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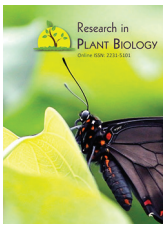

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Establishment of callus and cell suspension cultures of *Helicteres isora* L.

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ABSTRACT

Helicteres isora L. (Malvaceae) is a medicinal plant highly used in traditional therapeutic practices. It has shown wide-spectrum therapeutic activities including anti-plasmodial, hypoglycemic, hypolipidemic, hepatoprotective, antinociceptive, antioxidant and anti-HIV. Present investigation was undertaken with an objective of establishment of cell suspension cultures of this plant which can be further used for in vitro production of desired secondary metabolites and their further scale-up. Seed dormancy was broken using sulfuric acid and seedlings were raised in vitro. MS medium supplemented with 2,4-D (0.5 mg/L) produced maximum callus from the nodal explants. The callus produced was used as an explant for the establishment of suspension cultures. MS medium without any supplement was proved best for the establishment of cell suspension cultures of *H. isora*. To the best of our knowledge, this is the first report on *H. isora* cell suspension culture establishment.

KEYWORDS: *Helicteres isora*, cell suspension cultures, medicinal plant, plant growth regulators, auxins, 2,-4-D

INTRODUCTION

Helicteres isora L. (Malvaceae) is a traditional medicinal plant, commonly referred as Indian Screw Plant. It is a deciduous shrub which can be spotted throughout the central and western India. As per the Ayurvedic medicinal system, almost all the parts of this plant are applicable in treatment of various conditions such as cough, asthma, diarrhea, urinary astringent, colic as well as snake bite etc. [1]. The pods of this shrub have demonstrated many valuable activities including anti-plasmodial activity [2], hypoglycemic activity, hepatoprotective activity [3], antihyperglycemic and hypo-lipidemic activities [4,5], antinociceptive activity [3], antioxidant potencies [6], antimicrobial resistance reversal activities [7] and anti-HIV activity [8]. This plant is also being reported to be applicable in type II diabetes treatment [9]. The phytochemical investigation of this plant have revealed wide range of medicinally important chemical entities such as phytosterols, isorin, cucurbitacin B, isocucurbitacin B, betulic acid, rosmarinic acid derivatives, daucosterol, lupeol, amyrin, fridelin, neolignans, taraxerone, diosgenin and volatile oil [5,9]. However, heavy rate of exploitation of this plant from the native wild conditions along with lower rate of natural seed germination and pollination inadequacy leads to the low obtainability of this plant. By considering the availability of rich phyto-constituents, there is an urgent necessity for germplasm conservation, optimization of the conditions for production of active biomolecules as well as further improvement and propagation of this plant. Owing to the medicinal and commercial importance of this plant, suspension cultures might prove the ideal method for propagation as well as phytochemical production [1].

Cell suspension cultures are defined as individual cells or group of cells growing in the liquid medium. The suspension culture denotes the homogenous population of the growing cells which are exposed to the available nutrients as whole cell surface is in contact with the liquid. Compared to the callus cultures on the solid medium, suspension cultures allow the recovery of higher quantities of cellular biomass, from which a substance of interest (secondary metabolites or enzymes) can be isolated in higher quantities as well [10]. Due to such advantageous culture conditions, elicitation of the secondary metabolites production is usually achieved in cell suspension cultures. Improved production of such secondary metabolites has been accomplished in many plants via suspension cultures. Some examples include production of Abietane diterpenoids in *Cephalotaxus fortune* suspension cultures [11], Gymnemic acid in *Gymnema sylvestre* suspension culture [12], Hypericin in *Hypericum perforatum* suspension culture [13], Jaceosidin in *Saussurea medusa* suspension culture [14], Resveratrol in *Vitis vinifera* suspension culture [15], Sanguinarine in *Papaver somniferum* suspension culture [16]. Though the callus culture from different explants as well as hairy root cultures have been reported by our group previously [1,17] from *H. isora*, however,

suspension cultures are not yet reported, and an attempt was made in this investigation to optimize the suspension culture establishment in this important medicinal plant. The intense commercial and medicinal significance of *H. isora* implies the necessity of the establishing of the cell suspension culture of this plant for biomass as well as secondary metabolite production. Hence we report herein the efficient procedure to attain healthy cell suspensions from callus of *H. isora* which can be explored for *in vitro* production and extraction of secondary metabolites.

