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Research article

Contribution of environmental factors on *in vitro* culture of an endangered and endemic mangroves *Heritiera fomes* Buch.-Ham. and *Bruguiera gymnorhiza* (L.) Lam.

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Abstract: Importance and destruction of mangroves have appeared in some recent surveys. So their restoration through tissue culture study is urgently required because *in vivo* propagation is plagued with unforeseen obstacles. This study describes for the first time *in vitro* approach for threatened species *Heritiera fomes* and *Bruguiera gymnorhiza* through callus. For initiation of callus modified MS medium was formulated for each species which correlated with soil conditions of Sundarban mangrove forest. For both species the auxin NAA, nodal or shoot tip explants and rainy season were found to be most suitable for callusing. NaCl at the concentration of 20 mM and 60 mM promoted growth for *H. fomes* and *B. gymnorhiza* callus respectively which was found to be comparative for their growth *in vivo* as in Sundarban. Histological study indicated morphogenicity of callus. Previous *in vitro* studies on mangroves were mostly based on the effect of variety of hormones and different sea salts. However this present study clearly indicates that the *in vitro* studies of mangroves not only depend on these factors but greatly influence by soil condition of their habitual environment, seasonal condition etc. From this study it seems that more and more *in vitro* studies of mangroves are possible if researchers focus on their habitual environmental conditions as many mangrove species remains recalcitrant for *in vitro* study. The present research clearly indicated that the species may be restored in low saline or non-saline land as land destruction is another vital reason for mangrove extinction.

Keywords: *Bruguiera gymnorhiza* - *Heritiera fomes* - Mangrove - Callus culture - Seasonal influence.

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INTRODUCTION

Mangrove ecosystems are found in tropical and subtropical muddy beaches worldwide. The importance and threats to mangrove ecosystem have been discussed by various authors (Al-Bahrany & Al-Khayri 2003, Ren *et al.* 2009). Because of their importance and destruction, mangroves have attracted attention for their conservation and preservation (Al-Bahrany & Al-Khayri 2003). Problems for restoration of mangroves arise mostly in the form of shortage of seeds or viviparous seedlings and the disturbed soil conditions (Ohnishi & Komiyama 1998, Feller *et al.* 2003). 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, detailed knowledge of the plant material and its requirements for callus initiation is necessary before mass *in vitro* propagation can

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become a reality. However scanty literature is available for few mangrove species because it is recalcitrant to tissue culture study (Al-Bahrany & Al-Khayri 2003, Fukomoto *et al.* 2004, Kawana & Sasamoto 2008). During *in vitro* culture of mangroves explants frequently turn brown or black and eventually die shortly after inoculation (Kawana & Sasamoto 2008, Arumugam & Panneerselvam 2012) as it excretes high tannin and phenol or phenolic compound. Besides, it is very difficult to maintain their habitual environment. Moreover, success of in vitro response does depend not only on the plant genotype but also is strongly affected by environmental conditions.

Preventing or avoiding microbial contamination is the basis of successful plant tissue cultures. Endogenous microbial contamination is known to be one of the most serious threats in plant tissue culture, especially in tropical species (Kneifel & Leonhardt 1992). Various literatures are available indicating the association of microbes and mangroves in root, bark, leaves etc (Gupta *et al.* 2009). Among them Uchino *et al.* (1984) identified some entophytic microbes in *Bruguiera gymnorhiza* species from its aerial parts.

Bruguiera gymnorhiza (L.) Lam. (Rhizophoraceae) is a multipurpose true mangrove species found in all over the world. The fruits and bark of the whole plant have been used for treating diarrhoea, fever, malaria, shingles and eye diseases (Naskar & Bakshi 1987, Bandaranayake 1998). The durable wood is used for making boat, house, poles, beams etc (Naskar & Bakshi 1987). Natural products of this plant have anti-tumor activity and antibacterial activity (Naskar & Bakshi 1987, Bandaranayake 2002).

Heritiera fomes Buch.-Ham. is a true mangrove tree from family Sterculiaceae, known as Sundari in Bengali, found mainly in Southeast Asia (Naskar & Bakshi 1987, Ali *et al.* 2011). The largest deltaic mangrove forest, Sundarban is derived from its Bengali name (Naskar & Bakshi 1987, Gopal & Chauhan 2006). The wood of this species is used for making boat, raft, house and charcoal (Naskar & Bakshi 1987, Ali *et al.* 2011). Besides, various parts of the tree are used as folk medicine for heart diseases, diabetes, pain, diarrhoea, skin disorders, hepatic disorders, and goiter (Ali *et al.* 2011). Ethanolic extract of stem bark showed antioxidant, lipoxygenase inhibitory, antihyperglycemic, antinociceptive effects and antibacterial activities (Wangensteen *et al.* 2009, Ali *et al.* 2011). Due to its medicinal and economical values and increasing environmental stress (Various salt concentrations, global warming etc), this species is being exploited indiscriminately since a very long time and it is considered as a threatened plant according to IUCN red list 2013 (Naskar & Bakshi 1987, Gopal & Chauhan 2006).

Keeping the deforestation, tissue culture problem and multiple utility of these two species in mind, we describe here a preliminary study of micropropagation through callus culture for preservation and production of micropropagated plant for future restoration of degraded mangrove forest areas. To our knowledge, this is the first report of callus initiation as well as *in vitro* investigation of *Heritiera fomes* and *Bruguiera gymnorhiza*.

MATERIALS AND METHODS

Plant materials

Different explants were collected from an respective 8–10 years old tree at various seasons all over the year from Gosaba region (88° 39′46" East and 22° 15′45" North) of Indian Sundarban Mangrove forest.

