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Full Length Research Paper

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

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***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. crenulata* (Roxb.) Nicolson 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 vulnerable species. A callus induction and *in vitro* plantlet regeneration system for *N. crenulata* (Roxb.) Nicolson was optimized by studying the influence of explants type (leaf, nodal segment and shoot tip) and different concentrations of plant growth regulators. Callus formation and shoot differentiation was initiated on Murashige and Skoog's (MS) medium containing different 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-benzylaminopurine) and 2.0 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 organogenic callli with 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. Elongation and further 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 existence of high inter-explant variability in response to growth regulators. *In vitro* rooting of shoots was achieved on ½ strength MS medium supplemented with IBA. Best rooting was achieved on ½ strength 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 four weeks old callus 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 rapid regeneration of *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 for their primary health care (Mousumi et al., 2007). The success of any health care system depends on the availability of suitable drugs on a sustainable basis. Natural medicine improves the inner 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 *Hesperethusa crenulata* (Roxb.) M. Roem or *Limonia crenulata* Roxb.

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

The plant is known by a wide range of common names in almost four languages across India – Beli (Hindi), naibela (Kannada), bilvaparni (Sanskrit), and narivala (Tamil). Most of 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, sudorific and employed for 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 smallpox, malignant and pestilent fevers and is also considered an excellent antidote to various poisons. The bark is aromatic and cooling and is useful in vitiated conditions of pitta (Pullaiah and Chennaiah, 1997). The plant shows anti- inflammatory activity. Its powered stem wood is used traditionally as a natural skin conditioner especially as facial cosmetics in Myanmar and some parts of Northern Thailand. Intensive and unabated collection and exploitation of this plant has declined its natural population number and density to the 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 tissue culture studies (Francisco et al., 1992) which describes the non embryogenic callus induction of 28 citrus relatives and an attempt was made to isolate protoplast from resulting callus lines. Ramani et al. (2010) carried out the pharmacognostical, phytochemical and anthelmintic evaluation of leaves of *N. crenulata.* Plant tissue culture techniques for micropropagation alone or in combination with genetic transformation can be useful as tools for crop improvement and for investigating the production of important secondary metabolites. Callus induction and subsequent green plant regeneration is genotype specific. It is mentionable that the success of plantlet regeneration under *in vitro* culture system depends upon the 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 remove stress and to increase tolerance to stress, the activity of antioxidant enzymes, such as guaiacol peroxidase, superoxide dismutase, catalase, ascorbate peroxidase and glutathione 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* propagation particularly during different stages of callogenesis, where cultures are maintained for longer time under *in vitro* conditions. Therefore this study was undertaken to compare the regeneration capacities of different explants types and to compare the responses of different explants to various plant growth regulator (PGR) treatments, thereby describing a rapid regenerative and efficient protocol for regeneration of this threatened species. It also describes the changes in peroxidases and total soluble protein contents during different stages of callus growth and regeneration.

MATERIALS AND METHODS

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 expanded leaves of 1 cm^2 rectangular segments, 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 half an hour. They were then treated with 2% (v/v) Tween 20 (a commercial grade detergent) followed by several rinses in sterile distilled water. The disinfected explants were surface sterilized under 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 were then immersed in 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) alone or supplemented 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 leaf explants Kn (0.5 to 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 20 min. Cultures were 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 repeated thrice with 5 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 carefully removed from the 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 (systemic fungicide) for 10 seconds to minimize the microbial infection. Again a second wash was given with sterile distilled water. The treated plantlets were then transferred aseptically to small earthen pots containing mixture of vermiculite and sterilized soil in growth chamber with controlled temperature, light and humidity to accli-matize with the outside environment. Half strength liquid medium was added periodically. The plantlets were covered with polythene bags to ensure a relative humidity of 70 to 80%. The acclimatized complete plantlets 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 pestle and mortar. The 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 solution was made up to 1.0 ml with distilled water, protein contents were estimated by Lowry's method (1951). For peroxidase enzyme, 500 mg sample was prepared by homogenizing plant material (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 the method given in 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 deviation.