MATERIALS AND METHODS

Collection of Plant Material and *In Vitro* Seed Germination

Mature pods (fruits) of *H. isora* were collected from wild conditions from Khopoli, Maharashtra, India. The samples were authenticated at Anantrao Pawar College, Pune and specimen voucher (No. APCP/21) was submitted. Seeds were separated from mature pods and used to raise the seedlings vie *in vitro* seed germination. Isolated seeds were allowed to react with concentrated sulfuric acid for 4 min to break the seed-dormancy. Excess acid was then removed aseptically by washing the treated seeds with sterile double distilled water for five times. Acid-treated seeds were then placed on solid MS Medium [18], containing sucrose (3%), agar (0.8%) at pH 5.8 (autoclaved at 121°C, 15 psi for 25 min). Inoculated seed were then incubated for 3-4 days in dark, and transferred in white light condition after emergence of radical and plumule, at 25-27°C. Cultures were continued for 3-4 week, and nodes excised from young shoots (8–9 cm) from in vitro raised seedlings served as the source for callus induction.

Callus Induction

Nodal explants (approximately 1 cm) from 3–4 week old *in vitro*-raised plantlets were used for callus induction. The effect of auxins and/or cytokinins (separately and/or in combination) was checked on induction of callus initiation and proliferation. Excised nodal explants were inoculated in the MS Medium containing Sucrose (3%), Agar (0.8%), fortified with plant growth regulators; either in single [auxins such as 2,4-D (0.5-3 mg L^{-1}) and cytokinins such as BA (0.5-3 mg L^{-1}) and Kin (0.5-3 mg L^{-1}) alone] or in combination [IAA + BA, IAA + Kin, and BA + Kin]. Inoculated explants were maintained at 25-27°C with 16-h/d photoperiod. Subculturing was achieved on the fresh media of the same composition after each 20 days. Formation of the callus along with its biomass was recorded after 30 days on inoculation.

Establishment of Cell Suspension Cultures

Fresh and white calli obtained after 2 to 3 subcultures were used for the establishment of suspension cultures in MS medium without agar. Approximately 3.5 g of callus tissue was aseptically inoculated in the MS media (50 mL) with the composition as: Plain MS; MS + 2,4–D 0.5 mg L^{-1} (H1); MS + 2,4–D 1 mg L^{-1} (H2); and MS + 2,4–D 2 mg L^{-1} (H3). The cell suspension cultures were maintained on rotary shaker at 50-60 rpm (25-27° C, 16-h/d photoperiod). After 7-8 days in liquid culture, released/newly generated callus biomass was provided with fresh MS media of same composition. Healthy callus aggregates were repeatedly subcultured in new media for three cycles (total 21 days). After this period, biomass estimation was performed to compare the growth of callus in different media composition.

Statistical Analyses

All the experiments were performed in triplicates with at least 10 explants per treatment before conducting the statistical analyses. The results are presented as mean ± standard error.

RESULT AND DISCUSSION

The acid treatment positively resulted into breaking of seed dormancy which provided increased germination rate of the seeds, as reported by our group previously [1]. The calculated germination percentage was 45.33%. Inoculated nodal explants from 3-4 week old plantlets showed callus induction in all the PGR treatments (singly or in combination) (Table 1). However, maximum callus induction was observable in MS media fortified with 2,4-D singly, with 100% callus induction was achieved at the concentrations 0.5 and 1 mg L^{-1} of 2,4-D. At the same time, maximum biomass production was checked which revealed that 0.5 mg L^{-1} of 2,4-D was responsible for highest biomass production. A total of 0.82 g fresh weight per explant was recorded after 30 days of inoculation on solid MS media fortified with 0.5 mg L^{-1} of 2,4-D (Fig. 1). As proved to be the highest biomass producer, the callus generated at this concentration was further carried for establishing the suspension culture. 2,4-D has been reported previously responsible for callus induction and proliferation in various medicinal plants, six different species of *Ocimum* [19], *Carthamus tinctorius* [20] as well as in crop plants including *Oryza sativa* [21], *Saccharum* spp. [22], and *Miscanthus lutarioriparius* [23]. All these reports indicated importance of 2,4-D for callus induction and proliferation as in the present report.