Preparation of explants

Firstly explants were washed with running tap water, then dipped in 2% teepol solution for 8 min and washed two to four times with sterile distilled water. The explants were then surface sterilized with 0.1–0.2% $HgCl_2$ (w/v) solution for different time duration to standardize the surface sterilization protocol. Thereafter they were dipped in 70% ethanol for 1–2 minute and finally they washed three times with sterile distilled water to remove any traces of the $HgCl_2$ and ethanol.

Culture Media and Conditions

For *Heritiera fomes* surface sterilized segments (1.0–1.5 cm long) were cultured on modified MS medium (Murashige & Skoog 1962) having complete omission of ammonium nitrate and half concentration of potassium nitrate with 3% (w/v) sucrose for callus initiation and further experiments. On the other hand for *Bruguiera gymnorhiza* surface sterilized segments (1.0–1.5 cm long) were cultured on another type of modified MS medium having slight modifications including thrice addition of micro salts and addition of beef extracts, yeast extracts and casein hydrolysates into medium at the concentration of 50 mg l^{-1} each with 3% (w/v) sucrose for callus initiation. For further experiments the sucrose concentration were altered to design the experiment. The

pH of the medium was adjusted to 5.6–5.8 before autoclaving. To eliminate browning problem polyvinyl pyrrolidone (PVP) was used to treat explants at the concentrations of 1gm l^{-1}. The explants initially were implanted vertically on the culture medium and plugged tightly with non-absorbent cotton. All the cultures were kept under cool fluorescent light (16 h photo period 40 µmol m^{-2} s^{-1} at 25±2 °C) and 60–70 % relative humidity. For this study 2, 4-D and NAA in combination with BAP were used. For induction of callus and determining the degree of salt tolerant, 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 planting. Callus growth was measured by increment in fresh weight. In the present study the callus growth was measured after 2 months of inoculation. Culture tube containing medium was weighed just before and after inoculation. The difference in weight gave the fresh weight of the inoculated tissue. In successive cultures the total amount of tissues were transferred to pre-weighed fresh medium. 50% excess was taken for sacrificing due to contamination though all the experiments were done under aseptic condition in laminar air flow chamber.

