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Impact of various factors on *in vitro* investigation of rare mangrove species *Sonneratia apetala* Buch.-Ham. (Myrtales: Lythraceae) and *Suaeda maritima* (L.) Dumort (Caryophyllales: Amaranthaceae)

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Abstract. Recent studies have showed the importance and destruction of mangroves. So their restoration through tissue culture study is urgently required because *in vivo* propagation is plagued with unforeseen obstacles. *In vitro* investigation of mangroves found to be suitable material for salt tolerant mechanism studies and anti stress gene isolation. This study describes for the first time *in vitro* approach for rare species *Sonneratia apetala* Buch.-Ham. (Myrtales: Lythraceae) and herb species *Suaeda maritima* (L.) Dumort (Caryophyllales: Amaranthaceae) through callus. For this investigation, as a source of explants various part (leaf, intermodal and nodal segments) of both mangroves were used. These explants were cultured in various types of media (MS, LS, WPM, X and B_5) with different combination of phytohormones (2, 4-D and NAA with BAP in combination). We also examined the effect of NaCl and seasons on callus initiation and growth. The highest rate of callus formation was obtained with nodal explants in MS medium supplemented with 1.5 mg.L^{-1} NAA and 0.5 mg.L^{-1} BAP in combination and 1 mg.L^{-1} NAA and 0.5 mg.L^{-1} BAP in combination for *Sonneratia apetala* and *Suaeda maritime*, respectively. We also found that callus initiation rate and growth decreased with increasing NaCl concentration higher than 80 mM and 120 mM for *Sonneratia apetala* and *Suaeda maritima*, respectively in MS media. This study also found that monsoon season was best time for *in vitro* investigation of mangroves. The results presented here give an insight into the development of *in vitro* investigation suitable for mangroves. The initiated callus could be restored in low salaine or devoid of salaine land.

Keywords: Culture media screening; Callus culture; Mangrove; Salt tolerance; *Sonneretia apetala*; *Suaeda maritima*.

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Introduction

Mangrove is a typical ecosystem in the intertidal area from tropical to subtropical muddy beaches worldwide. It is considered as low-cost and high-efficiency wetland systems for the treatment of municipal wastewater (Tam and Wong,

1996) and for remediation of heavy metal-polluted marine environments (MacFarlane et al., 2003). It has critical ecosystem functions such as coastal protection, land stabilization and CO_2 fixation (Ren et al., 2009). However destruction of mangrove forest is noticed due to natural and anthropogenic uses (Alongi, 2002). For mangrove restoration many have planted propagules and seeds which depend on natural yields. Therefore it is not possible to get enough numbers of good propagules and seeds all over the year. Recently there is shortage of mangrove seed source (Komiyama et al., 1996). In these circumstances, the alternative method is tissue culture study to get enough number of micropropagated plantlet. Mangrove species are physiologically unique in their adaptations to such water logged and saline condition. Crop scientists, studying the unique adaptation pattern of mangroves, are keen to impart these unique characters in food crops by breeding or biotechnological means (Fukomoto et al., 2004) as salinity and water logging are among the major environmental threats with serious implication on food, fuel and fibre production, especially in arid and semiarid regions (Dagar, 2005). Besides, about one-third of all agricultural lands are becoming saline (Dagar, 2005). To understand the salt and water logging tolerance theoretically or biochemically, callus or cell culture of mangroves may provide promising result. However there are scanty literatures are available on some mangrove species because they are much recalcitrant for tissue culture study (Fukomoto et al., 2004, Kawana and Sasamoto, 2008). During *in vitro* studies explants frequently turn brown or black shortly after inoculation and result in tissue death (Kathiresan, 1990) due to presence of high amount of tannin and phenolic compounds in mangroves (Ravi and Kathiresan, 1990).