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, that is, NAA (0.5 to 5.0 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 and shoot tips. These 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 callusing response was 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. However, callusing rate 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 callogenesis and shoot 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 hormone free medium (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 amount of callus which 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 on shows rhizogenic 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) induced green, hard 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 concentration being 2.0 mg/L. 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 rhizogenic response with 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 growth. On 2, 4-D (0.5 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 of leaf explants was 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 that among the various explants (leaf/shoot tip/ nodal segment) tried, leaf explants proved to be the best and among the various auxins tried, NAA was the most suitable auxin at 2.0 mg/L 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 enhanced by the addition of 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 explants types as callus regenerating in the absence of NAA turned brown/ black and showed no further growth. From the combination of PGR's tested, callus production was maximum at BAP (0.5 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) and NAA (0.5 mg/L) (Table 2).The callus so produced was green, compact, healthy and fast growing. Similar results were reported by Pathak and Heble (2002), Rajeshwari and Paliwal (2008), 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 concentrations of BAP (0.5 to 5.0 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 obtained on MS medium 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 shoot tip 10±0.3 (Table 2). Addition of lower quantities of NAA along with higher concentration of BAP seemed to have a positive response on the organogenic callus from leaf explants. When the 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, compact and could not be separated easily.

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; R, rhizogenic callus.

Kinetin did not prove to be beneficial for producing green and healthy callus. The callus produced on kinetin supplemented medium was brown, watery and slow growing. Thus, 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, neither BAP nor Kn alone 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 concentration being 2.0 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 to 6 weeks. Since the 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 bud induction medium’. This synergistic effect of BAP and auxin has been demonstrated in many plants