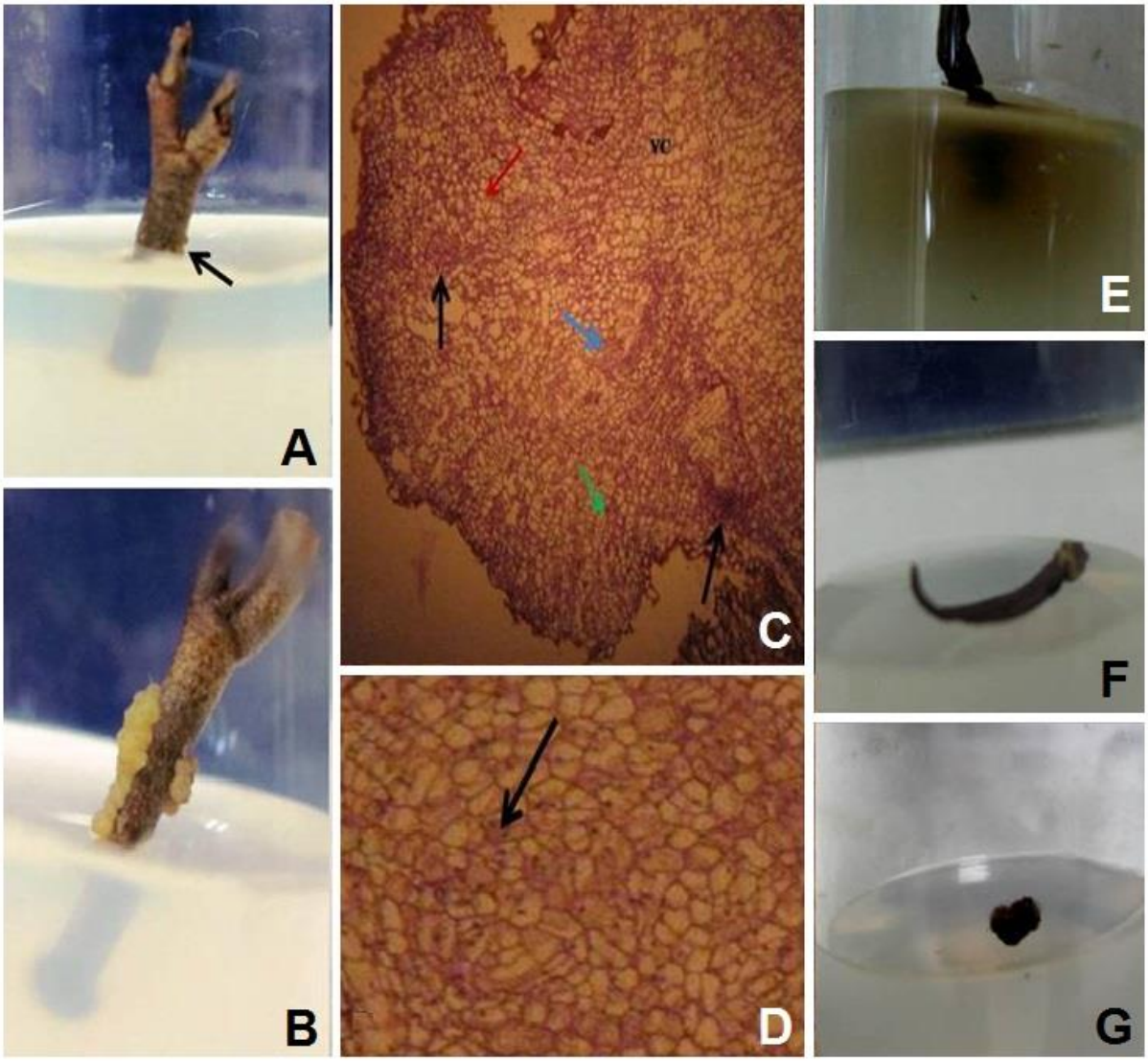

Figure 1. Callus initiation, *in vitro* sprouting of different explants, histological section and media discoloration of *Heritiera fomes*: **A,** Callus initiation site after 10 days of explants inoculation using NAA and BAP combination of *H. fomes*; **B,** Yellow and light brown callus formation after 2 weeks of inoculation of explants of *H. fomes*; **C,** General view of 3 week old protuberance produced at the proximal part of the explant (Vetical section), VC- Large vaculated cells, Black arrow indicates calli at inner region containing both small meristematic cells with highly-stained nucleus in mitotic cells zone (MCZ), Green arrow indicates embyogenic cells where red arrow indicates non embryogenic cells; **D,** Close view of embryogenic cells on callus where black arrow shows the embryogenic cells; **E,** Media discoloration caused by secretion of tannin or phenolic compounds after five days of inoculation; **F,** In vitro sprouting of leaf using NAA and BAP in combination; **G,** Deep brown small callus formation in combination of 2, 4-D and BAP.

Histological preparation

For histological studies, the explants were fixed in FAA (formaldehyde: acetic acid: ethanol; 100:50:50) solution for 10 days. The fixed samples were washed for 40 min, twice with double distilled water. After washing, the fixed samples were dehydrated through the ethanol series (30%, 50%, 70%, 80% and 90%) for 30 min at each stage. The samples were embedded in paraffin wax (melting point 58°C) and section vertically at 10µM thickness on a rotary microtome. The sections were mounted onto slides and allowed to dry for at least 10 min before staining. The specimens were stained with hematoxylin-eosin and counter stained with safranin solutions. The sections were then examined under phase contrast microscope.

Statistical Analysis

Experiments were set up in completely randomized design. Each experiment was repeated three times with 10 - 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 as it showed only *in vitro* sprouting for both the species. For *Heritiera fomes* callus was obtained from nodal and internodal segments only (Fig. 1) whereas for *Bruguiera gymnorhiza*, the callus formation was best observed with shoot tip (Fig. 2).

