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Full Length Research Paper

Effect of season, explants, growth regulators and sugar level on induction and long term maintenance of callus cultures of *Ficus religiosa* L.

Priyanka Siwach*, Anita Rani Gill and Kavita Kumari

Department of Biotechnology, Chaudhary Devi Lal University, Sirsa, Haryana, India.

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The effects of antioxidant treatments, 2,4-D concentrations and sucrose level were examined in order to optimize the induction and long term maintenance of callus cultures of *Ficus religiosa* L. from different explants including nodal segments, inter-nodal segments and shoot apices. The explants subjected to incubation for 40 min in antioxidant solution (2% ascorbic acid + 2% citric acid) exhibited maximum percent culture establishment (73.33%). Establishment was reduced when incubation time was longer than 40 min. The explants collected in May and June gave maximum response (100 %). The highest frequency of callus induction (100%) was observed in nodal segments on Murashige and Skoog medium supplemented with 2.26 µM 2,4-dichlorophenoxyacetic acid and 3% sucrose after an average of 8.38 days. Maximum callus proliferation, resulting in fresh weight of 4.29 g after four weeks of culture, was also observed for callus initiated from nodal segments on the earlier mentioned medium. Higher concentrations of 2,4-D and lower concentrations of sucrose were found to be unfavourable for callus induction as well as proliferation. Callus cultures, initiated from nodal segments, could be successfully maintained in healthy and proliferative form for consecutive nine months on the earlier said medium.

Key words: *Ficus religiosa* L., explants, callus induction, callus proliferation, callus maintenance, *in vitro.*

INTRODUCTION

Ficus religiosa L. is an ornamental and fuel wood tree, belonging to the family Moraceae. It is found wild or cultivated throughout India, Pakistan, Bangladesh and Nepal and has strong religious holdings for Hindus and Buddhists. The tree is large, heavily branched with long petiolated, long tipped, leathery, heart-shaped leaves. It is very popular as a shade tree because of its good form and easy adaptability to various soil conditions and so very commonly planted as an avenue or roadside tree. The fruits and tender leaf buds are occasionally eaten in times of scarcity. The fruits are eagerly devoured by birds.

Various preparations from *F. religiosa* exhibit important medicinal applications like bark extract is used in the treatment of diabetes, asthma, inflammations and glandular swelling, wound healing, stomatitis and skin diseases. The leaves are reported to be effective in the treatment of constipation as well as ear problems (Venkataswamy et al., 2010). Root bark is found good for cleaning ulcers and in the treatment of leucorrhoea (Jain et al., 2010). Recently, stem bark is also reported to have remarkable acetylcholinesterase inhibitory activity, the latter being very effective for the treatment of Alzeimer's disease (Vinutha et al., 2007). Some of the secondary metabolites responsible for the various biological activities have been identified and characterized like bergenin, lupin 3-one, methyl oleanolate, lanosterol, n-octacosanol, β-sitosterol, stigmasterol, caffeic acid, amides and flavanoids while many are still unexplored and uncharacterized. Mostly, the isolation and characterization of these metabolites is done from naturally occurring sources. Such sources are time and labour consuming and are influenced by environmental conditions and strong religious values associated with *F.*

*Corresponding author. E-mail: Psiwach29@gmail.com.

Abbreviations: MS, Murashige and Skoog; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **DMRT,** Duncan's multiple range test; **CRD,** complete random design; **ANOVA,** analysis of variance; **Fwi,** fresh weight increase.

religiosa L. Because of these reasons, the medicinal properties of this tree have not been harnessed fully and hence, commercial level processing demands an alternative source.

Plant cell cultures were introduced as an important tool for studying and producing plant secondary metabolites in the mid 1960s. Some highly effective secondary metabolites that are used in pharmaceuticals, food industry and cosmetics have been produced through plant cell cultures, callus cultures, shoot cultures and root cultures (Taha et al., 2008; Mathur et al., 2007; Zhao et al., 2001; Zhang et al., 2002). The direct manipulation of plant cell and tissue culture systems has many advantages over the conventional isolation of secondary metabolites. Such manipulations have been reported to enhance the secondary metabolites production in many species (Mathur et al., 2007; Zhang et al., 2002). Despite great advancement in plant tissue culture techniques, the success with woody plants, particularly of old age, has continued to be a challenging task (McCown, 2000). In some cases, there are hundred percent losses during *in vitro* establishment. The most frequent problems are infections, endogenous bacteria and browning of explants (Chandra et al., 2004). Season has also been reported to be an important factor for mature tree tissue culture (Parasharami et al., 2003).

