shoot bud elongation medium'. A similar observation was made by Gupta et al. (1994), Kaur et al. (1998), Mohasseb et al. (2009), Jain et al. Qaiser (2010).

Rooting

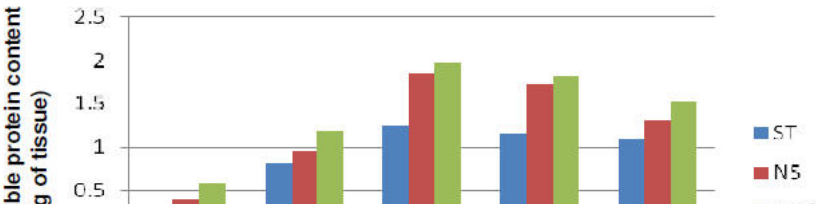
For rooting, individual shoots of 2 to 3 cm length were transferred to MS medium supplemented with IBA (0.5 to 2.5 mg/L) individually. In this study, rooting was observed on ½ MS medium supplemented with IBA (1.0 mg/L) and (2.0 mg/L). At IBA (2.0 mg/L) roots we to root obtained at IBA (1.0 mg/L). Similar results with IBA were reported by Sharma and Patni (2006) and Meena et al. (2010). Following this, the rooted pl and transferred to the soil.

The procedure described here is the first successful plant regeneration system for *N. crenulata* through indirect organogenesis using a wide range of expla of the present investigation reflect the existence of large inter-explants variability in callusing response.

Such variations can be attributed to the physiological condition of the explants, which is determined by genetic factors (Nagarathana et al., 1991). capacity of leaf explants in comparison to nodal segment and shoot tip has also been reported by Koroch et al. (2003) and Dhar and Joshi (2005). Acc Asahira (1980), intercalary meristems distributed in leaves might be responsible for the higher regeneration potential.

Biochemical investigations

Among different explants, the amount of total soluble protein varies with the age of callus and the highest amount of total soluble protein contents wer callus derived from leaf explants (1.96 mg g^{-1} of tissue) while node and shoot tip have 1.85 and 1.24 mg g^{-1} of tissue, respectively (Figure 2). In comparison of any age has more total soluble protein contents than any part of plant which is due to the protein synthesis during organ formation particularly during the s study, activity of peroxidases in different explants and in the calli of different ages was also estimated. Among different explants, leaf (callus at 4 week) h (0.88 unit^{-1} min^{-1} g^{-1} fresh weight of tissue) of peroxidases, and as the age of callus increased, the activity of peroxidases decreased and t observed in 8 week old callus. Similar results were observed in case of callus derived from nodal explants and shoot tip. In case of callus derived from nodal activity (0.45 unit^{-1} min^{-1} g^{-1} fresh weight of tissue) of peroxidases was estimated in 4.0 week old callus and in the case of shoot tip derived callus, the highe min^{-1} g^{-1} fresh weight of tissue) of peroxidases was estimated in 4.0 week old callus (Figure 3). The increase in the peroxidase activity can be correlated v plants are grown under *in vitro* conditions and exogenous growth regulators (auxins and cytokinins) are also present in growth medium, calli exhibit hig (Csiszar et al., 2003). As a result of ethylene production, defense mechanisms at a transcriptional level and generation of active oxygen species includin which result in increased peroxidase activities (Levins et al., 1995). Peroxidase isoenzymes are widely distributed among higher plants and are frequently o and due to these characteristics, different organs from same plant may show different peroxidase patterns (Thorpe et al., 1978; Asins et al., 1982). Appea persistent isoperoxidases during part of growth cycle has been reported by Balasimha and Subramanian (1983), Swarnkar et al. (1987) and Meena and Patn



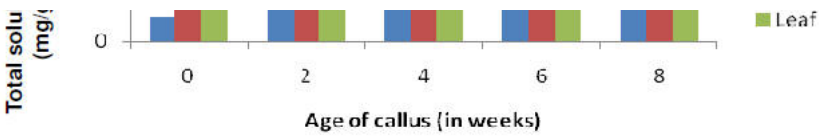


Figure 2. Estimation of total soluble protein contents (mg g^{-1} of tissue) in callus cultures of different ages.

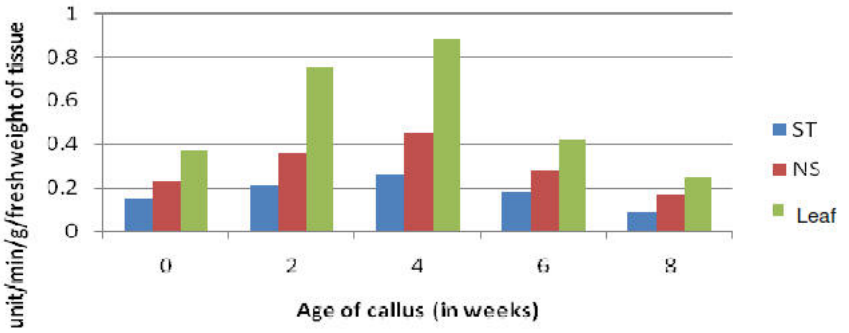


Figure 3. Estimation of peroxidase activity at different stages of callus growth.

Conclusion

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In conclusion, this study reports an efficient and easy to handle protocol for organogenesis through callus for vulnerable plant of semi- arid region of Ind soluble protein contents and peroxidase activity changes during different stages of callus growth and the type of explants used and this study can be used a of differentiation during morpho-genesis of callus and shoot formation. Leaf is the best explant source for callus induction. The present callus regeneration important for advanced studies on genetic improvement and in future, also has considerable potential as an alternative means for production of known metabolites.

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