The callus obtained at the previous step showed disaggregation of cell mass after approximately 5-7 days of inoculation in liquid MS medium. The disaggregated calli were then showed

Table 1: Effect of plant growth regulators (PGRs) on production of callus from nodal explants of *Helicteres isora* L

MS+PGR s	mg/L	Callus induction %	FW (g)	DW (g)
Control	--	--	--	--
2,4-D	0.5	100±1.5	0.82±0.04	0.05±0.009
	1.0	71.4±1.9	0.37±0.03	0.03±0.008
	2.0	100±2.1	0.54±0.04	0.04±0.008
	3.0	92.8±1.8	0.67±0.11	0.04±0.007
BAP	0.5	21.4±0.8	0.10±0.01	0.01±0.003
	1.0	42.8±1.4	0.40±0.02	0.04±0.002
	2.0	71.4±1.9	0.31±0.04	0.03±0.002
	3.0	28.6±2.6	0.07±0.01	0.01±0.004
Kin	0.5	64.3±1.8	0.08±0.01	0.01±0.003
	1.0	85.7±3.1	0.63±0.12	0.06±0.002
	2.0	64.3±2.9	1.34±0.14	0.14±0.009
	3.0	64.3±1.8	0.77±0.09	0.08±0.006

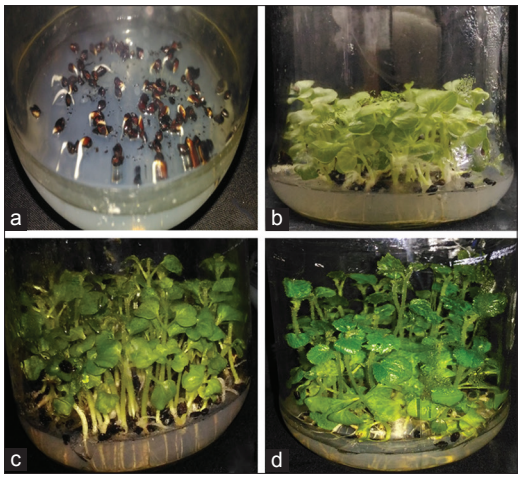

Figure 1: *In vitro* seed germination of *Helicteres isora* L. (A) Acid-treated germinating seeds after 4 days of inoculation; germinated seedling after (B) 12 days (C) 20 days (D) 25 days

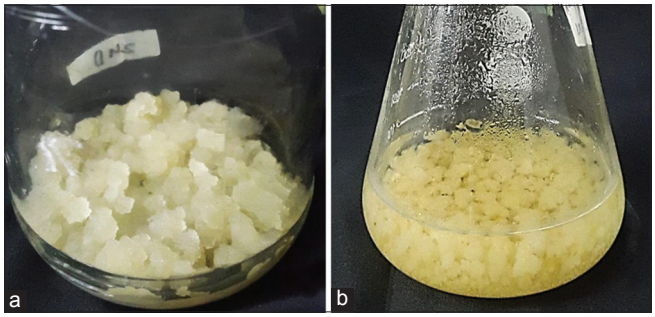

Figure 2: (A) Callus proliferation on MS +2,4-D (0.5 mg L^{-1}) after 30 days of subculture (B) Suspension culture of *Helicteres isora* in MS medium after 30 days of inoculation

Table 2: Effect of varying concentrations of 2,4-D on production of cell suspension cultures of *Helicteres isora* L. Samples were harvested after 21 days (3 cycles of 7 days each) for recording FW and DW

2,4-D (mg/L)	Callus inoculated (g)	FW of Suspension cultures (g)	DW of Suspension cultures (g)
0.0	3.5	17.75±1.7	0.96±0.09
0.5	3.5	17.43±1.6	0.82±0.07
1.0	3.5	10.78±1.2	0.35±0.05
2.0	3.5	5.24±0.9	0.31±0.05

increment in size with increase in the time. After 21 days of the inoculation, the total biomass analysis indicated that the suspension cultures growing in MS media without 2,4-D showed maximum biomass in terms of fresh as well as dry weight (Table 2). There was gradual decrease observed in the biomass produced with increase in the 2,4-D concentration (0.5-2 mg L^{-1}). Hence it was noted that maximum biomass production was attained in plain MS medium after 21 days of inoculation (Fig. 2). There are several reports on establishment and use of suspension cultures for production of large-scale secondary metabolite production in a sustainable and eco-friendly way. Orihara and Ebizuka [24] established shake-flask cell suspension cultures of *Olea europaea* for the production of triterpene acids. Chattopadhyay *et al.* [25] scaled up the cell suspension cultures of *Podophyllum* to bioreactor level for the production of podophyllotoxins. The present holds merit since optimal cell suspension cultures were obtained on plain MS without using any PGR, which adds to the cost effectiveness of the method and may prove to be beneficial for production of desired secondary metabolites from the cell suspension cultures.

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