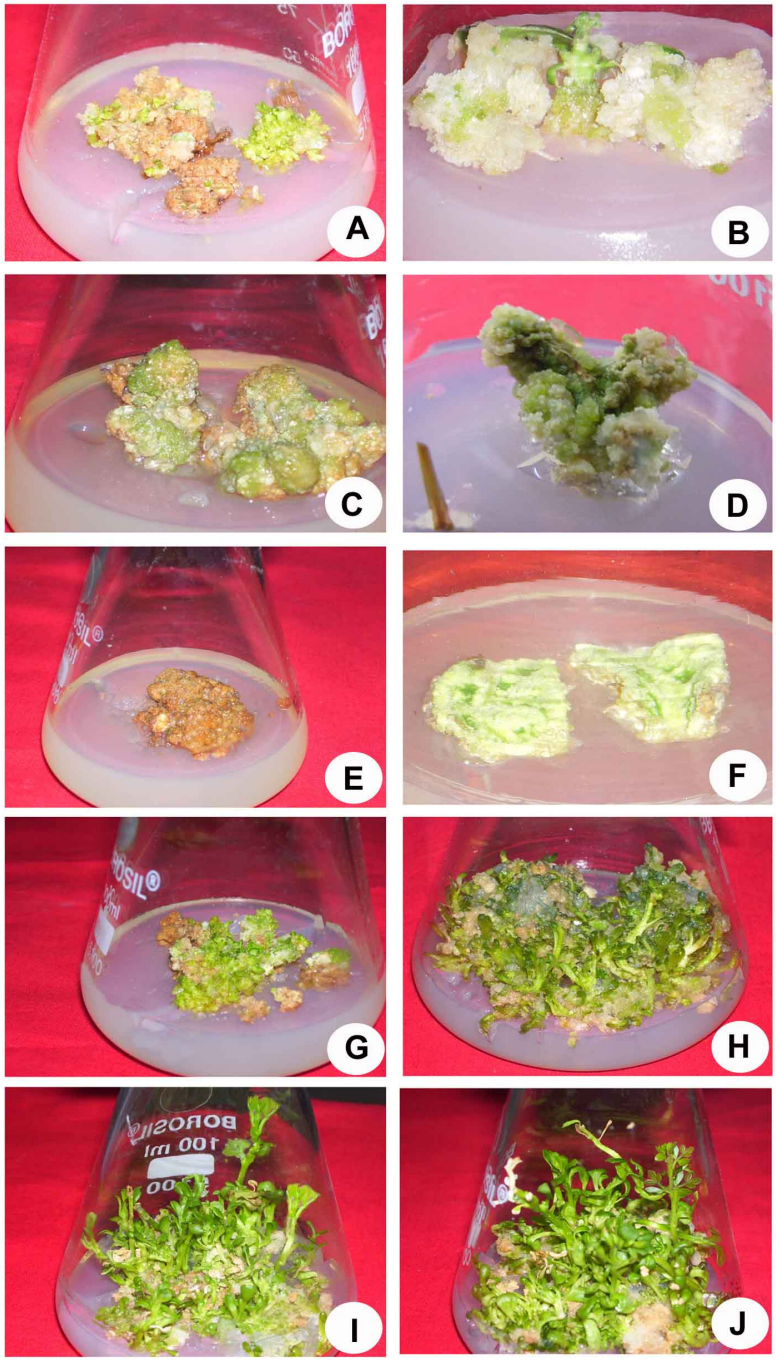

Figure 1. Callus induction, differentiation and elongation from nodal segment, shoot tip and leaf explants of *Naringi crenulata*. **A,** Callus formation from nodal segment supplemented with IBA (1.0 mg/l); **B,** Callus formation from shoot tip explant with IBA (1.0 mg/l); **C,** Callus induction from 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 *N. crenulata*.

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 and Kn.

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

by Swamy et al. (1992), Purohit et al. (1994), Casado et al. (2002), Fraternale et al. (2002), Salvi et al. (2002), Dode et al. (2003) and Ahmad et al. (2010).

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 medium. Therefore, for 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 1I and J and Table 3). BAP and Kn in combination, though not ideal for shoot bud induction, were effective in converting shoot buds into sturdy and healthy shoots. Therefore, this medium was 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. (2009) and Abbas and 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 were short as compared 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 plantlets were hardened 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 explants. Thus, the results 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). The high regeneration 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). According to Hosoki and 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

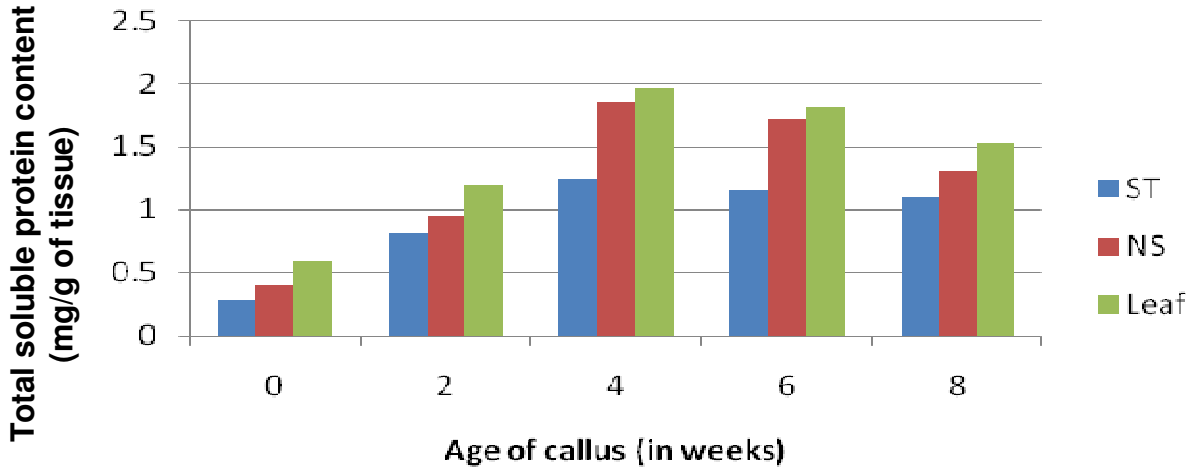

Figure 2. Estimation of total soluble protein contents (mg g^{-1} of tissue) in callus cultures of different ages.

