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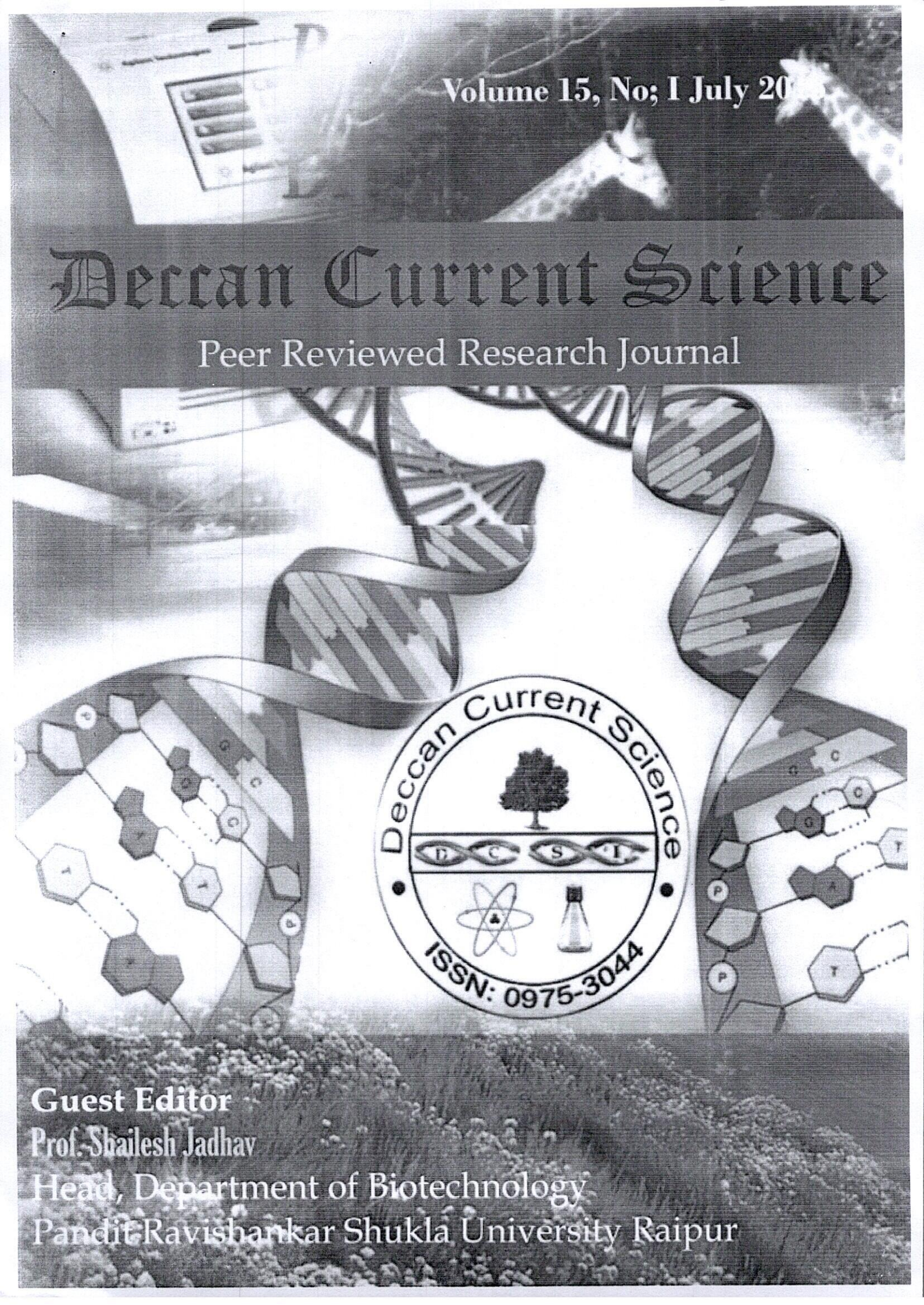

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Establishment of Callus and Cell Suspension Cultures of *Bryonialaciniosa*, A Medicinal Plant