Till date, there is no report regarding the use of any of the *in vitro* tissue of *F. religiosa* for secondary metabolite isolation and to the best of our knowledge, efficient induction and long term maintenance of callus cultures of *F. religiosa* L. for this purpose has not been reported, though there are a few reports about micropropagation (Deshpande et al., 1998). This study describes the optimization of the conditions for efficient long term *in vitro* callus growth of 45 to 50 year-old *F. religiosa* L. tree. Poor explants response, rapid explants browning and heavy microbial infestation are major hurdles faced by to successfully establish *in vitro* cultures. The callus cultures generated in this study can be easily used for effective screening, isolation and characterization of various secondary metabolites of *F. religiosa* L. for future applications in the pharmaceuticals.

MATERIALS AND METHODS

Plant material and explants preparation

The plant material used in this study was obtained from 45 to 50 year-old tree of *F. religiosa* L., growing near the campus area of the Chaudhary Devi Lal University, Sirsa, Haryana, India. Three explants including nodal segments, inter-nodal segments and shoot apices (each 3 to 4 cm in size) were excised from healthy and juvenile shoots and were washed under running tap water for 10 min to remove the traces of dust and dirt. These were then, swabbed with an alcohol (50% v/v) soaked muslin cloth followed by washing in tween-20 (2% v/v) for 10 min. After, these were subjected to surface sterilization by treating successively with bavistin (0.1% w/v) for 7 min and 0.1% (w/v) mercuric chloride solution for 8 min. After each surface sterilization treatment, the explants were thoroughly rinsed 5 times with sterilized distilled water. The explants were trimmed to a final size of 0.5 to 2 cm and were placed aseptically in 150 ml Erlenmeyer flasks/magenta boxes containing 35 to 40 ml medium.

Culture establishment

Effect of anti-oxidant solution on phenolic browning of explants

Preliminary trials had resulted in immediate explants and media browning. To overcome this, explants were incubated in anti-oxidant solution (2%, w/v, ascorbic acid + 2%, w/v, citric acid) on rotary shaker at 200 rpm for different time periods, just after washing under running tap water and then, were subjected to surface sterilization as explained earlier.

Effect of season on percent response of explants and frequency of contamination

Since woody perennial trees have endogenous infections and follow a periodical growth pattern, explants were collected and cultured every month from October 2008 to September 2009, to study the effect of season on percent response as well as on percent contamination of *in vitro* cultures.

The MS basal medium (Murashige and Skoog, 1962) supplemented with 2.26 µM 2,4-D and 3% w/v sucrose, pH of which was adjusted to 5.8 before being solidified with 0.8% w/v agar, was employed for both the earlier mentioned culture establishment study. The culture vessels containing the media were autoclaved at 15 lb and 121°C for 20 min. Cultures were maintained at 25 ± 2°C, at a photoperiod of 16 h (80 µmol m^{-2} s^{-1}) for 4 weeks.

Callus induction

Effect of 2, 4-dichlorophenoxyacetic acid (2,4-D) concentrations and explants nature for optimal proliferation

All the three types of explants were cultured vertically on MS medium supplemented with different levels of 2,4-D (2.26 , 4.52 , 6.79 , 9.05 , 11.31 and 13.57 µM) to select out the best explant and most favouring concentration of 2,4-D with regard to callus induction and proliferation. Medium sterilization and culture conditions were described earlier. After 4 weeks of culture, observations were made for percent callus induction, fresh weight of callus and callus morphology. Observations were made daily for noting the number of days required for callus initiation.

Effect of sugar concentration

Retaining the best explant and best 2,4-D level with regard to pe cent callus induction and fresh weight of callus, two more levels of sucrose (2 and 2.5%) were tested and observations were made as reported earlier. Medium sterilisation and culture conditions were described earlier.