The *Sonneratia apetala* Buch.-Ham. (Myrtales: Lythraceae) is a true mangrove tree from Sonneratiaceae family found in all over the mangrove region of the world and are considered as invasive species of mangrove (Tian et al., 2009, Naskar and Bakshi, 1987, Ren et al., 2009). This species is reported to improve soil fertility and having medicinal property like antioxidant, antimicrobial and cytotoxic activity (Ren et al., 2009). The leaf of this species is used to treat hepatitis (Banerjee et al., 2008, Jaimini et al., 2011). The fruit of this species is consumed by local people of mangrove region after cooking or other preparations. Additionally ripe fruits are used to treat parasites and half ripe fruit for cough (Banerjee et al., 2008 and Jaimini et al., 2011). Shete et al. (2007) reported that the aerial part of this species has the potentiality for the bioaccumulation of Pb and Zn.

The *Suaeda maritima* (L.) Dumort (Caryophyllales: Amaranthaceae) is an annual, succulent, salt-marsh mangrove annual herb, with semi-cylindrical leaves from Chenopodiaceae family grows in very alkaline and saline moist soils (Polić et al., 2009, Ravikumar et al., 2011). It is found in the sea cost of Asia, Europe, North and South America and Australia too (Polić et al., 2009). The leaf of this species is used to treat hepatitis and having medicinal properties like antiviral, antibacterial, antioxidant and hepatoprotective activity (Singh et al., 2012).

Keeping the deforestation, tissue culture problem, medicinal and economical value of these plants in mind, we described a preliminary study of micropropagation and degree of salt tolerant, through callus culture, for preservation and production of micropropagated plant for future restoration at degraded areas. To our knowledge, this is the first report of *in vitro* investigation of these species with systematic study.

Materials and methods

Plant material

Different explants from mature plants were collected at various seasons all over the year from Gosaba region (88° 39’ 46” E and 22° 15’ 45” N) of Indian Sundarban Mangrove Forest.

Surface sterilization of explants

The explants (1-1.5 cm long) were washed with 2 % (v/v) teepol for 10 min and rinsed thrice with distilled water, disinfected with 0.15 % (w/v) $HgCl_2$ for 10 min and again rinsed four times with sterile distilled water. There after the sterilized explants were cut into small segments and aseptically inoculated on different nutrient media. All the experimental procedures were carried out under aseptic conditions in a laminar airflow.

Culture conditions

The cultures were generally maintained at standard condition with a temperature of 25 ± 1 °C under 65 ± 5% relative humidity and 16 h photoperiod under 2000 lux intensity provided by white fluorescent lamps.

Culture medium and growth regulators used

Four different types of media were used for callus initiation like Murashiege and Skoog (MS) medium (Murashiege and skoog, 1962), Woody Plant medium (WPM, Lloyd and McCown, 1981), Linsmaier and Skoog (LS) medium (Linsmaier and Skoog, 1965) and X medium (Rao et al., 1998). Considering the herb species *Suaeda maritima* in mind we also included B5 (Gamborg et al., 1968) medium along with other mentioned medium. All media were supplemented with 3% (w/v) sucrose and 0.8% agar powder. The pH of the medium was adjusted to 5.7 before autoclaving. For induction of callus and determining the degree of salt tolerant mechanism, NaCl was added in the medium at various concentration of this experiment. The callus initiation rate (the ratio of the number of explants pieces having calli to total number of explants pieces planted in the same culture) was scored about one month after inoculation. To determine the callus growth on NaCl, fresh weight of calli growth (FWG) was measured 60 days after inoculation as (W_1-W_0) where W_0 is the initial inoculation weight and W_1 the final weight. For this study we observed two auxin like 2, 4-dichloro phenoxy acetic acid (2,4-D) and α-naphthalene acetic acid (NAA) in combination with one cytokinins i.e, 6-benzyladenine purine (BAP).

Data collection and statistical analysis

Each experiment was repeated three times with 13 replicates. Data were analyzed by one way analysis of variance (ANOVA) and the difference between means were scored using Duncan's Multiple Range Test $P \leq 0.05$ (Duncan, 1955) on the statistical package of SPSS (Version 10).

Results

Selection of explants for callus initiation

Among the different explants used, leaves were not found to be suitable for callusing. Callus was obtained from nodal and internodal segments (Figure 1 and 2).