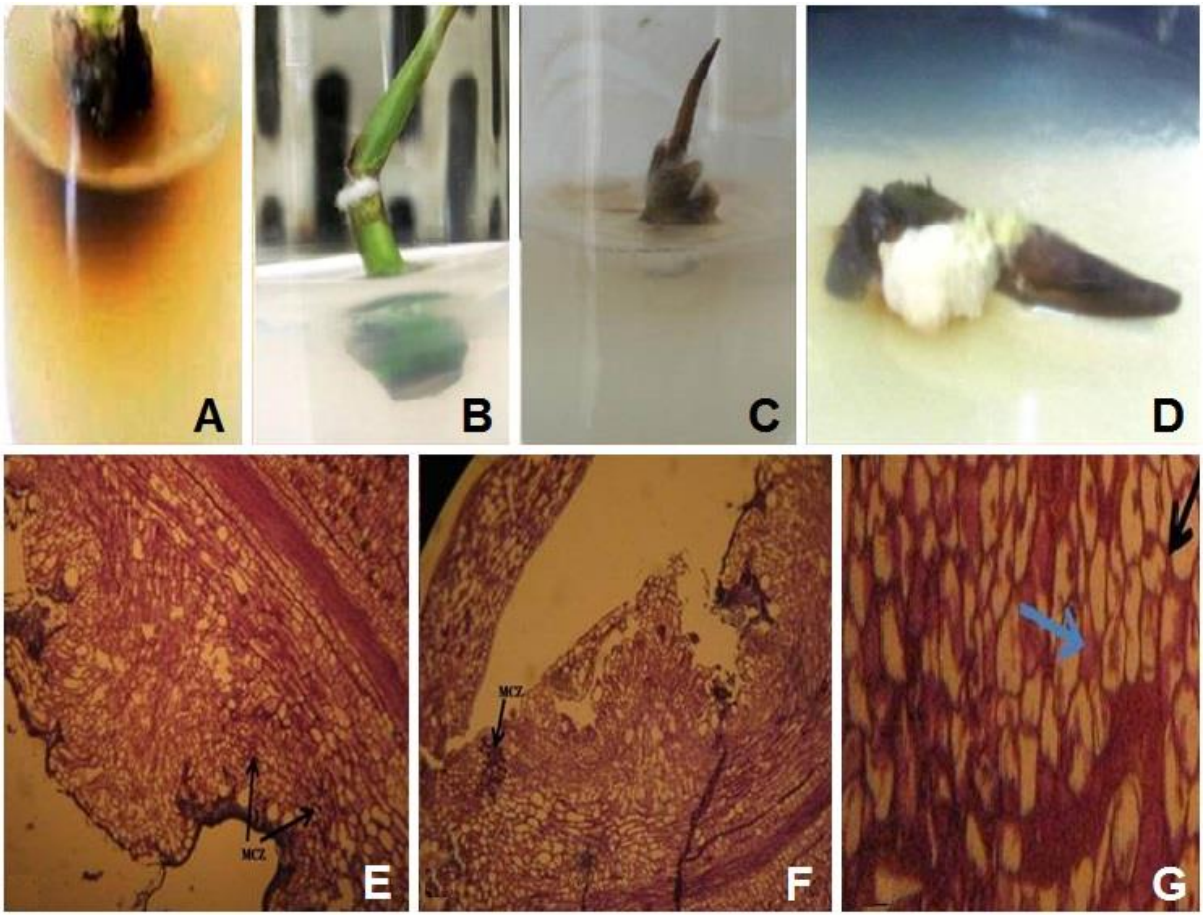

Figure 2. Callus initiation, histological section and media discoloration of *Bruguiera gymnorhiza*: **A,** Media browning by secretion of phenolic compounds of explants of *B. gymnorhiza*; **B,** Showing fungal contamination by explants; **C,** Showing bacterial contamination; **D,** White callus formation after 2 weeks of inoculation of explants; **E,** General view of 4-week-old protuberance produced at the proximal part of the explants (Vertical section) with wound callus formation; **F,** Showing extensive enlargement of scutellar parenchyma cells and the formation of peripheral pockets of dividing scutellar cells. Calli inner region containing both small meristematic cells with highly-stained nucleus in mitotic cells zone (MCZ) and vacuolated large cells; **G,** Higher magnification of the MCZ: Blue arrow indicates accumulation of soluble carbohydrate (sucrose and/or glucose) or tannin and Black arrow indicates 4 thick-walled proembryonic cells.

Standardisation of surface sterilization protocol for explants

Approximately 70% of successful sterilization was achieved from 0.1% $HgCl_2$ (w/v) solution treatment for 10- 15 minute incubation period for *H. fomes*. However for surface sterilization of *B. gymnorhiza* 0.2% $HgCl_2$ (w/v) solution treatment for 15-20 minute incubation period were required (Table 1) for efficient sterilization. Although initially some other treatments showed promising result however after 2 to 3 weeks of incubation, some cultures that had showed no contamination was found to be contaminated (Fig. 2).

Table 1. The effect of different concentration of $HgCl_2$ solution for different periods of time for removal of microbial contamination for *Bruguiera gymnorhiza*.

Percentage of $HgCl_2$ Solution (w/v)	**Incubation Period (minutes)**	**Aseptic inoculation (%)**
0.10	10	5.58 ± 0.00^{d}
0.10	15	7.68 ± 4.43^{cd}
0.10	20	30.25 ± 5.12^{cd}
0.15	10	25.38 ± 4.43^{cd}
0.15	15	30.50 ± 2.56^{c}
0.15	20	51.02 ± 2.56^{b}
0.20	10	46.15 ± 7.69^{b}
0.20	**15**	**76.66 ± 6.78^{a}**
0.20	**20**	**76.66 ± 6.78^{a}**

Note: Values in the last column are Mean ± SE of Mean followed by the letters within the column indicating the level of significance at P≤0.05 by Duncan's Multiple Range Test (same letter within the column of the treatments indicates the absence of difference; different letters indicate the significant difference; and combination of letters indicate no significant difference).

Elimination of browning problem

Generally media browning is caused by the secretion of phenolic compounds and its callus inhibition activity was discussed by various authors (Gill *et al.* 2004). Before inoculation and after sterilization the explants were treated with PVP solution at the concentration of 1gm/L (W/V) for 45 min and kept the culture after inoculation was kept in darkness for seven days which was found to remove media discoloration sufficiently (Figs. 1 & 2).

Effect of sucrose on callus initiation

To check the effect of sucrose concentration on callus initiation, we incubated the cultures on different sucrose concentration containing medium like 1%, 2%, and 3%. Among them, 3% (w/v) sucrose containing medium gave best results for *H. fomes* while for *B. gymnorhiza* gave best result on 2% (w/v) sucrose containing (Fig. 3).

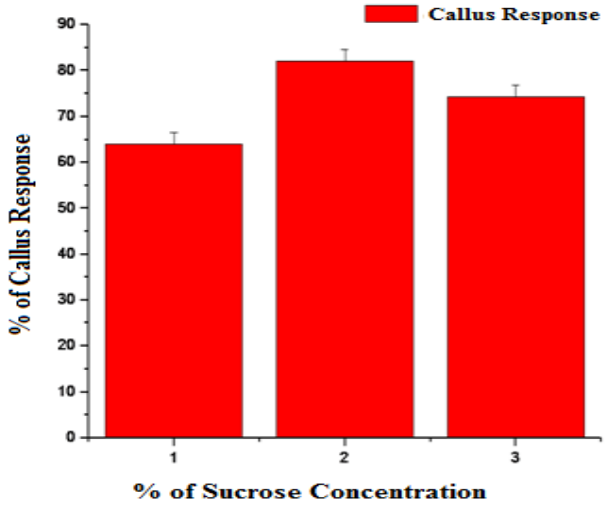

Figure 3. Effect of sucrose concentration on callus response of *Bruguiera gymnorhiza.*

Callus initiation rate between different hormone concentrations

For both the species, callus initiation was observed within 2 to 3 weeks after inoculation. Higher rates of callus initiation were obtained at combinations of 2 mg l^{-1} and 0.5 mg l^{-1} BAP and NAA respectively for *H.*

fomes (Table 2) and for *B. gymnorhiza* it was 1.5 mg l^{-1} and 0.5 mg l^{-1} NAA and BAP respectively (Table 3). Before fixing in FAA for histological preparation, the *B. gymnorhiza* callus showed white, compact and almost smooth nature while the *H. fomes* callus was yellow or light brown in colour and compact and nodular in nature in respective hormone combination (Figs. 1 & 2). It was observed that 2, 4-D failed to initiate callus on *B. gymnorhiza*. The action of 2, 4-D on callus initiation showed slow growth rate for *H. fomes* (Table 3), the callus being deep brown in colour (Fig. 1) and dormant in nature. From these result it seems that 2, 4-D was not suitable for callus culture for these species. During this study it was observed that the rate of callusing response of mangroves were very low 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. Similar results were obtained by Mimura *et al.* (1997).