Callus maintenance

Calli were routinely sub cultured for consecutive eight months, each sub culture after a regular interval of 30 days on three media formulation. These three media had the same 2,4-D concentration (the most suitable one with regard to maximum fresh weight increase, Fwi) but differed in sucrose level (3, 2.5 and 2%). For

Table 1. Effect of incubation time in anti-oxidant solution on initiation of browning and percent establishment of explants of *F. religiosa* L., cultured on MS medium supplemented with 2.26 µM 2,4-D.

Incubation time (min)	Day of initiation of browning of explant (Mean ± SE)	Percent establishment* (Mean ± SE)
0 (Control)	02.14 ± 00.09^{f}	16.66 ± 03.33^{e}
5	03.19 ± 00.35^{f}	20.00 ± 05.77^{de}
10	05.33 ± 00.35^{e}	43.33 ± 03.33^{bcd}
20	07.11 ± 00.43^{d}	50.00 ± 10.00^{abc}
30	10.48 ± 00.28^{c}	60.00 ± 05.77^{ab}
40	14.77 ± 00.42^{b}	73.33 ± 12.01^{a}
60	14.99 ± 00.61^{b}	70.00 ± 00.00^{a}
80	18.06 ± 00.29^{a}	60.00 ± 05.77^{ab}
100	19.13 ± 00.47^{a}	33.33 ± 14.53^{cde}

Means within columns followed by the same letters are not significantly ($P < 0.05$) different as determined using Duncan's multiple range test. *Percent establishment refers to fraction of the explants showing green and healthy state out of the total explants, cultured on a particular treatment (as some explants become damaged by the anti-oxidant solution, exhibiting wrinkled appearance with no sign of any growth).

each treatment, calli pieces weighing 20 to 25 mg (fresh weight), obtained by division of larger callus, were inoculated on fresh medium and allowed to proliferate for 30 days. On 30th day, fresh weight increase (Fwi) was calculated as the difference between the final and the initial fresh weight. Fresh weight recording was done under aseptic conditions.

Experimental design and statistical analysis

All the experiments were conducted using a complete random design (CRD). There were 10 replicates per treatment and each treatment was repeated in three sets. Data were subjected to analysis of variance (ANOVA) using the SPSS version 12. The significant differences among the treatments were compared using Duncan's multiple range tests (DMRT). Treatment values were represented as the mean ± SE.

RESULTS AND DISCUSSION

Culture establishment

Effect of anti-oxidant solution on phenolic browning of explants

The browning and subsequent death of explants during preliminary studies can be attributed to the oxidation of polyphenols which exist in high amount in *F. religiosa* (Bushra et al., 2009). Treatment of the explants with antioxidants like ascorbic acid and citric acid seems to inhibit polyphenoloxidase enzyme (it converts the phenols to typical brown polymers) and so is one of the effective strategy to control the phenolic browning of *in vitro* cultures (Pizzocaro et al. 2007). In this study, incubation of explants in anti-oxidant solution (2% ascorbic acid + 2% citric acid) on rotary shaker at 200 rpm for different time period significantly affected the day of initiation of browning ($P < 0.05$) as well as explants establishment ($P < 0.05$) (Table 1).

The initiation of browning was significantly delayed by increase in incubation time, maximum delay being for 80 and 100 min (Table 1). However, delaying of browning beyond 40 min incubation time did not have any positive impact on explants establishment, rather explants viability was affected negatively due to excess exposure of anti-oxidant solution. The maximum culture establishment (73.33%) was observed on 40 min incubation time and this was significantly not different from that with 20, 30, 60 and 80 min, when compared using DMRT. However, visual observations for repeated treatments indicated that, 20 and 30 min incubation time resulted in remarkable explants and media browning, while 60 and 80 min incubation time resulted in unhealthy explants with vitrified appearance. So the incubation time of 40 min was selected as the most favouring one for culture establishment. To make it more efficient, cultures were sub cultured on the fresh medium on the 19th day and it resulted in hundred percent culture establishments at the end of the fourth week. Such frequent transfer of explants to fresh medium has been reported by many workers as a favouring factor towards culture establishment (Tiwari et al., 2002). This practice of incubating the explants for 40 min followed by sub culturing on the 19th day was followed in the following experiments.