Screening for suitable culture media and hormonal combinations

Callus initiation was observed within 2 to 3 weeks after inoculation using different combinations of auxin (NAA) and cytokinin (BAP) in all the media however X medium required 4 to 5 weeks for callus initiation for both species (Figure 1 and 2). The highest rate of callus formation was obtained in MS medium supplemented with 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP in combination and 1 mg L^{-1} NAA and 0.5 mg L^{-1} BAP in combination for *S. apetala* (Table 1) and *S. maritima* (Table 2), respectively. We also tried to initiate callus on 2, 4-D alone or in combination with BAP but there were no response for *S. apetela.* The 2,4-D containing medium showed low callus initiation rate, slow growth and dark brown nature callus for *S. maritima* (Figure 2). The *in vitro* shoot induction was obtained from 1 mg L^{-1} BAP along with both the auxins i.e., NAA and 2,4-D at the concentrations of 1 mg L^{-1} (Figure 2, Table 2).

From the result it seems that MS medium is superior to other medium for

callus formations from both species (Figure 1 and 2) similar findings were reported by various author for tissue culture study of other mangrove species (Singh et al., 2004, Kawana and Sasamoto, 2008). Rao et al. (1998) prepared X medium for the cultivation of *Excoecaria agallocha* a mangrove species but it was not found to be suitable for callus initiation from both plants.

Table 1. Rate of callus initiation of *S. apetala* in MS medium at different concentrations of plant hormones (mg/L).

NAA	BAP	% of callus response	Nature of callus
1.5	**0.5**	**64.09±2.56^{a}**	Hard, Compact, Deep brown
	1.0	51.27±5.12^{abcd}	Hard, Compact, Deep brown
	1.5	41.02±2.56^{cdef}	Hard, Compact, Deep brown
2.0	0.5	32.04±10.01^{ef}	Hard, Compact, Deep brown
	1.0	28.20±5.13^{f}	Hard, Compact, Deep brown
	1.5	41.02±6.78^{cdef}	Hard, Compact, Deep brown
2.5	0.5	35.89±5.13^{def}	Hard, Compact, Deep brown
	1.0	43.58±5.12^{bcdef}	Hard, Compact, Deep brown
	1.5	58.97±5.13^{ab}	Hard, Compact, Deep brown
3.0	0.5	48.71±5.12^{abcde}	Hard, Compact, Deep brown
	1.0	48.71±2.56^{abcde}	Hard, Compact, Deep brown
	1.5	53.84±4.43^{abc}	Hard, Compact, Deep brown

Means sharing the same letter are not significantly different (P≤0.05) using Duncan's multiple range test.

Table 2 Hormonal combinations (mg/L) of *Suaeda maritima* callus on MS medium.

Medium	Response percentage	Nature of Callus
2,4-D+ BAP		
0+0	00.00±0.00^{g}	-
1.0+0	15.38±8.87^{fg}	Hard, Compact, deep brown
1.5+0	20.51±11.17^{ef}	Hard, Compact, deep brown
2.0+0	35.89±6.78^{cdef}	Hard, Compact, deep brown
1.0+0.5	48.71±2.56^{abc}	Hard, Compact, deep brown
1.5+0.5	46.15±4.43^{abcd}	Hard, Compact, deep brown
2.0+0.5	33.32±6.78^{cdef}	Hard, Compact, deep brown
1.0+1.0	**35.89±5.13^{cdef}**	Shoot formation
1.5+1.0	46.15±4.43^{abcd}	Hard, Compact, deep brown
2.0+1.0	25.63±2.56^{def}	Hard, Compact, deep brown
NAA+ BAP		
1.0+0	33.32±10.25^{cdef}	Compact, Granular, White
1.5+0	38.45±4.44^{bcde}	Compact, Granular, White
2.0+0	41.12±6.76^{bcde}	Compact, Granular, White
1.0+0.5	**64.09±6.78^{a}**	Compact, Granular, White
1.5+0.5	43.58±9.24^{abcd}	Compact, Granular, White
2.0+0.5	38.55±5.4.44^{bcde}	Compact, Granular, White
1.0+1.0	**58.97±5.13^{ab}**	Shoot formation
1.5+1.0	48.71±6.78^{abc}	Compact, Granular, White

Means sharing the same letter are not significantly different (P≤0.05) using Duncan's multiple range test.