Table 2. Rate of callus initiation of *Heritiera fomes* in modified MS medium at different concentrations of plant hormones (mg l^{-1}).

Hormone Concentrations (mg l^{-1})			Callus response (%)	Nature of Callus
NAA	**2, 4-D**	**BAP**		
0	0	0	0.00 ± 0.00^{h}	-
0.5	-	0.5	17.94 ± 5.12^{defg}	Soft, Granular, Yellow
		1.0	23.07 ± 6.28^{def}	Soft, Granular, Yellow
		2.0	$\mathbf{69.25\pm4.44^{a}}$	Soft, Granular, Yellow
1.0	-	0.5	23.22 ± 4.30^{def}	Soft, Granular, Yellow
		1.0	25.63 ± 2.56^{cde}	Soft, Granular, Yellow
		2.0	15.38 ± 4.43^{efgh}	Soft, Granular, Yellow
1.5	-	0.5	28.19 ± 2.56^{cde}	Soft, Granular, Yellow
		1.0	41.02 ± 6.78^{bc}	Soft, Granular, Yellow
		2.0	20.50 ± 5.12^{def}	Soft, Granular, Yellow
2.0	-	0.5	33.32 ± 6.78^{cd}	Soft, Granular, Yellow
		1.0	17.94 ± 2.56^{defg}	Soft, Granular, Yellow
		2.0	28.20 ± 6.78^{cde}	Soft, Granular, Yellow
2.5	-	0.5	28.20 ± 5.13^{cde}	Soft, Granular, Yellow
		1.0	51.27 ± 2.56^{b}	Soft, Granular, Yellow
		2.0	23.07 ± 4.43^{def}	Soft, Granular, Yellow
-	0.5	0.5	0.00 ± 0.00^{h}	-
		1.0	7.69 ± 4.43^{fgh}	Hard, Deep brown
		2.0	2.56 ± 2.56^{gh}	Hard, Deep brown
-	1.0	0.5	17.94 ± 6.78^{defg}	Hard, Deep brown
		1.0	23.07 ± 4.43^{def}	Hard, Deep brown
		2.0	15.38 ± 4.43^{efgh}	Hard, Deep brown
-	1.5	0.5	28.20 ± 5.13^{cde}	Hard, Deep brown
		1.0	41.02 ± 6.78^{bc}	Hard, Deep brown
		2.0	23.07 ± 4.43^{def}	Hard, Deep brown
-	2.0	0.5	20.51 ± 9.24^{def}	Hard, Deep brown
		1.0	28.20 ± 5.13^{cde}	Hard, Deep brown
		2.0	20.50 ± 6.78^{def}	Hard, Deep brown
-	2.5	0.5	19.22 ± 3.84^{defg}	Hard, Deep brown
		1.0	28.20 ± 5.13^{cde}	Hard, Deep brown
		2.0	23.22 ± 4.30^{def}	Hard, Deep brown

Note: Values in the second last column are Mean ± SE of Mean followed by the letters within the column indicating the level of significance at $P\leq0.05$ by Duncan's Multiple Range Test (same letter within the column of the respective hormone indicates the absence of difference; different letters indicate the significant difference; and combination of letters indicate no significant difference).

We also tried to initiate callus on different media like MS, Woody Plant medium (WPM, Lloyd & McCown 1981), Linsmaier and Skoog (LS) medium (Linsmaier & Skoog 1965), X medium (Rao *et al.* 1998) and Amino Acid (AA) medium (Thompson *et al.* 1986) but there were no response found for these species.

Histological study of initiated callus

The histological study showed the production of protuberance at the proximal part of the explant (horizontal section) of *H. fomes*. This study of both species showed the formation of mitotic cells zone (MCZ) i.e., parenchymatous cells with nucleus and dense cytoplasm dividing actively (Figs. 1& 2). It also noted that in the intercellular spaces of callus tissue there was a more or less dense fibrillar or reticular network which is complexes of polysaccharide polymers having a microfibrillar network texture (Fig. 2) or accumulation of tannin. *B. gymnorhiza* callus also indicated extensive enlargement of scutellar parenchyma cells and the formation of peripheral pockets of dividing scutellar cells. Two types of cells present in callus might be distinguished: embryogenic and nonembryogenic (Figs. 1 & 2).

Table 3. Rate of callus initiation of *Bruguiera gymnorhiza* in modified MS medium at different concentrations of plant hormones (mg l^{-1}).