Effect of season on percent response of explants and frequency of contamination

The heavy infestation of microbes is a major problem to *in vitro* establishment of old age woody plants (Nejad, 2005). The surface sterilization protocol being followed during this study was in fact selected after vigorous

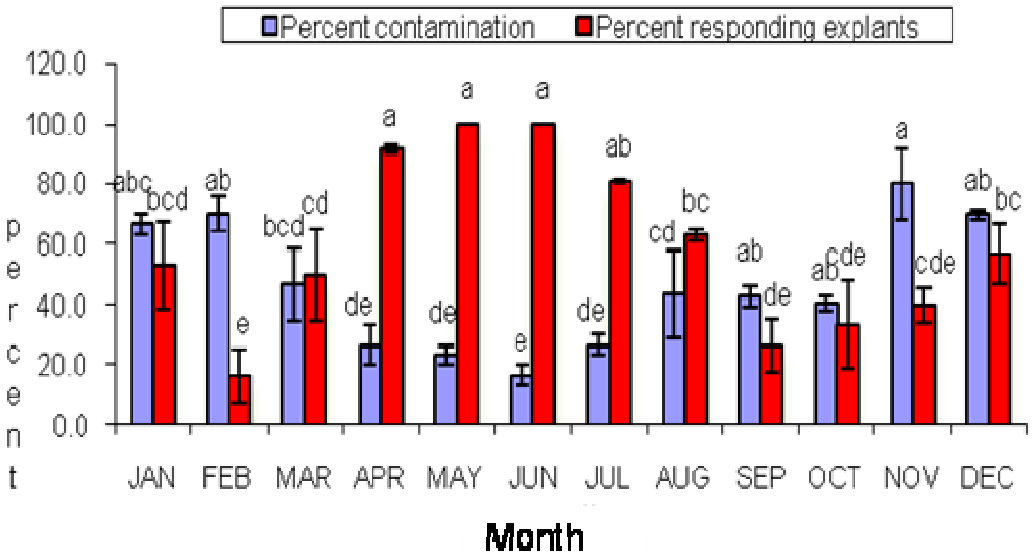

Figure 1. Effect of season on percent response and percent contamination of explants of *Ficus religiosa*, cultured on MS medium supplemented with 2.26 µM 2,4-D. Bars represent, Mean ± SE (Means within columns followed by the same letters are not significantly ($P < 0.05$) different as determined using Duncan's multiple range test).

exercises of trying out more than fifteen different protocols (Data not shown). The frequency of contamination was significantly ($P < 0.05$) low for April, May, June and July, minimum being 16.67% for the month of June, while it was very high for November, December, January and February (Figure 1). Other modifications in the protocol affected the viability of explants profoundly, without controlling the contamination effectively (data not shown). Studies are also going on towards identification of the microbial contaminants so that a specific treatment may be formulated for commercial use (not completed yet).

The plant material collected in the months of April to July exhibited a significantly ($P < 0.05$) higher response, maximum 100% for the months of May and June (Figure 1). In central northern India, April to July is the active growth period of *F. religiosa* and the axillary buds are in actively growing state. This better response of actively growing shoot buds over dormant buds is in agreement with several other reports for woody perennials (Siril and Dhar, 1997).

Callus induction

Effect of 2,4-D concentrations for optimal proliferation

The use of plant growth regulators is of fundamental importance in directing the organogenic response of any plant tissue/organ under *in vitro* conditions (Che et al., 2002; Sugiyama and Imamura, 2006). Studies have shown that, 2,4-D is one of the most effective auxins for the induction and growth of callus (Lee et al., 2004; Burbulis et al., 2007). In this study, effect of six concentrations of 2,4-D on callus induction and proliferation from three different explants of *F. religiosa* was investigated. The three way ANOVA for the differences between various concentrations of 2,4-D, between explants and between sets with regard to percent callus induction, number of days for callus induction and fresh weight of callus indicated that, all the three responses were significantly affected by the explants ($P < 0.01$), the concentration ($P < 0.01$) and explants X concentration interaction ($P < 0.01$).