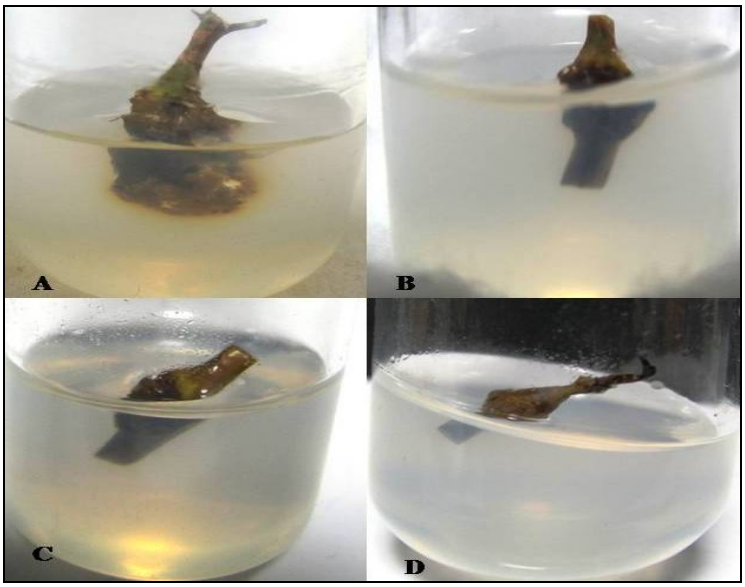

Figure 1. Callus initiation on different medium. A-Callus initiation on MS, B- Callus initiation on LS, C-Callus initiation on WPM, D- Callus initiation on X.

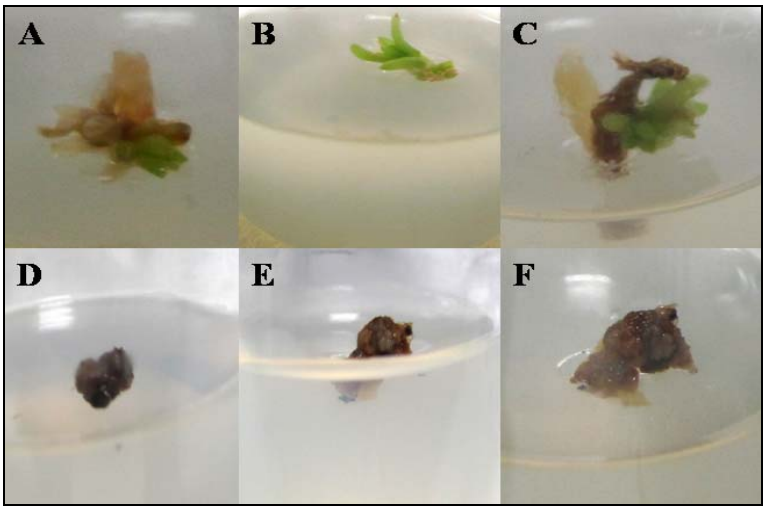

Figure 2. A, B, and C showing Shoot or leaf like structure formation of *Suaeda maritima* on MS medium, D, E and F showing deep brown callus formation of that species on same medium.

Prevention of browning problem in medium

Generally media browning is caused by the secretion of phenolic compounds (Roy and Sarkar, 1991) and its callus inhibition activity was discussed by Gill et al. (2004). Browning can be minimized by adding antioxidants or phenol absorbents into medium (Romano and Loucao, 1992) or by transferring explants into new culture media at regular intervals according to some literature (Romano and Loucao, 1992; Altan et al., 2010). However observation revealed during this experiment that addition of extra compounds made the medium complex which interfered with callusing and in latter case the process became time consuming and laborious. So keeping these constraints in mind cultures were kept in dark initially for 7 days and yielded satisfactory result for both the species.