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ABSTRACT

An efficient procedure has been developed for callus induction and cell suspension cultures of *Bryonialaciniosa*for the first time. Explant selection was carried out among leaf, stem and root to select a suitable type of explants capable of higher callus formation. Leaf explants thus selected showed maximum response to callus induction (67.1%). Modified B5 medium supplemented with 0.5 mg/l 2,4-D plus 2 mg/l BA was the most favorable medium for callus formation with the highest induction rate (94.8%) and greatest fresh weight of callus (1.7 g per explant). Cell suspension cultures were established by transferring 2-8 g fresh callus to 80 ml liquid B5 medium. An inoculum size of 8 g produced the greatest biomass accumulation which was 13.1 g/l. In response to various sucrose concentrations from 10 g /l to 80 g/l, cultures with 60 g sucrose lg/l not only produced the highest dry biomass (18.5 g/l).These prepared cell suspension cultures provided a useful material for further regulation of Secondary metabolite biosynthesis and for enhanced production of valuable products on a large scale.

Keywords: BA- 6-benzyladenine; DW- dry weight; 2,4-D- 2,4-dichlorophenoxyacetic acid; B5- Gamborg's medium.

Introduction:

*Bryonialaciniosa*syn*Bryonopsislaciniosa* (Cucurbitaceae) locally known as 'Shivlingi' and 'Gargumaru' is distributed throughout India. It is an annual climber with bright red fruits and is reported to be highly medicinal(Supe, 2011). Locally in India its seeds are being used for promoting conception in women. It was thought that seeds may be containing some hormone like substance or substances that may help in the secretion of hormones in the human system or may be possessing substance which may be having strengthening effect on uterine muscle. Ayurvedic literature survey indicated the use of entire plant is bitter tonic, hepatoprotective, antipyretic, laxative and used to correct the metabolic abnormalities. Plant is also used against snake-bite(Paul and Raychaudhuri, 2010). Its leaves are used on inflammation (Nadakarni 1982; Kirtikar and Basu, 1987). Fruits are recommended in liver disease, leucoderma, inflammation and abdominal disease(Rai,1989,Sathish Kumar 2010) Fruits are used as blood purifier. Root extract is having

diuretic, hepatoprotective and kidney stone removing properties. From leaves, a bitter principle bryonin has been reported (Singh and Malviya, 2006). From seeds, saponin molecules are identified with the help of TLC and antibacterial, antifungal, anti-inflammatory and diuretic activities have been reported (Sivakumar et al., 2004).

With an increasing demand for those products, the plant cell culture provides an attractive alternative source that could overcome the limitations of extracting useful metabolites from limited natural resources. And it is now widely being employed as a model system to investigate the production of specific secondary metabolites because it offersexperimental advantages both to basic and applied research and to the development of models with scale-up potential (Buitelaar and Trapmer, 1992; Chang and Sim, 1995). Additionally, cell cultures exhibit advantages for studies of elicitor-induced responses (Stella and Braga, 2002) which are otherwise difficult to be carried out in the intact plant. Although eneration of callus and suspension cultures ofBryonia have been described (Supe,2013., Singh, 2006). There is little information available in literature with respectto *Bryonialaciniosa*. Realizing the importance of*Bryonialaciniosa*and its extracts in clinical medicine, we attempted to apply this strategy to establish callus and cell suspension cultures of *Bryonialaciniosa*acting as source for the production of valuable secondary metabolites. In the present paper, the initiation of callus and cell suspension cultures of *Bryonialaciniosa* and their characteristics are described. Moreover, special alkaloid productions are also investigated by manipulating inoculum size and sucrose concentration in 250 ml flasks.