The maximum percent callus induction for each explant, nodal segments (100%), inter-nodal segments (42%) and shoot apices (60%), was observed on 2.26 µM level (Table 2). The frequency of callus induction decreased with the increase in the concentration of 2,4-D, for each explant (Table 2). Synthetic auxins like 2,4-D may possess herbicidal property at high concentrations which might inhibit callus formation (Evans et al., 2003). Similar observation of low level of 2,4-D favouring the callus formation has been made in *Gymnera sylvestre* also (Gopi and Vatsala, 2006). An early response was observed on 2.26 µM 2,4-D, the increase in concentration of 2,4-D causing significantly delayed response, for all the three explants (Table 2). The callus formation initiated from the cut edges, touching the medium, of the explants and was brownish in colour. Callus, from each type of explants, proliferated with different growth rate on each level of 2,4-D. The maximum proliferation resulting in higher fresh weight of callus after four weeks of culture, from each explant (4.29 g from nodal segment, 0.55 g from inter-nodal segments and 3.55 g from shoot apices) was observed on 2.26 µM 2,4-D, (Table 2) (Figure 2).

Table 2. Percent callus induction, fresh weight of callus, after four weeks of culture, and number of days for callus initiation, recorded from three different explants on various concentrations of 2,4-D.

2,4-D conc. (µM)	Percent callus induction (Mean)			Fresh weight of callus (gm) (Mean)			Number of days for callus initiation (Mean)		
	Nodal segment	Inter-nodal segment	Shoot apice	Nodal segment	Inter-nodal segment	Shoot apice	Nodal segment	Inter-nodal segment	Shoot apice
2.26	100.0^{a}	42.0^{a}	60.0^{a}	4.29^{a}	0.55^{a}	3.55^{a}	8.38^{f}	10.16^{d}	6.94^{e}
4.52	72.0^{b}	35.0^{a}	50.0^{ab}	2.54^{b}	0.46^{b}	0.53^{b}	13.24^{e}	13.81^{c}	13.00^{d}
6.79	52.0^{c}	23.0^{b}	38.0^{b}	2.05^{c}	0.39^{bc}	0.46^{b}	17.09^{d}	17.14^{b}	17.17^{c}
9.05	40.0^{c}	18.0^{b}	23.0^{c}	1.63^{d}	0.36^{c}	0.38^{b}	20.00^{c}	21.18^{a}	18.79^{b}
11.31	23.0^{d}	3.0^{c}	12.0^{cd}	0.77^{e}	0.33^{c}	0.38^{b}	21.15^{b}	22.00^{a}	22.00^{a}
13.57	13.0^{d}	0.0	0.0	0.44^{f}	-	-	23.37^{a}	-	-
Mean	50.0	20.00	31.0	2.41	0.46	1.32	15.33	14.53	13.87

Means within columns followed by the same letters are not significantly ($P < 0.05$) different as determined using Duncan's multiple range test.