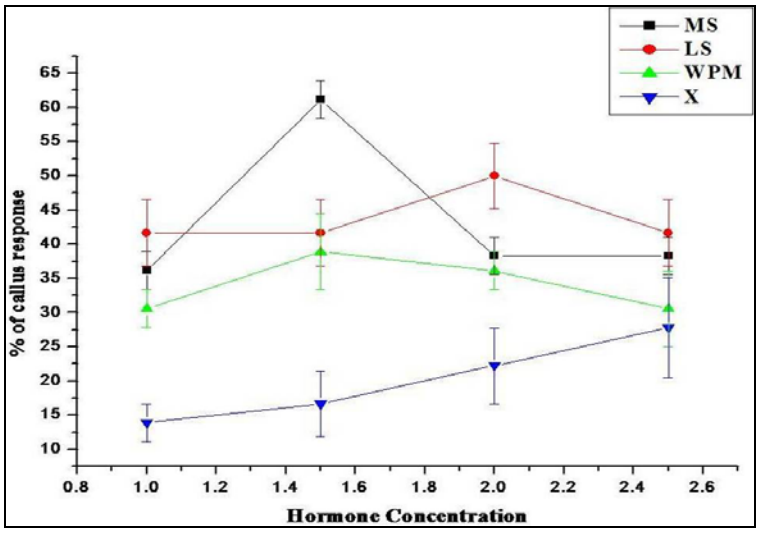

Figure 3. Callus initiation rate of *Sonneratia apetala* in four different medium under four constant hormone concentrations (mg.L^{-1}).

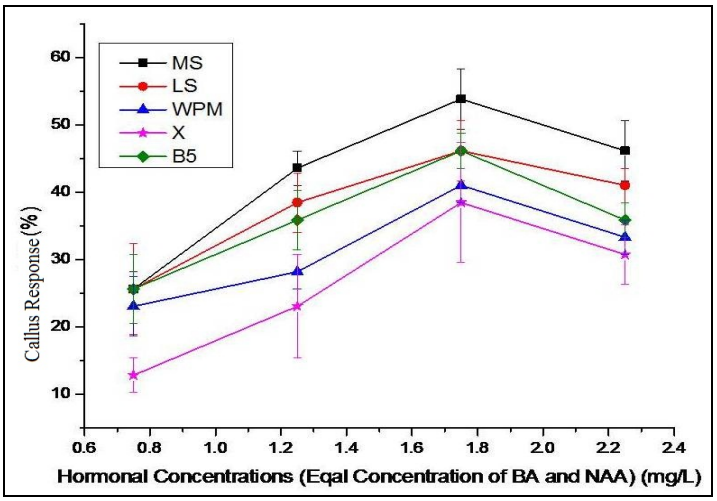

Figure 4 Callus initiation rate of *Suaeda maritima* in five different medium under four constant hormone concentrations (mg.L^{-1}).

Effect of salt concentration on callus initiation

The most important characteristic of mangrove plants is their salt tolerance. We examined the effects of the NaCI concentration on the callus initiation rate. The effects of NaCI concentration on the callus initiation were compared 60 days after initiation (Figure 3). From this study it was found that the herb mangrove species *Suaeda maritima* could gave callus well in 120 mM NaCl (Figure 5). *Sonneratia apetala* gave highest callus response at the concentration of 80 mM NaCl (Figure 6).

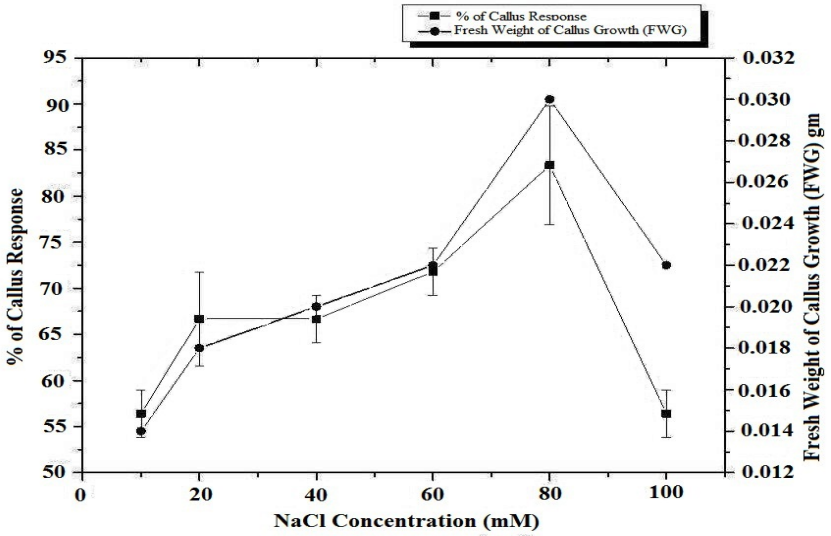

Figure 5. Effect of salt concentration on callus initiation and growth in MS medium using 1.5 mg.L-^{1} and 0.5 mg.L^{-1} NAA and BAP respectively with nodal and intermodal segments of *Sonneratia apetala* in MS medium.

