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EVALUATION OF CALLUS BROWNING AND DEVELOP A STRATEGICALLY CALLUS CULTURING OF *BOERHAAVIA DIFFUSA* L.

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Abstract: Callus browning is a major problem in *Boerhaavia diffusa*. This phenomenon was investigated in present study by evaluating major reason for callus browning, develop a strategy for the survivals of callus and study the accumulation of secondary metabolites. Torpedo shaped embryos were cultured on semisolid MS basal medium supplemented with n various combinations of hormones, with and without adjuvants. After a particular time callus used for cytological, fresh viz dry weight studies and later used for the secondary metabolite study by HPTLC method. Cytological studies of the callus were performed to understand the reason for low survival of the callus. Over a culture period of 30 days revealed that the callus was made up of three types of cells: small isodiametric cells, elongated cells and elongated enucleated cells. The isodiametric cells were meristematic and predominant during the initial days of the culture and subsequently their number decreased and elongated nucleated and enucleated cells increased. Towards the latter part of the culture period the enucleated cells were predominant. The increase in elongated cells coincided with increased browning of the callus and peroxidase activity. The HPTLC of extracted callus with different precursors confirmed the presence of some flavonoids likes kaempferol, quercetin, myrecetin. A strategic subculturing method was developed where in the small cells were isolated and subcultured every three weeks and the life of callus could thus be prolonged to almost 30-36 weeks. Based on these studies conclude that the life of callus could be prolonged to almost 30-36 weeks by strategic subculturing method. This study is important because as plant has various medicinal properties so its secondary metabolites can be collected by invitro callus production at particular time period.

Key words: *Boerhaavia diffusa*, callus browning, peroxidase, strategic subculture

Introduction

Boerhaavia diffusa is an important medicinal plant, known as punnarnava in Sanskrit and is used in a wide number of Ayurvedic preperations. The plant harbours a large number of secondary metabolites such as geranylacetone, limonene, indoleresorcinol monoacetate, vanilin, eugenol and kaempferol 3-O-robinobioside in leaves and quercetin 3-O-robinobioside, caffeoyltartaric acid, eupalitin 3-O-galactosyl (1-2)-glucoside, and isomenthone in roots [PEREIRA & al. 2009]. It has been in use to alleviate a large number of ailments such as liver disorders, dyspepsia, jaundice, enlargement of spleen, and abdominal pain [KIRTIKAR & BASU, 1956; RAWAT & al. 1997; MALIK, 1980].

Plant cell cultures are proving to be effective alternative for producing *in vitro* secondary metabolites [ROBERTS & KOLEWE, 2010; LEE & al. 2010]. In this regard a study was undertaken to establish callus cultures of *Boerhaavia diffusa* for *in vitro* secondary metabolite production. However, a major problem was the sustenance of callus

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for long periods due to browning and early senescence of callus. This interfering phenomenon is known to be tissue browning due to the production of phenolic compounds. Now days it is consider being serious problem because tissue browning event involves many toxic compounds through the phenolization process eventually resulting in the necrosis of cells [BANERJEE & al. 1996; MURATA & al. 2001; WU & LIN, 2002; CHEN & al. 2012].

Browning of callus is a major problem for growth and long term maintenance of callus and in many cases inhibits adventitious shoot formation [LEE & WHITAKER, 1995]. This problem has been encountered in several plants such as peach [LEE & al. 1990], sugarcane [CHEN & al. 1990], *Taxus cuspidata* [FETT-NETO & al. 1992, 1993], oak [TOTH & al. 1994], *Zea mays* [DOWD & NORTON, 1995], *Pinus sylvestris* [LAUKKANEN & al. 2000], *Taxus chiensis* [CHOI & al. 2000], guava, date palm [DAAYF & al. 2003], *Taxus media* [BAEBLER & al. 2005], cotton [OZYIGIT & al. 2007], *Cicer arietinum* [NAZ & al. 2008], *Jatropha curcas* [HE & al. 2009], *Nigella glandulifera* [ZHOU & al. 2010], *Taxus brevifolia* [KHOSROUSHAHI & al. 2011] and *Senna occidentalis* [ISAH & MUJIB, 2013), *Taxus chinensis* [NAN & al. 2015].