NAA (mg l^{-1})	BAP (mg l^{-1})	Callus Response (%)	Nature of Callus
0	0	0.00 ± 0.00^{f}	-
0.5	0.5	12.81 ± 5.12^{e}	Hard, Compact, White
	1.0	5.12 ± 2.56^{bcef}	Hard, Compact, White
1.0	0.5	53.84 ± 7.69^{b}	Hard, Compact, White
	1.0	28.20 ± 5.13^{d}	Hard, Compact, White
1.5	0.5	**74.35 ± 2.56^{a}**	Hard, Compact, White
	1.0	56.40 ± 5.12^{b}	Hard, Compact, White
2.0	0.5	56.40 ± 2.56^{b}	Hard, Compact, White
	1.0	38.88 ± 2.77^{cd}	Hard, Compact, White
2.5	0.5	51.27 ± 2.56^{bc}	Hard, Compact, White
	1.0	43.58 ± 2.56^{bc}	Hard, Compact, White

Note: Values in the second last column are Mean ± SE of Mean followed by the letters within the column indicating the level of significance at P≤0.05 by Duncan's Multiple Range Test (same letter within the column of the respective hormone indicates the absence of difference; different letters indicate the significant difference; and combination of letters indicate no significant difference).

Effect of salt concentration on callus initiation and growth

Bruguiera gymnorhiza gave maximum callus response and growth at the concentrations of 60 mM NaCl (Fig. 4) concentrations whereas endangered and endemic *H. fomes* gave best callus response and growth at the concentration of 20 mM NaCl (Fig. 5).

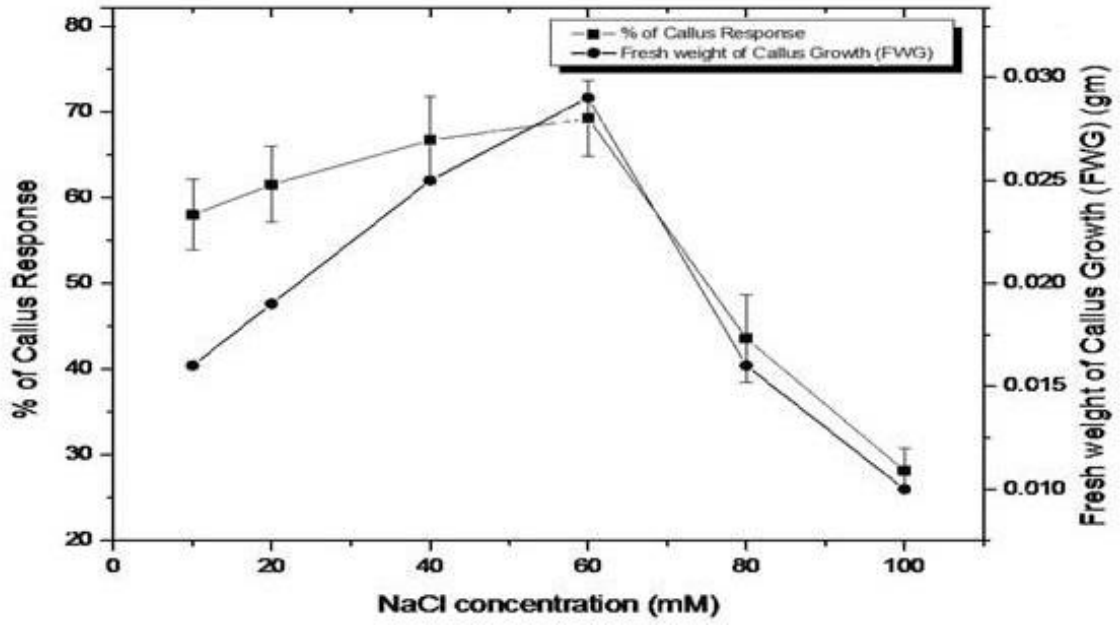

Figure 4. Effect of salt concentration on callus initiation and growth of *Bruguiera gymnorhiza.*

Seasonal effect on callus formation

This investigation was carried out in different seasons viz., rainy season, winter season and summer season 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 (Fig. 6) as compared to other seasons which generally showed explants dormancy and excretion of phenolic compound vigorously.

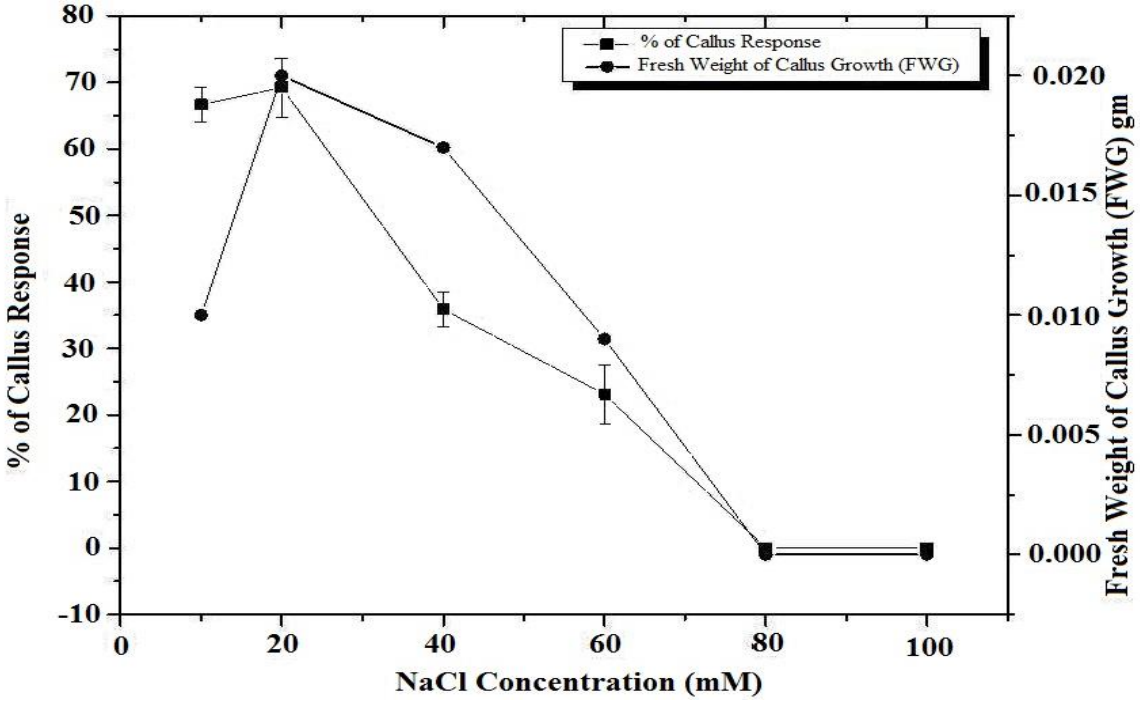

Figure 5. Effect of salt concentration on callus initiation and growth of *Heritiera fomes.*