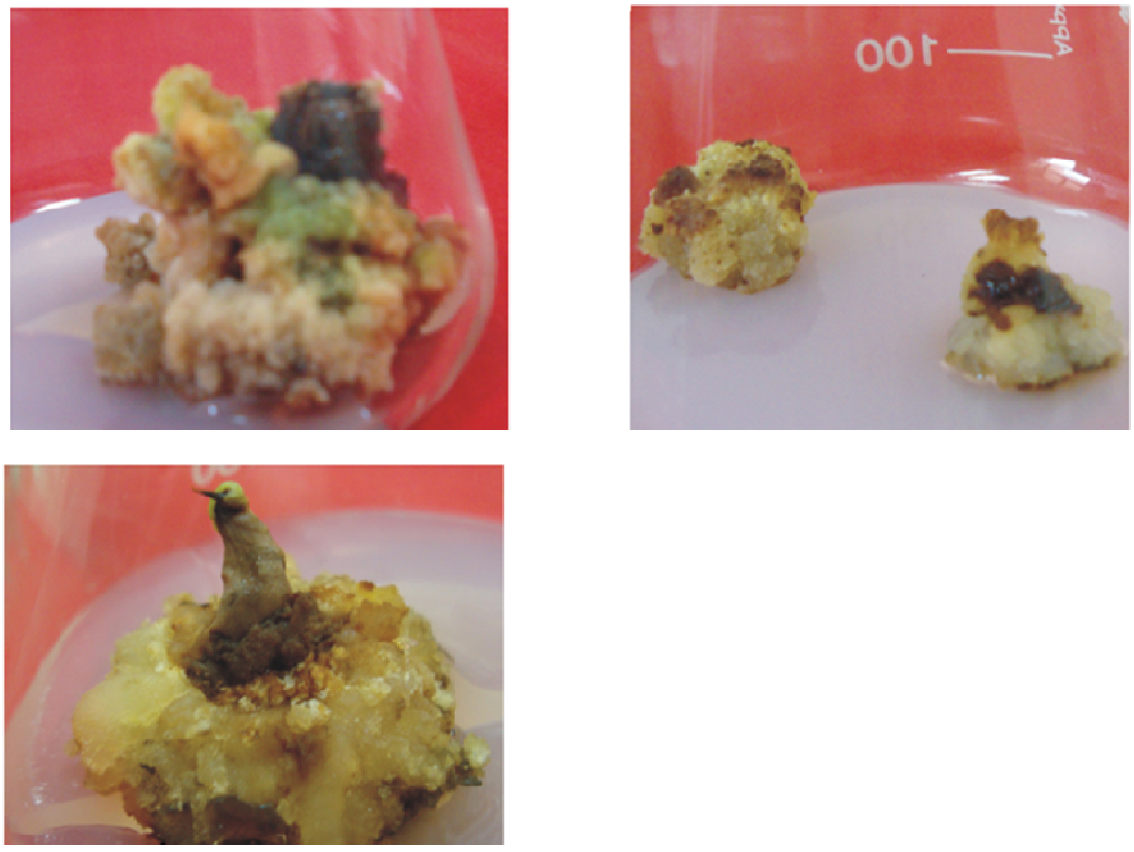

Figure 2. Proliferated callus, after 28 days, from (a) nodal segments, (b) inter-nodal segments and (c) shoot apices.

The nature of explants has a profound impact on the response (Ishii et al., 2004). Averaging the results from all the concentrations of 2,4-D together, nodal segment, of the three explants used, comes out to be the best explant with regard to maximum percent callus induction and maximum proliferation of callus after four weeks of culturing, followed by shoot apices and inter-nodal segments (Table 2). Callus forming ability of explants would be explained by differential reactivity to media components (Ikram, 2005). Nodal segments have active meristematic cells in the axils which may be responsible for better performance of the explants. Poor performance

Table 3. Effect of sucrose concentration on percent callus induction and fresh weight of callus from nodal segment cultured on 2.26 µM 2,4-D supplemented MS medium.

Sucrose level (%)	Callus induction (%)	Fresh weight of callus (g)	Callus morphology
3 (control)	100[a]	4.290[a]	Compact, brownish yellow colour
2.5	96.67[a]	4.147[a]	Compact, brownish yellow colour
2	66.67[b]	3.927[a]	Watery, pale yellow in colour

Means within columns followed by the same letters are not significantly ($P < 0.05$) different as determined using Duncan's multiple range test.

Table 4. Effects of sucrose concentration on callus proliferation after repeated sub-culturing, on 2.26 µM 2,4-D supplemented MS medium.

Sucrose level (%)	On 28th day	Fresh weight increase (gm) (Mean)								Mean
		1st Sub culture	2nd sub culture	3rd sub culture	4thSub culture	5thSub culture	6th sub culture	7th sub culture	8th sub culture	
3	4.290	3.435	3.721	3.881	4.140	4.332	4.438	4.597	4.695	4.0339[a]
2.5	4.147	2.227	2.220	2.221	2.017	1.951	1.909	1.867	1.717	2.0292[b]
2	3.927	1.770	1.908	2.045	1.936	1.815	1.776	1.744	1.724	1.7631[c]

Means within columns followed by the same letters are not significantly ($P < 0.05$) different as determined using Duncan's multiple range test.

of shoot apices when compared with nodal segments may be attributed to different endogenous level of various hormones. Poor response of inter-node explants for callus induction has previously been reported in *Acacia mangium* (Xie and Hong, 2001).