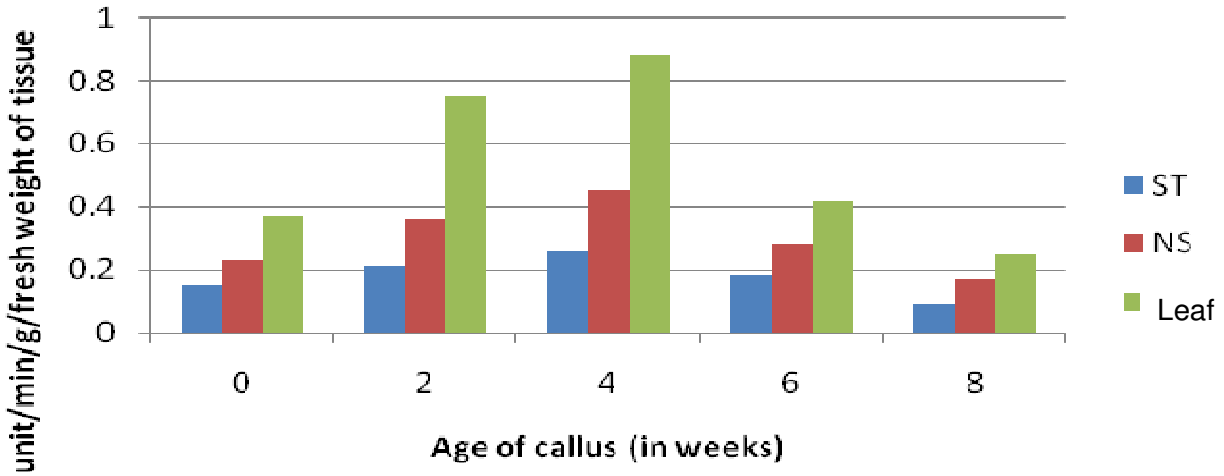

Figure 3. Estimation of peroxidase activity at different stages of callus growth.

protein varies with the age of callus and the highest amount of total soluble protein contents were found in 4 week old 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, *in vitro* raised callus 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 shoot induction. In this 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) had the highest activity (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 the lowest activity was 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 explants, the highest 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 highest activity (0.26 $unit^{-1}$ 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 with the fact that when plants are grown under *in vitro* conditions and exogenous growth regulators (auxins and cytokinins) are also present in growth medium, calli exhibit high ethylene production (Csiszar et al., 2003). As a result of ethylene production, defense mechanisms at a transcriptional level and generation of active oxygen species including H_2O_2 are activated, which result in increased peroxidase activities (Levins et al., 1995). Peroxidase isoenzymes are widely distributed among higher plants and are

frequently organ or tissue specific and due to these characteristics, different organs from same plant may show different peroxidase patterns (Thorpe et al., 1978; Asins et al., 1982). Appearance of transient and persistent isoperoxidases during part of growth cycle has been reported by Balasimha and Subramanian (1983), Swarnkar et al. (1987) and Meena and Patni (2007).

Conclusion

In conclusion, this study reports an efficient and easy to handle protocol for organogenesis through callus for vulnerable plant of semi- arid region of India, *N. crenulata.* Total 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 as biochemical marker of differentiation during morphogenesis of callus and shoot formation. Leaf is the best explant source for callus induction. The present callus regeneration system may also be important for advanced studies on genetic improvement and in future, also has considerable potential as an alternative means for production of known and new secondary metabolites.

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