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Rajneesh Kumar
Genetics & Tree Improvement Division, Institute of Forest Productivity, Lalgutwa, Ranchi, Jharkhand, India

Animesh Sinha
Genetics & Tree Improvement Division, Institute of Forest Productivity, Lalgutwa, Ranchi, Jharkhand, India

Pradip Kumar Sarkar
ICAR-Research Complex for Eastern Region, Farming System Research Centre for Hill and Plateau Region, Plandu, Ranchi, Jharkhand, India

Jai Kumar
Department of Forestry, Birsa Agricultural University, Kanke Road, Ranchi, Jharkhand, India

Corresponding Author:
Rajneesh Kumar
Genetics & Tree Improvement Division, Institute of Forest Productivity, Lalgutwa, Ranchi, Jharkhand, India

In vitro callus induction from nodal and leaf explants of *Heritiera fomes* Buch - Ham: An endangered mangrove

Rajneesh Kumar, Animesh Sinha, Pradip Kumar Sarkar and Jai Kumar

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Abstract
Heritiera fomes (an important threatened mangrove) belongs to the family Sterculiaceae, is well known to be used as folk medicine for curing heart diseases, diabetes, pain, diarrhoea, skin disorders and hepatic disorders. Moreover, the ethanolic extract of stem bark had been reported to have the properties like antioxidant, lipoxygenase inhibitory, antihyperglycemic, antinociceptive effects and antibacterial activities. Being an important species, its multiplication is must. But in general, its multiplication through seeds is difficult due to having problems of seed collection, habitat type, seed viability and short storage life of seeds. In contrary, *in vitro* micropropagation could be one of the options. Hence, a study was conducted to investigate the callus induction from nodal and leaf explants of *H. fomes* under *in vitro* micropropagation for development of a protocol for future work. The result revealed that, the MS (Murashige and Skoog) medium containing BAP (2.0 mg/l) + NAA (1.0 mg/l) was found to be the most suitable for callus induction and mass of callus (*i.e.*, fresh weight and dry weight) from nodal explants and the MS medium containing BAP (2.0 mg/l) + NAA (2.0 mg/l) was best for the callus induction and mass of callus (*i.e.*, fresh weight and dry weight) from leaf explants of *H. fomes.*

Keywords: *Heritiera fomes*, mangrove, callus, micropropagation, *in vitro*, fresh weight, dry weight, BAP, kinetin, NAA and 2,4-D

Introduction
Mangroves are the woody plants that naturally grow at the border between land and sea in tropical and sub-tropical climate under extreme environmental conditions such as high salinity, extreme tides, strong winds, high temperature and soil conditions like muddy, anaerobic soils [1] and regular flooding [2]. The importance and threats to mangrove ecosystem had already been reported by various authors [3, 4]. Because of their great importance and destruction of their habitat, the species had attracted attention for their conservation and management [3]. Mangroves present particular problems of seed collection due to the nature of the habitat, seed viability and short storage life of seeds. The survival of mangrove seedling in the field is also generally poor because of the dynamic nature of their habitat, thus replacement planting often needs to be undertaken for up to three years [5].
Among all the mangrove species, *Heritiera fomes* Buch. -Hum is a true mangrove tree, belongs to the family *Sterculiaceae* and is known as Sundari in Bengali, mainly in Southeast Asia [6, 7]. The wood of this species is used for making boat, raft, house and charcoal [6, 7]. Besides, all the plant parts are used as folk medicine for curing heart disease, diabetes, pain, diarrhoea, skin disorders, hepatic disorders and goitre [7]. Due to its medicinal and economical values and increasing environmental stress (various salt concentrations, global warming, *etc*.), this species was being exploited indiscriminately since a very long time and it was considered as a threatened plant according to ICUN red list 2013 [6, 8].
Micropropagation is one of the most useful and widely used technologies in tree improvement programme. Sharp [9] reported that micropropagation may be useful for forest trees characterised by poor seed set, absence of uniform seed production, and seed prone to genetic damage or loss of viability during storage-features common in mangroves [10]. A good number of endangered and threatened species have been successfully regenerated using *in vitro* culture methods using nodal segments. Many studies reported that, mangrove species are recalcitrant to tissue culture studies [3, 11, 12]. During *in vitro* culture of mangrove plants frequently turn

brown or black and eventually die shortly after inoculation [12, 13], as it excretes high tannin and phenolic compound. Though, *in vitro* propagation in *Heritiera fomes* has not yet done and may the best alternative method for propagating of this species. With this background, a study was undertaken to investigate the callus induction from nodal and leaf explants of *H. fomes* under *in vitro* micropropagation for development of a protocol for future work.