Materials and methods:

Plant material and culture conditions for callus induction

Materials of *Bryonialaciniosa were* collected from Botanical garden, St. Thomas College, Bhilai in November 2008. Fresh, healthy and mature explants obtained from the plant were cleaned under running tap water and surface-sterilized by placing them in 70% ethanol for 1 min, followed by two treatments of 10 min rinse in 0.1% mercuric chloride solution and five times wash with sterile distilled water. After surface sterilization, explants were cut into about 1 cm2 for leaves and 1 cm length for stems and roots. Then they were placed separately on 30 ml B5 basal medium in 150 ml flasks supplemented with 3% sucrose and different cytokinin/ auxin combinations solidified with 1% agar.The media used for all the experiments were autoclaved at 121^{0}C for 20 min. The pH value of the media was adjusted to 6.0 prior to autoclaving. All the cultures were maintained under 16-h photoperiod illumination. The incubation temperature was kept at 25^{0}C.

Suspension cultures and cell growth determination

Suspension cultures were developed by transfer of 2-8 g fresh weight (FW) friable calli into 250 mlflasks containing 80 ml B5 liquid medium supplementedwith 0.5 mg /l 2,4-D (2,4-dichlorophenoxyaceticacid) and 2.0 mg /l BA (6-benzyladenine),the best exogenous hormone combinationdetermined for callus formation. All the cell suspensioncultures were placed on a rotary shakerwith a speed of 130 rpm at (25^{0}C inthe dark. The cells wereharvested from suspensioncultures by filtration via a Buchner funnelwith a filter paper, washed with distilled water toremove residual medium, and then filtrated againunder vacuum. After filtration the cells were driedat 50^{0}C to constant dry weight (DW). Cell growthwas measured on DW basis.

Results and discussion:

Effects of different types of explants oncallus induction

Different types of explants were placed on basalB5 medium containing 1 mg /l 2,4-D, 1 mg /l BAand 1 mg /l kinetin for 2 months of cultivation.Leaf explants exhibited highest frequency of callusformation (67.1%), which was almost 2- and 12-fold greater than that obtained in stem and rootexplants, respectively. These results obtained hereindicated that leaf and stem explants were suitablefor callus induction of *Bryonialaciniosa rather* than rootexplants. Similar results were reported in callus inductionof*Tanacetumbalsamita* L Shoja et al., 2010, in which it wasdescribed that callus could be induced successfully (Aslam, et al., 2006). Owing to the capacity of higher callus formation, hence, leaf explants were used for further experiments.

Effects of exogenous plant growth regulators on callus formation

Calli were successfully generated in all combinations of 2,4-D and BA two months after inoculation. But no callus as induced by basal B5 medium only (Table I). This indicated that exogenous hormone was essential to callus formation of Bryonia*laciniosa*. The exclusive presence of BA in the medium, regardless of its concentration, was less Satisfactory for callus initiation. While in the absence of BA, the cultures containing corresponding concentrations of 2,4-D showed higher callus weight in a range from 0.25 g to 0.63 g FW per explant and higher response from 27.7% to 8.3% (Table I). This phenomenon suggests that 2,4-D played a more important role in callus formation from leaf explants compared to BA. However, the combinations of auxin and cytokinin were found to produce more callus than auxin or cytokinin alone. One combina

tion containing 0.5 mg /l 2,4- D and 2 mg /l BA, was proved to be the most efficient in promoting callus development (1.68 g) from leaf explants with 94.8% response, followedby 1 mg /l 2,4-D + 2 mg /l BA (1.40 g) and then0.5 mg /l 2,4-D + 1 mg /l BA (1.35 g). In addition,most of the cultures containing 2,4-D (0.5-1 mg /l) and BA (0-2 mg /l) produced friableyellowish calli, which initiated at cut surfaces ofthe explants about 20 d after inoculation and proliferatedquickly. However, cultures with BA onlyresulted in less friable calli and white roots germinatedfrom those compact calli after 3 months ofcultivation. This observation was similar to callusdevelopment of *Holostemmaadakodien*(Martin,2003).A plant growth regulator is a key factor responsiblefor callus initiation and development in plantcell cultures. Our results indicate that the combinationof 2,4-D and BA was effective to satisfactorilyinduce calli from leaf explants of *Bryonialaciniosa*.The medium used for callus formation of *C. Platycarpa*Makino and *C. ochotensis*var. *raddeana*,which are also relatives of *Corydalis*, contained alow level of 2,4-D (1 mg /l) (Iwasa*et al.*, 2003). In *C. ambigua*, Hiraoka*et al.* (2001) described that the medium including2,4-D and BA could induce callus on the explants surface. The stimulative effect of 2,4-D incombination with BA on callus formation has alsobeen reported for *Oryzasativa*(An *et al.*, 2004).During following subcultures, healthy andyoung calli cultured on B5 + 0.5 mg /l 2,4-D +2 mg /l BA showed a bright yellow color, butsome of them turned brownish as they grew older.