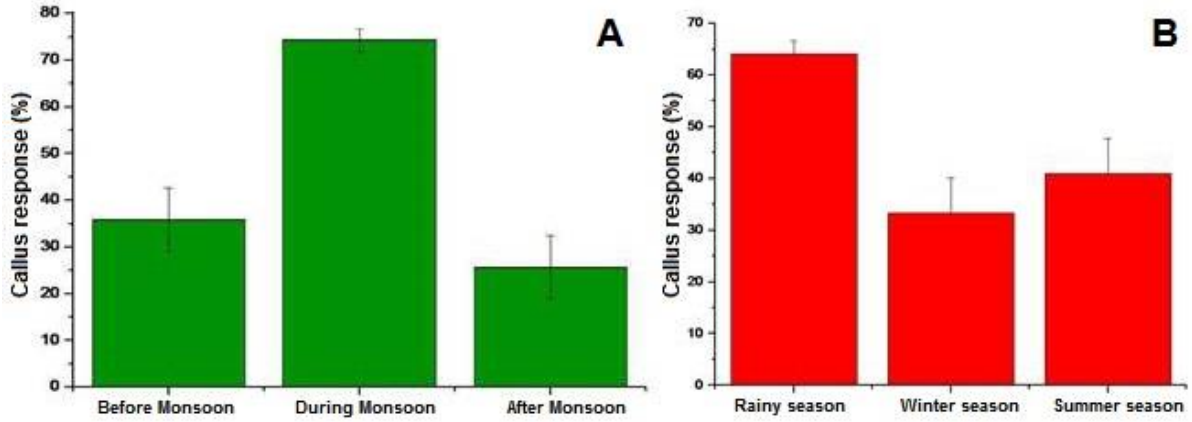

Figure 6. Effect of various seasons on callus initiation in modified MS medium: **A,** *Bruguiera gymnorhiza*; **B,** *Heritiera fomes.*

DISCUSSIONS

Literature studies indicated that the leaves of mangrove species are excellent source for callusing or cell culture studies (Mimura *et al.* 1997, Hayashi *et al.* 2009). However in this study both the species failed to produce callus from leaf. They gave callusing response either on shoot tip or nodal and intermodal segments which was not previously reported for any mangrove species. This may be due to the selection of explants, their size, age and ecological factors, which greatly influence the success of the *in vitro* culture which varies widely from plant to plant (George 1993).

In this study mercuric chloride was used as surface sterilant as it is the mostly used surface sterilant for tissue culture. Here for surface sterilization of *Bruguiera gymnorhiza* 0.2% $HgCl_2$ (w/v) solution treatment for

15- 20 minute incubation periods was required.

In vitro response of most of the mangrove species till now reported maximum were done with MS medium *viz* Yasumoto *et al.* (1999) reported for *Sonneratia alba*; Al-Bahrany & Al-Khayri (2003) reported for *Avicennia marina;* Ogita *et al.* (2004) reported for *Kandelia candel*; Kawana & Sasamoto (2008) reported for *Sonneratia alba* and *Bruguiera sexangula;* Hayashi *et al.* (2009) reported for *Avicennia alba, Avicennia marina, Sonneratia alba* and *Bruguiera sexangula.* In this present work for callus culture of *Bruguiera gymnorhiza* and *Heritiera fomes,* the MS medium was modified. Similar findings of use of modified MS medium for cultivation of mangroves were also reported by Rao *et al.* (1998), formulated a new medium named 'X' for *Excoecaria agallocha*; Vartak & Shindikar (2008) for *Bruguiera cylindrica*. Besides for optimum growth of *B. gymnorhiza* yeast extract and casein hydrolysates were added. It may happen because mangroves are unique in their physiological adaptations. They can tolerate various nutrient statuses, water logging and various levels of sea salts (Feller *et al.* 2002). Similarly plant tissue culture depends on media composition with special emphasis to growth regulators, carbon source and organic additives, the genotype and the source of explants. In this study we found that *B. gymnorhiza* gave no callus response in MS media but when the media was formulated with thrice micro nutrients and addition of organic substances *i.e.,* yeast extracts and casein hydrolysate the callus initiation occurred. It may have happened because they are naturally adapted to higher micronutrients and organic matter, particularly for Sundarban forest (Naskar & GuhaBakshi 1987, Ramanathan *et al.* 2008). In Sundarban mangrove region sulphates are higher (Naskar & GuhaBakshi 1987, Ramanathan *et al.* 2008). In our study, addition of thrice concentration of micro nutrient was actually the addition of higher sulphates as micronutrient to MS. It is well known that in plant tissue culture media, casein hydrolysate is a rich source of different amino acids (Shahsavari 2010, Talapatra & Raychaudhuri 2012). Addition of casein hydrolysate in the medium is required because Mimura *et al.* (1997) showed that the Amino Acid medium (Thompson *et al.* 1986) enhanced the rate of callus initiation of *Bruguiera sexangula* which is another species of the *Bruguiera* genus. From this study and from the study of Mimura *et al.* (1997) it seems that somehow amino acids play a crucial role for *in vitro* response particularly for this genus and casein hydrolysate was found to be fit for this type of study.