Materials and Methods

Plant material

Seedlings of *Heritiera fomes* were collected from Sundarbans Mangrove forest of India and grown at the experimental garden of Institute of Forest Productivity campus, Ranchi during monsoon season of the year (since May-June, 2014), which served as the source of explants used in the study. The present study was carried out in the Tissue-culture laboratory of Institute of Forest Productivity, Ranchi (Jharkhand).

Apical leaf explants

The Apical leaf explants were cut into small pieces from the 2-3-year-old plant and washed with running tap water for 30 minutes. The explants were then washed with 0.1% Bavistin solution for 30 minutes and then surface sterilised with 0.1% (w/v) mercuric chloride for 3-7 minutes, followed by washing them 3-4 times with sterile double distilled water under laminar bench and inoculated on agar-solidified MS (Murashige and Skoog) medium supplemented with different concentrations of BAP and Kinetin alone and the combination of BAP with 2,4-D and NAA. The pH of the medium is adjusted to 5.6-5.8 before sterilization. Cultures were maintained at 25±2°C with a photoperiod.

Nodal segments

Nodal segment (1-2 cm) was excised from the growing tips of 2-3 old tree of *Heritiera fomes* used as explants and washed with running tap water for 30 minutes. The explants were then treated with 0.1% Bavistin solution for 30 minutes and then surface sterilised with 0.1% (w/v) mercuric chloride for 3-11 minutes, then washed 3-4 times with sterile double distilled water under laminar bench and inoculated on agar-solidified MS (Murashige and Skoog) medium supplemented with different concentrations of BAP and Kinetin alone and the combination of BAP with 2,4-D and NAA. The pH of the medium is adjusted to 5.6-5.8 before sterilization. Cultures were maintained at 25±2°C with a photoperiod.

Observations

In the present the percentage of callus initiation was recorded after one month of inoculation. Callus growth was measured by increment in fresh weight.

Statistical analysis

Data recorded, were analysed using Systat-12 software [14] for computation of descriptive statistics (*i.e.*, mean, standard deviation and critical difference). The Completely Randomized Design (CRD) was followed for statistical analysis.

Result and discussion

MS medium supplemented with different concentrations of 2,4-D, Kinetin, BAP and NAA, either alone or in combination were used for callus induction. Within six to ten days of culture, callus induction started in nodal explants and within fifteen to twenty days in leaf explants, when cultured on MS medium supplemented with BAP (2.0 mg/l) and NAA (1.0 mg/l) gives highest 80% of callus induction from nodal explants. Callus obtained from these explants was yellowish in colour and very soft in nature (Fig.1). The highest fresh weight and dry weight of callus in nodal explants were recorded as 717.33 ± 14.25 mg and 112.67 ± 6.96 mg, respectively (Table 1). The highest percentage of callus induction from leaf explants was recorded as 60% on the medium containing BAP (2.0 mg/l) + NAA (2.0 mg/l), highest fresh weight and dry weight of callus in leaf explants was 959.00 ± 5.86 mg and 220.33 ± 2.73 mg, respectively (Table 2). Moreover, the study showed a slow growth rate while compared with other territorial plants. Furthermore, only 15-20% callus formation from nodal explants was recorded when experimented and inoculated on different other media like WPM (Woody Plant Medium) and B5 (Gamborg) medium. Many literatures revealed that, leaves of Mangrove species are excellent source for callusing [15, 16]. Moreover, callusing only from nodal explants of *H. fomes* was reported by Kader *et al.* [17].

However, the present study revealed that, callusing from leaf explants of *H. fomes* under *in vitro*, which was not reported earlier, can now form the base and the developed protocol can be used further by the researchers or academicians to carry forward the work for any future studies.