Effect of sucrose concentration

Sucrose is the main source of energy for *in vitro* plant tissue cultures as these have insufficient autotrophic ability. Sucrose not only acts as an external energy source but also contributes to the osmotic potential of the medium (Nowak et al., 2004) which would permit the absorption of mineral nutrients present in medium, essential to the cells growth and so the optimal osmotic pressure required for optimal proliferation, may vary from culture to culture. A significant effect of carbon source concentration, in culture media on the frequency of callus formation has been noticed in many plants like rice (Shahnewaj and Bari, 2004) and olive (Gracia et al., 2002). Retaining the best explant (nodal segments) and best 2,4-D level (2.26 µM), with regard to maximum callus initiation and proliferation (Table 2), two lower levels of sucrose (2.5 and 2%) were used for studying the effect on the earlier stated responses (Table 3). Since 100% callus induction with good proliferation was achieved with 3% sucrose, the aim of this objective was to know whether at low level, we can achieve considerably similar percent callus induction with the same or reasonably higher proliferation.

When the responses on 2.5 and 2% sucrose were compared with that of the control (3%), the frequency of callus induction did not differ significantly on 2.5% sucrose while on 2% level, it was significantly lower. On the other hand, fresh weight of callus (after four weeks of culture), was found to be significantly similar on all the three levels of sucrose as analysed by DMRT (Table 3). This indicates that, the optimal osmotic pressure range required by *in vitro* cultures of *F. religiosa* is well covered by the three concentrations of sucrose used in this study. Callus morphology was found to be affected remarkably with sucrose concentration. Callus on 3 and 2.5% was compact in appearance with brownish green colour, while on 2%, it was watery and flaccid in appearance with pale yellow colour.

Callus maintenance

Calli obtained from nodal segments were maintained for consecutively nine months on the MS medium supplemented with 2.26 µM at three different levels (3, 2.5 and 2%) to define the best conditions for long term maintenance and proliferation of callus cultures (Table 4).

Calli maintained on 3% sucrose proliferated with a higher average monthly Fwi of 4.0339 g when compared with Fwi of 2.0292 and 1.763 g on 2.5 and 2% sucrose, respectively (Table 4). This was in contrast to the observations on callus maintenance of *Taxus baccata* L., where 3% sucrose reduced the callus growth, while 2% sucrose favoured the fast growth of callus, during long term maintenance (Mihaljevic et al., 2002). Fresh weight of calli lowered after first sub-culture on all the three

Figure 3. Callus, initiated from nodal segments, with dark brown patches, after third sub-culture.

Figure 4. Proliferative and healthy callus, on MS medium with 2.26 µM 2,4-D and 3 % sucrose, after eight sub-culture.

media but lowering of weight was more drastic on 2.5 and 2% supplemented media (Table 4). However, on 3% sucrose fresh weight started to increase after second sub-culture and this process continued till eighth sub-culture (Table 4). This increase in proliferation rate indicate towards the habituation phenomenon where some of the cultured cells may develop the auxin biosynthetic pathways in prolonged cultures (Dodds and Roberts, 1995), though the actual mechanism underlying habituation is not well understood. A continuous decline in fresh weight till eighth sub-culture was observed on 2 and 2.5% sucrose, these levels seem to provide an inadequate osmotic pressure for the proliferation of callus cultures of *F. religiosa* L. After each sub-culturing, calli obtained were more friable in nature than that of previous month. On 2% sucrose, callus appeared unhealthy with swollen surfaces. Patches of dark brown colour were visible on the callus, raised on 3 and 2.5% supplemented media, after third sub-culture (Figure 3) indicating the production of secondary metabolites and area of these patches increased with subsequent sub-culturing. Calli after eighth sub-culture, were brownish in colour (with patches of dark and light colour) and highly friable in nature (Figure 4).

Conclusions

We have established an efficient and simple protocol for the long term proliferation of callus cultures initiated from explants of mature tree of *F. religiosa* L. Callogenesis showed range of responses depending upon season, explant, 2,4-D concentration and sucrose level. It was resulted from this study that, April to July is the most favou-ring months for callus induction. Nodal segments were selected out as the best explants for this study. MS medium supplemented with 2.26 µM 2,4-D and 3% sucrose, favoured the maximum percent callus induction as well as the maximum proliferation during repeated sub-culturing. As the study on the secondary metabolites of *F. religiosa* L. is still limited, callus tissue raised during the present study can serve as an alternative source of identification, characterization and commercial level isolation of secondary metabolites. Further experiments may be carried out for the enhancement of certain secondary metabolites as well as the production of some novel ones, with the aim to understand and utilise the medicinal properties of this perennial plant species.

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