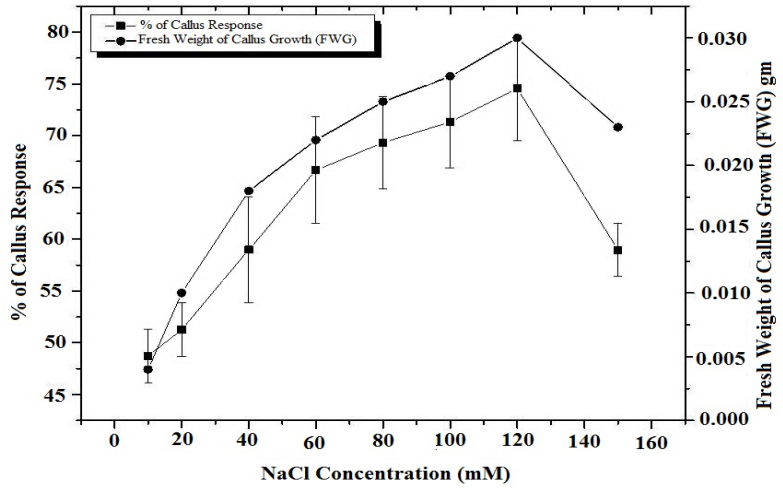

Figure 6. Effect of salt concentration on callus initiation and growth in MS medium using 1 mg.L^{-1} NAA and 0.5 mg.L-^{1} Bap with nodal and intermodal segments of *S. maritima*.

Effect of salt concentration on callus growth

NaCl effect, on callus growth was determined after 60 days of inoculation. From this experiment it was noticed that the effective NaCl concentration that gave best callus imitation response was also find to be best for callus growth for each species in this study (Figure 5 and 6).

Seasonal effect on callus formation

This investigation was carried out in different seasons viz., monsoon (July-September), after monsoon (November-February) and before monsoon (March-June) to check the seasonal effect for callus formation. From this experiment it was found that for callus culture of this two

species rainy season was best time (Figure 7 and 8) as compared to other seasons which generally showed explants dormancy and excretion of phenolic compounds vigorously.

Discussions

It is well developed fact that mangrove callus and suspension cultures are useful in stress related physiological studies (Yasumoto et al., 1999, Kura-Hotta et al., 2001). The present research has opened the way to study the salt tolerance as well as salinity tolerance mechanisms and genetic transformation of mangrove plants at cellular level. It was also noticed in this study that the callusing response of mangrove was very low and maximum callus initiation occurred at lower surface of explants as reported by Singh et al. (2004) and generally initiated callus showed slow growth than other territorial plants and it may be because of their fluctuating and extreme environment of their habitat.

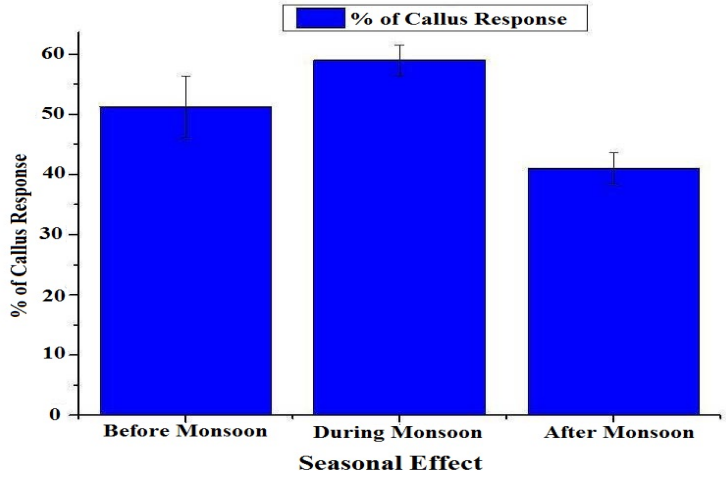

Figure 7. Effect of various seasons on callus initiation of *S. apetala* in MS medium.