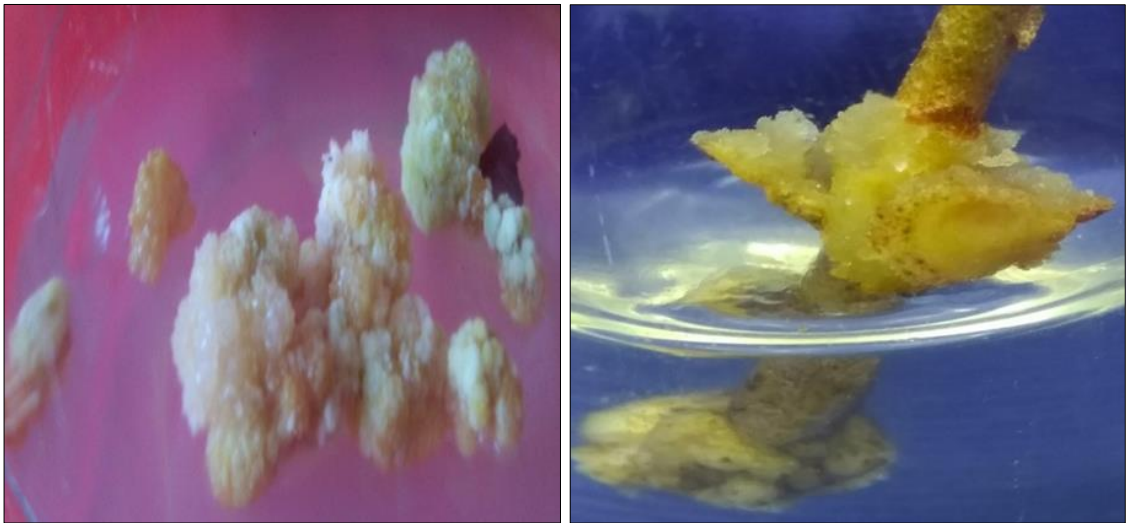

Fig 1: A, callus initiation in leaf explants of *H. fomes* after 20 days; B, callus induction in nodal explants of *H. fomes* after 10 days.

Table 1: Effect of growth regulators on induction of callus and biomass production in *H. fomes* from nodal explants

Hormone concentration (mg/l)	% of callus induction	Days for callus imitation	Fresh weight of callus (mg)	Dry weight of callus (mg)
Control	15.00 ± 2.89	24	67.67 ± 1.45	12.67 ± 1.45
BAP 0.5	20.00 ± 0.58	21	89.00 ± 4.58	16.00 ± 3.46
BAP 1.0	30.00 ± 2.89	17	136.67 ± 7.69	19.33 ± 2.33
BAP 2.0	43.33 ± 0.88	14	158.00 ± 1.73	21.67 ± 2.03
BAP 3.0	43.33 ± 4.41	11	142.33 ± 1.45	20.33 ± 0.88
KIN 0.5	20.00 ± 2.89	19	122.33 ± 2.91	16.00 ± 0.58
KIN 1.0	25.00 ± 5.00	21	113.67 ± 2.07	22.00 ± 1.16
KIN 2.0	38.33 ± 3.33	18	150.33 ± 6.69	13.67 ± 2.03
KIN 3.0	25.00 ± 2.52	17	143.67 ± 0.88	13.67 ± 0.67
BAP 2.0 + NAA 0.5	50.00 ± 2.52	12	214.67 ± 37.78	52.67 ± 2.33
BAP 2.0 + NAA 1.0	80.00 ± 2.52	6	717.33 ± 14.25	112.67 ± 6.96
BAP 2.0 + NAA 2.0	70.00 ± 1.16	6	576.33 ± 6.33	74.00 ± 0.58
BAP 2.0 + NAA 3.0	55.00 ± 0.00	8	437.67 ± 6.74	66.67 ± 3.53
BAP 1.0 + NAA 0.5	58.33 ± 2.03	10	421.33 ± 4.48	66.67 ± 2.67
BAP 1.0 + NAA 1.0	45.00 ± 2.89	15	314.00 ± 13.12	60.00 ± 2.52
BAP 1.0 + NAA 2.0	40.00 ± 2.89	17	265.33 ± 5.93	40.00 ± 1.16
BAP 1.0 + NAA 3.0	35.00 ± 2.89	22	224.00 ± 6.43	34.33 ± 2.33
BAP 2.0 + 2,4-D 0.5	30.00 ± 1.73	19	157.00 ± 11.68	33.33 ± 0.88
BAP 2.0 + 2,4-D 1.0	20.00 ± 1.16	25	142.00 ± 4.93	30.00 ± 1.16
BAP 2.0 + 2,4-D 2.0	15.00 ± 2.89	21	87.67 ± 5.04	14.33 ± 0.33
BAP 2.0 + 2,4D 3.0	10.00 ± 1.16	25	66.67 ± 3.53	12.00 ± 1.16
BAP 1.0 + 2,4-D 0.5	20.00 ± 1.16	20	65.67 ± 2.33	13.00 ± 0.58
BAP 1.0 + 2,4-D 1.0	15.00 ± 2.89	20	55.33 ± 7.69	12.00 ± 0.58
BAP 1.0 + 2,4-D 2.0	10.00 ± 0.00	21	41.67 ± 4.41	9.33 ± 0.33
BAP 1.0 + 2,4-D 3.0	10.00 ± 0.58	24	42.33 ± 6.74	9.33 ± 0.67
S.E. (m)	*2.49*		*9.92*	*2.21*
C.D. at 5%	*7.10*		*28.25*	*6.30*
C.V. (%)	*13.10*		*8.67*	*12.04*