The main reason for tissue browning of *in vitro* tissues has been correlated with excessive accumulation of phenolics [LEE & al. 1990; DOWD & NORTON, 1995; LAUKKANEN & al. 2000; DUBRAVINA & al. 2005; ISAH & MUJIB, 2013]. Plant phenolics are chemically active because of the presence of a hydroxyl functional group [SREENIVASULA & al. 1989]. Phenolic compounds though cause browning in exposed areas but play many important functions in higher plants. They may combine with proteins either reversibly by hydrogen bonding or irreversibly by oxidation. Phenolics modulate plant development by regulating indole acetic acid catabolism [ARNALDOS & al. 2001]. They are effective in plant growth regulation, cell differentiation and organogenesis [OZYIGIT & al. 2007]. Phenols on oxidation form compounds called quinones that polymerize to impart the characteristic brown colour and are inhibitory to plant cellular growth [MAYER & HAREL, 1979]. Phenol oxidation may be catalyzed by polyphenol oxidases or peroxidases [VAUGHN & DUKE, 1984; KE & SALTVEIT, 1988] which act synergistically. Polyphenol oxidase promotes peroxidase activity by generating H_2O_2 through the oxidation of phenolic compounds [RICHARD-FORGET & GAUILLARD, 1997].

Studies in Scots Pine have shown that browning is primarily because of peroxidase activity [LAUKKANEN & al. 2000], though, in *Jatropha* polyphenol oxidase has been reported to play a more important role [HE & al. 2009]. Peroxidase has been also reported to be associated with degradation of chlorophyll and peroxidation of lipid in senescing plant tissues [CAMPA, 1991]. In *Pinus virginiana* callus browning, an increased polyphenol activity was associated with a concomitant decrease in the antioxidant enzymes ascorbate peroxidase, glutathione reductase and superoxide dismutase [TANG & NEWTON, 2004].

Several methods have been adopted to alleviate browning of *in vitro* callus or shoot cultures. Chief among these have been the use of antioxidants as ascorbic acid – *Taxus* sp. [FETT-NETO & al. 1992]; *Magnolia officinalis* [FU & al. 2009] or cysteine – *Taxus* sp.[FETT-NETO & al. 1992]; growth adjuvants as casein hydrolysate – *Taxus brevifolia* [GIBSON & al. 1993]; activated charcoal – *Taxus* sp. [FETT-NETO & al. 1992]; *Magnolia officinalis* [FU & al. 2009]; polyvinyl pyrolidine – *Rollinia mucosa* [FIGUEIREDO & al. 2001]; *Magnolia officinalis* [FU & al. 2009]. Other strategies to reduce *in vitro* browning

have been to lower the salt strength of MS medium to half or one-fourth as in *Nigella* [ZHOU & al. 2010] and *Actinidia arguta* [HAN & al. 2010], or by replacing high salt strength medium MS with that of moderate strength medium of Schenk and Hildebrandt in *Taxus* [FETT-NETO & al. 1993].

To alleviate the problem of callus browning in *B. diffusa*, the present study growth dynamics of the callus with respect to variation in the cell types, their growth rates, changes in fresh and dry weights of callus was correlated with variation in peroxidase activity of the callus. Based on these results a strategy for subculture of callus was developed which prolonged the life of callus *in vitro* to several subcultures. Secondly, as plant cell cultures are useful for producing *in vitro* secondary metabolites so in this regard a study was undertaken to establish callus cultures of *B. diffusa* for *in vitro* secondary metabolite production and to identify some of important secondary metabolites from *in vitro* production of cell culture by HPTLC.

Material and methods

Plant material: young immature fruits were collected from the Botanical garden of the Institute, Dayalbagh Agra.

Sterilization of fruits: fruits were collected, washed under running tap water for about 30 min followed by a quick rinse in ethanol then surface sterilized with 0.1 % mercuric chloride for 8 min under aseptic conditions.

Embryo isolation: the sterilized young immature fruits were dissected under aseptic condition and embryos were cultured on appropriate medium.