Those calli were also observed to favor light condition(16-h photoperiod) rather than completedarkness. Calli cultivated under complete darkcondition more frequently turned into brown ordark in comparison to those calli under light condition.

Therefore, the homogenous calli selectedfrom cultures were maintained on B5 medium containing0.5 mg /l 2,4-D and 2 mg /l BA underlight condition for a long repeated subcultureprocess. Development of fast-growing

homogeneouscallus was achieved after about one year.

Effects of inoculum size on suspension cultures

Suspension cultures were initiated by transferring2-8 g fresh friable calli into 80 ml B5 liquidmedium in 250 ml flasks. In the case of 8 g, cellgrowth increased in the initial 12 d followed by aremarkable decrease. This biomass decrease could be caused by some cells which couldn't adapt to the change from solid to liquid medium and graduallywent to death. After that, biomass increased rapidly and reached the highest value (13.1 g /l) on day 21, with about 6 times increase compared to that at the beginning (2.1 g /l). Similar development of 6 g callus was demonstrated and the stationary phase was reached after 21 d. As to 4 g, a lag phase of about 12 d was observed, followed by a slower growth. However, there was no marked growth for 2 g during the whole cultivation and an appreciable amount of cell debris accumulated in the media in 30 d. (Table 2). The results show that cell growth requires a certain initial density of cells and lower inoculum size is inhibitory to growth of suspension cultures. Similar growth inhibition has also been reported for transformed cell suspension cultures of *Coleus forskohlii*(Mukherjee *et al.*, 2000). The inoculum size had a positive effect on biomass and metabolite production up to an optimum concentration (Gorret*et al.*, 2004). In this study, the optimal inoculums size for biomass accumulation which is comparable to those obtained by Chiou*et al.* (2001) for production of polyunsaturated fatty acids in cell suspension cultures of *Marchantiapolymorpha*.

Effects of sucrose concentrationon suspension cultures

High sucrose concentration usually resulted in high biomass accumulation and secondary metabolite production of plant cell cultures. In response to increased sucrose concentration from 10 g /l to 60 g /l, cultures not only accumulated more biomass but also produced alkaloids. On the contrary, biomass accumulation andalkaloid production were significantly decreased when sucrose concentration increased from 60to 80 g /l (Table 3). This phenomenon suggests that the cell growth was suppressed by relatively higher initial sucrose concentration, which contributed to higher osmotic pressure (Zhang *et al.*, 1996). Similar suppression was also reported in cell cultures of *Panax ginseng* (Akalezi*et al.*, 1999). The optimal sucrose concentration for maximal biomass was 60 g /l. In all cases of sucrose concentrations, the pH value of medium presented a similar profile to biomass. The results demonstrate that manipulation of sucrose concentration is an effective method to improve alkaloid production in this cell culture, which is in agreement with Pasqua*et al.* (2005). In conclusion, callus and cell suspension cultures of *Bryonialaciniosa*were achieved for the first time and provided a homogenous material for the production of secondary metabolites. Remarkable improvements of biomass accumulation were successfully obtained by manipulating inoculum size and sucroseconcentration. The optimal inoculum size and sucrose concentration were 10% (FW, w/v) and 60 g /l, respectively. This work may be beneficial for further regulation of alkaloid biosynthesis and for enhanced production of valuable alkaloids on a large scale.

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