Table 2: Effect of growth regulators on induction of callus and biomass production in *Heritiera fomes* from apical leaf explants

Hormone concentration (mg/l)	% of callus induction	Days for callus imitation	fresh weight of callus (mg)	Dry weight of callus (mg)
Control	0.00 ± 0.00	No callus	0.00 ± 0.00	0.00 ± 0.00
BAP 0.5	10.00 ± 0.58	25	65.00 ± 4.04	12.67 ± 1.20
BAP 1.0	15.00 ± 0.58	25	76.67 ± 6.57	15.67 ± 0.33
BAP 2.0	35.00 ± 0.58	21	251.00 ± 18.68	59.33 ± 1.76
BAP 3.0	30.00 ± 1.16	20	118.33 ± 5.24	43.67 ± 0.88
KIN 0.5	0.00 ± 0.00	No callus	0.00 ± 0.00	0.00 ± 0.00
KIN 1.0	15.00 ± 1.16	21	80.00 ± 4.16	10.00 ± 0.58
KIN 2.0	15.00 ± 1.16	25	85.67 ± 6.44	10.67 ± 0.67
KIN 3.0	25.00 ± 1.16	21	124.33 ± 1.20	14.00 ± 0.58
BAP 2.0 + NAA 0.5	30.00 ± 0.58	21	384.33 ± 5.78	63.00 ± 1.53
BAP 2.0 + NAA 1.0	45.00 ± 2.89	25	727.33 ± 5.46	128.67 ± 1.33
BAP 2.0 + NAA 2.0	60.00 ± 2.89	19	959.00 ± 5.86	220.33 ± 2.73
BAP 2.0 + NAA 3.0	50.00 ± 2.89	15	836.33 ± 12.55	153.67 ± 3.76
BAP 1.0 + NAA 0.5	30.00 ± 1.73	19	269.33 ± 4.06	68.33 ± 0.88
BAP 1.0 + NAA 1.0	35.00 ± 1.73	19	308.33 ± 15.90	71.33 ± 0.67
BAP 1.0 + NAA 2.0	20.00 ± 1.16	21	224.67 ± 2.60	60.00 ± 1.16
BAP 1.0 + NAA 3.0	15.00 ± 0.58	25	113.67 ± 3.67	30.33 ± 0.33
BAP 2.0 + 2,4-D 0.5	10.00 ± 0.58	30	59.00 ± 3.79	10.67 ± 0.67
BAP 2.0 + 2,4-D 1.0	10.00 ± 0.00	21	51.67 ± 11.67	13.00 ± 0.58
BAP 2.0 + 2,4-D 2.0	0.00 ± 0.00	No callus	0.00 ± 0.00	0.00 ± 0.00
BAP 2.0 + 2,4D 3.0	0.00 ± 0.00	No callus	0.00 ± 0.00	0.00 ± 0.00
BAP 1.0 + 2,4-D 0.5	15.00 ± 1.16	30	69.33 ± 7.06	9.33 ± 0.67
BAP 1.0 + 2,4-D 1.0	10.00 ± 0.58	30	78.00 ± 5.29	11.00 ± 1.00
BAP 1.0 + 2,4-D 2.0	1.67 ± 1.67	30	50.67 ± 0.67	6.33 ± 0.33
BAP 1.0 + 2,4-D 3.0	0.00 ± 0.00	No callus	0.00 ± 0.00	0.00 ± 0.00
S.E. (m)	*1.33*		*7.13*	*1.22*
C.D. at 5%	*3.78*		*20.30*	*3.48*
C.V. (%)	*12.07*		*6.26*	*5.22*

Conclusion

The callusing was formed from both nodal and leaf explants inoculated on MS medium supplemented with combination of growth regulators *i.e.*, 2.0 mg/l of BAP and 1.0 mg/l of NAA for nodal explants and 2.0 mg/l of BAP and 2.0 mg/l of NAA for leaf explants. This study was proposed to develop a standard protocol for induction of callus from both nodal and leaf explants followed by standardization of media and

concentration and combinations of plant growth regulator for *Heritiera fomes.*

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