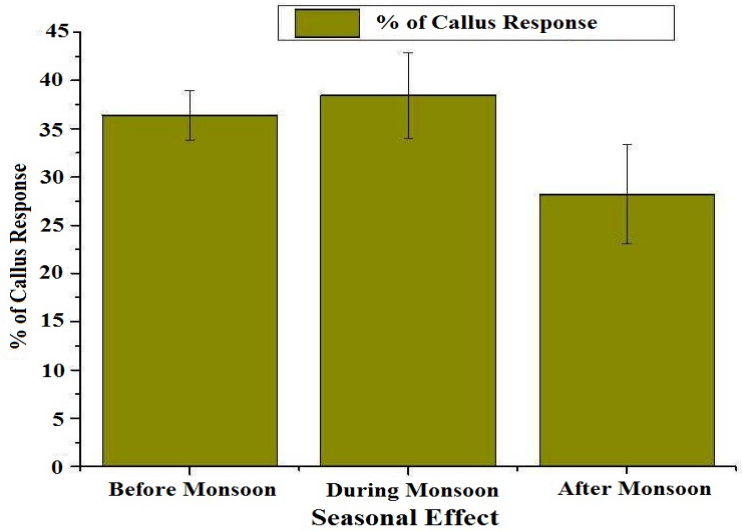

Figure 8. Effect of various seasons on callus initiation of *S. maritima* in MS medium.

In this experiment two different mangroves showed different pattern of NaCl tolerance. The literature studies also indicated that *S. maritima* could tolerate more salt concentrations than *S. apetala*. Khan et al. (2000) showed that this genus can grow naturally at 200-400 mM NaCl concentrations. It may be the reason that species is common on the side of creeks and river beds and plays a dominant role for its better adaptation to the higher degree of salinity and tidal influences (Naskar and Guha Bakshi, 1987). From this experiment it was found that for tissue culture of these species rainy season was best time as compared to other seasons which generally showed explants dormancy and excretion of phenolic compound vigorously. Many tree species which are collected during rainy season (active growth time) shows tremendous growth in *in vitro* conditions because physiological state of tissue of tree species varies due to variation of season (El-Morsey and Millet, 1996). During winter season the explants showed low viability i.e., dormant in nature and exude maximum phenolic compounds. This may be because the cytosolic ribosome contents are altered in winter metabolism at cellular level in tree species (Haggman, 1986).

The present investigation is a preliminary study for micropropagation and salinity tolerance of *S. apetala* and *S. maritima* as this is the first documentation of callus induction of these mangroves. However, the efficiency of plant regeneration from callus of mangrove plants is very low (Rao et al., 1998; Kura-Hotta et al., 2001; Al-Bahrany and Al-khayri, 2003; Hayashi et al., 2009) and it is also very difficult to establish cell culture systems in mangroves, except for few species (Kawana and Sasamoto, 2008). At present we were involved in production of micropropagated plantlets from this callus by transferring them into medium containing high concentration of cytokinin and low concentration of auxin and this may give promising result as the low auxin concentration turns off a certain number of genes which favour embryonic programme (Gill et al., 2004). The shortage or destruction of land in coastal region is another important factor for mangrove destruction (Komiyama et al., 1996). In this regard the present research clearly indicated that the species may be restored in low saline or devoid of saline land as callus is being extensively used for afforestation programmes (Ahuja, 1991). Callus culture give tools for genetic cell transformation by somaclonal variation, induced mutagenesis and genetic engineering which are not only much more rapid than conventional breeding but can also give rise to novel genes and genotypes rather than other traditional methods like mass selection, inbreeding and hybridization which is laborious and time consuming depending on environmental conditions and existing gene pool(s) for plant development (Ahmad et al., 2010). This study can give such type of opportunities for these important mangrove plants.

The results presented here give an insight into the development of *in vitro* investigation suitable for mangroves. Potentially, higher callus efficiency may be achieved through investigating medium components, hormones other than used in this study, sea salts other than examined in this study.

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Conflict of interests

The authors declare that they have no conflict of interests.

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