Preparation of medium: for all studies MS basal medium [MURASHIGE & SKOOG, 1962] was used supplemented with various growth regulators such as 2,4-D, BAP or NAA in different combinations with various growth adjuvants such as silver nitrate, casein hydrolysate or activated charcoal. To increase secondary metabolite contents some precursors such as phenyl aniline, t-cinnamic acid were also added.

Callus establishment and maintenance: young immature embryos were cultured on MS basal medium supplemented with 2,4-D alone at 0, 0.5, 1, 1.5, 2, 2.5 and 3 mg/l. The resulting callus was subculture to their respective hormone combination media. Callus was subcultured every 20 days. Besides, the callus was also transferred to MS basal medium supplemented with NAA (0.5, 1.0, 1.5, 2.0, 2.5 and 3 mg/l) + BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l). The NAA+BAP medium was also supplemented with various adjuvants: silver nitrate (5, 10 and 15 mg/l), casein hydrolysate (25, 50 and 100 mg/l), activated charcoal (5, 10, 20 mg/l) or proline (10, 25 and 50 mg/l).

Cell type composition of the callus: the cell type and composition of callus was studied in the 3% sucrose solution. Types of cells and their numerical variation were counted using a haemocytometer. Dimensions such as length and breadth of the cells were measured using the software package NIS Elements-D version 3.1 (Nikon, Japan).

Spatial distribution of cells in callus: spatial distribution of different types of cells in the callus was determined by cutting thin free-hand vertical sections of the callus. The sections were stained with acetocarmine and the distribution of different types of cells from portion proximal to medium to the portion distal from medium was studied in Nikon Stereozoom Microscope (SMZ 800) attached with Nikon digital camera.

Temporo-numerical variation in different type of cells in the callus: callus cultured on MS + 1 mg/l 2,4-D + 0.5 mg/l BAP was used for this study. Small isodiametric cells were carefully isolated and inoculated on fresh medium. Subsequently, differentiation into various cell types and their numerical variation over a period of four weeks was recorded at 5-day intervals for a culture period of 30 days.

Fresh and dry weight measurements: fresh and dry weights of callus from MS + 1 mg/l 2,4-D + 0.5 mg/l BAP + 25, 50, 100 mg/l casein hydrosylate, 25, 50, 100 mg/l phenylanaline and t-cinnamic acid were recorded every fifth day starting from day 0 to day 25. To get dry weight callus was dried in an oven maintained at 60 °C for 48 h or till the weight became constant.

Peroxidase assay: peroxidase activity of callus from day 0 to day 30 at five day intervals was assayed by guaiacol method[38].

Preparation of callus extract: callus obtained from immature embryos was extracted for quantifying their secondary metabolite content [CHAUDHARY & al. 2012].

Preliminary tests for secondary metabolites: Shinoda test conducted for flavonoid and Dragendorff test for alkaloids [SVENDSEN & VERPOORTE, 1983].

Preparation of standard solution and HPTLC conditions: a 100 ppm stock solution of standards kaempferol, quercetin, mercetin and leuteonine (Sigma-Aldrich, St. Louis) were prepared and scanned the samples according to HPTLC standard conditions (CAMAG, France) [CHAUDHARY & al. 2012].

Results

Callus study: MS basal medium without 2,4-D the embryos neither grew nor survived while lower concentrations of 2,4-D (0.5, 1 and 1.5 mg/l) supported good callus formation than the higher concentrations. Best callus initiation and growth from the torpedo embryos was obtained in MS + 0.5 mg/l 2,4-D. The callus was creamish, compact and friable (data not shown). The callus from initiation phase could not survive beyond 15 days when transferred to a medium containing either 2,4-D or BAP alone at all concentrations combinations. Presence of both 2,4-D and BAP and their concentration was critical for survival of the callus in subsequent subcultures. Concentration of 2,4-D with BAP at 1 and 0.5 mg/l supported the best callus growth (Fig. 1). Till the third subculture, the callus was healthy, creamish and friable. In subsequent cultures the callus slowly turned watery, brown and by fifth and sixth subculture (150-180 days) the entire callus became brown and died. MS medium supplemented with various growth adjuvants such as casein hydrolysate, activated charcoal, ascorbic acid, silver nitrate and proline did not improve callus growth but callus turned brown and died within 2-3 weeks of transfer (data not shown). Reducing MS salt strength to half also did not help reduce browning of the callus.