The physiological state of the donor plants on growth depends on environment, which affects the response of explants under *in vitro* conditions (Jahne & Lorz 1995). The MS medium presented here for *H. fomes* had low nitrogen level as compared to other plant medium which supports the low nitrogen level in mangrove region (Feller *et al.* 2002, Feller *et al.* 2003) as well as Sundarban mangrove forest as there is no humus deposition in the soil (Naskar & Bakshi 1987, Ramanathan *et al.* 2008). So it seems that mangrove species can grow *in vivo* in low level of nitrogen, which was also retained in tissue culture conditions. Similarly *in vitro* response to low level of nitrogen was also reported by Mimura *et al.* (1997), Rao *et al.* (1998) and Arumugam & Panneerselvam (2012). The complete omission of ammonium nitrate may be essential as it has toxic effect in many higher plant species which inhibit plant growth (Britto & Kronzucker 2002, Shanjani 2003).

In this study *B. gymnorhiza* respond and grew better in 2% sucrose concentration in respective medium. In mangrove ecosystem carbon source are varied and in Sundarban the parcentage of organic carbon source was too low (Naskar & GuhaBakshi 1987). In the present study with *B. gymnorhiza* we found that callus initiation rate was highest in 2% (w/v) sucrose containing medium which correlate with nutrient status of Sundarban mangrove ecosystem.

From this experiment it was found that the NAA could alone or in combination with BAP initiate callus from mangrove species. However in case of 2,4-D it failed to initiate callus alone or in combination with with BAP from *Bruguiera gymnorhiza.* It may be because recent study showed that 2,4-D has toxic effect on plants i.e., it alters the chlorophyll, protein and phenol content (Peixoto *et al.* 2007). Generally high concentrations of cytokinin and low concentrations of auxins favour shoot response. Here in case of *Heritiera fomes* this type of ratio too favoured callus initiation from this species.

In this experiment two different mangroves showed different pattern of NaCl tolerance. Based on this study it was found that the *Bruguiera gymnorhiza* gave best callus initiation and growth at 60 mM NaCl concentration. Mimura *et al.* (1997) found that seedling callus of *Bruguiera sexangula* gave highest growth at 100 mM NaCl. Sundarban this species is very 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 & GuhaBakshi 1987). *Heritiera fomes* calli showed better response and growth at 20 mM NaCl concentration.

Hossain *et al.* (2014) showed that *Heritiera fomes* prefers extremely low saline condition for their survival and growth. Recent studies on palynological evidence and salinity influences have clearly showed that *Heritiera fomes* has declined relatively recently as the salinity has increased in Indian Sundarban regions which are also retained in this experiment (Naskar & GuhaBakshi 1987, Gopal & Chauhan 2006, Mitra & Banerjee 2010). Under natural conditions, mangroves exhibit clear tolerance of salts differences among species. Findings of the present study are comparable with naturally relative tolerance levels of two different mangrove species.

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 & Millet 1996). During winter season the explants showed low viability *i.e.*, dormant in nature and exuded maximum phenolic compounds. This may be because the cytosolic ribosome contents are altered in winter metabolism at cellular level in tree species (Haggman 1986).

Land destruction in coastal region is another important factor for mangrove extinction (Ohnishi & Komiyama 1998). In Indian Sundarban region approximately 50% of land has been destroyed for human habitation and settlement, agricultural development and brackish water fisheries (Ramanathan *et al.* 2008). The present study clearly indicates that these species may be restored in low saline or none of saline land as callus as it is being extensively used for afforestation programmes (Ahuja 1991) and tapping useful compounds from plants. The present investigation is a primilary study for micropropagation of mangrove species as most mangrove species remain very hard to be established through cell culture systems or callus culture (Kawana & Sasamoto 2008). The totipotent character of plant cells and tissues can be expressed by their ability to regenerate into plants via embryogenesis or organogenesis and for this histological study of callus is very much needed. Both processes lead to *in vitro* regeneration and are a major prerequisite for genetic transformation study. However, the widespread application of gene transfer techniques for crop improvement cannot be successfully achieved if the processes leading to morphogenesis are not well understood. 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 thus provide opportunities of micropropagation of these multipurpose mangrove plants.

According to UNESCO there are approximately 50 different types of true mangrove species are found worldwide. Among them less than 10 true mangrove species were reported for *in vitro* study. It seems from this study that different environmental condition or stress which promotes the growth of mangroves *in vivo* greatly influences the *in vitro* culture of mangroves. Previous *in vitro* studies on mangroves were mostly based on the effect of variety of hormones with their different concentration taken and different effect of sea salts taken with their different concentration. However this present study clearly indicates that the *in vitro* studies of mangroves not only depend on variety hormones or different sea salts but greatly influence by soil condition of their habitual environment, seasonal condition etc. From this study it also seems that more and more *in vitro* studies of mangroves are possible if researchers focus on their habitual environmental conditions. The results presented here give an insight into the *in vitro* studies suitable for mangrove species which is correlated with various environmental factors of mangrove ecosystems. Potentially, higher callus efficiency may be achieved through investigating medium components other than this study, hormones other than used in this study and use of various sea salts other than used in this study.

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