Fresh and dry weight changes in callus: a typical sigmoidal growth curve was observed for the weight changes. The lag phase lasted for the first five days, thereafter, fresh weight showed a consistent increase till day 25 beyond which weight became stationary. Dry weight increase followed almost a similar pattern except that dry matter accumulation was sluggish till day 20. Thereafter, in the next five days dry matter accumulation increased exponentially, and then became stationary in the final period of 25-30 days (Fig. 2A).

Types of cells in the callus: callus from the medium MS + 1 mg/l 2,4-D + 0.5 mg/l BAP at the end of 30 day growth period was composed of three types of cells: (i) small, isodiametric cells with centrally placed nucleus and intensely staining cytoplasm (Fig. 3A), (ii) elongated cells with sparsely stained cytoplasm and nucleus drifted to one side (Fig. 3 B & C) and, (iii) elongated cells with sparse cytoplasm without traceable nucleus. The distribution of these three types of cells in callus at the end of 30 days was shown in Figure 3 D. In a total of 3400 cells 72% were nucleated and only 28% of the total cells were enucleated (the enucleated cells were elongated). Of the nucleated cells 46% were small, isodiametric with a centrally placed nucleus and intensely staining cytoplasm. The remaining 54% of cells are elongated; their cytoplasm stained light and had a peripheral nucleus. The only cells that appeared to be meristematic were the small isodiametric cells as these were present in clumps. The elongated cells and the enucleated cells were mostly present as separate individual cells. The area of small cells doubled during the 30 day period while the area of elongated cells increased by slightly more than 10% (Fig. 2 B). A perusal of Fig. 2D indicates that balance sheet of small cells was mostly negative throughout culture period except on day 15 when a slight increment of about 50 cells was recorded.

Peroxidase activity: the callus turned brown with passage of time and at the end of subculture period entire callus became brown and died. Peroxidase activity at different intervals during callus growth and has been overlapped on the results of numerical variation in the cell types (Fig. 2C). The long as the small cells were in sufficient numbers (day 10) and actively dividing the peroxidase activity was almost absent, however, as the number of small cells start to decline and the number of elongated and enucleated cells increased the peroxidase activity increases and on day 20 the enzyme activity peaks, thereafter, by day 25 and 30 its activity declines. On day 20, corresponding to the peak activity of peroxidase, only a few small cells were present and the cumulative numbers of elongated and enucleated cells were sufficiently large. Thereafter, there was a rapid decline in the number of small cells and an increase in number of elongated and enucleated cells. Peroxidase activity though was minimum on last two intervals (day 25 and 30) of culture but it never became zero. This activity of peroxidase paralleled increased browning of callus and also increased dry weight. As based on the results obtained it could be argued that so long as a reasonable population of small meristematic cells was present the callus continued to proliferate.

Fresh and dry weight: The callus attains a maximum fresh and dry weight between the second and the third week of culture. Incidentally, it was during this period that the population of small cells stabilizes and peroxidase activity also peaks. Thus, a strategy for subculturing such a callus should aim at that period of callus growth which has the maximum number of senescing cells and optimal gain in fresh and dry weight. Such a stage in callus cultures of *B. diffusa* as revealed by the present study was in the second to third week of culture. Therefore, callus at the end of third week was subcultured using the portion proximal to the medium as it had the maximum number of small meristematic cells. The steps involved in the strategy for subculture of callus were: (a) subculture of callus to fresh medium was done after every three weeks instead of the earlier four weeks, (b) only the portion of the callus rich in small, isodiametric cells was transferred to fresh medium, and (c) the callus was cleaned of all the elongated, brown and dead cells. Following this procedure carefully the life of callus could be increased to almost 30 to 36 weeks.

The dried callus were fractionated and analysed on HPTLC against these flavonoids standards. It was estimated that the callus of *B. diffusa* contained 0.36 µg/µl of flavonoids as quercetin at maximum (0.95 µg/µl) followed by kaempferol (1.5 µg/µl) and myrcetin was the least (0.95 µg/µl) For details refer previous paper [CHAUDHARY & DANTU, 2011].

Discussion

The present study revealed that it was possible to establish healthy growing callus from young embryos of *B. diffusa*. However, the callus could not be maintained beyond five subcultures as it becomes brown and eventually dies. Cytological studies revealed that the well established callus was made up of three types of cells: small, isodiametric meristematic cells with central nucleus, elongated cells with peripheral nucleus and enucleated elongated cells. The study also revealed that the small cells are continuously differentiated into nucleated and enucleated elongated cells. Microscopic examination showed depositions of optically dense substances on the cell wall of the elongated cells (Fig. 3). Presumably these morphological changes and possible biochemical changes (as revealed by change in colour of the cells and depositions on cell walls) could be resulting in browning and early apoptosis of the callus cells. Further, it was also noted that increase in peroxidase activity in the callus cultures was associated with increase in browning of the callus and elongated cells and concomitant decrease in small cells. Accumulation of secondary metabolites in the callus cells has been implicated in browning and early death due to apoptosis [SOLOMON & al. 1999; KNIGHT & al. 2001; QIAO & al. 2003].

Morphological and biochemical changes in *Taxus chinensis* var. *meirei* cells occurred mainly in the non-dividing cell clusters indicating that the cells died by apoptosis. These authors found a close relationship between cell apoptosis and Taxol formation. Taxol concentration increased with increased number of apoptotic cells and reached a maximum after 23 days of culture which corresponded to a maximal ratio of apoptotic to total cells to about 13%. That the apoptotic cells mainly occurred in the cell clusters of brown colour was also observed by EXPOSITO (2009). Permanent loss of cell viability was observed in callus cells of *Taxus cuspidata* P991 that were producing high levels of Taxol upon elicitation with methyl jasmonate [KIM & al. 2005]. *B. diffusa* plants are known to be rich in flavonoids and other secondary metabolites [PEREIRA & al. 2009]. It could be possible that callus cultures of *B. diffusa* are actively producing some secondary metabolites [PEREIRA & al. 2009; MURTI & al. 2010; GOYAL & al. 2010]. The elongated nucleated cells probably undergo differentiation to produce some secondary metabolites and excessive accumulation of which lead to browning and eventual death of these cells.

Callus browning has often been associated with an increase in accumulation of phenolics such as lignin [LAUKKANEN & al. 2000]. This increase in phenolics has been linked to increase in polypenol oxidase (POP) and peroxidase (POD) activity in *Malus sylvestris*, *Heva brasiliensis*, *Panax ginseng*, *Camellia sinensis* [BERGER & al. 1985; HOUSTI & al. 1992; KORMUTAK & VOOKOVA, 2001; BONFILL & al. 2003; AOSHIMA & TAKEMOTO, 2006]. Present study also revealed an increase in peroxidase activity as callus browning intensified. In callus cultures of *B. diffusa*, present study, decrease in actively dividing small cells and concomitant increase in non-meristimatic elongated cells caused untimely senescence and increase in population of brown cells. Decrease in regenerability, poor growth and eventual death because of callus browning has

been observed in several species [BERGER & al. 1985; DOWD & NORTON, 1995; LAUKKANEN & al. 2000; KAWAOKA & al. 2003; HE & al. 2009; HAN & al. 2010]. *In vitro* browning of callus has been overcome in many species by changing the composition of the basal medium, or by reducing the salt strength, or by adding adjuvants such as casein hydrolysate, silver nitrate, proline or activated charcoal. Interestingly, in the present study, the callus of *B. diffusa* could not be prevented from browning either by changing composition of basal medium or by changing growth regulators or by adding any of the adjuvants mentioned.

The decline in peroxidase activity towards the end of culture period in callus cultures of *B. diffusa* could be attributed to decrease in small meristematic cells that were continuously differentiating into nucleated and enucleated cells. These elongated cells are continuously becoming brown and dying. The differentiation of the small meristematic cells into non-dividing elongated cells (nucleated and enucleated) and the consequent decrease in the number of small cells by the 15th day of culture resulted in senescence of the callus. This was reflected by browning of callus and an increase in the peroxidase activity. A subculturing strategy was developed where in the small meristimatic cells proximal to the medium were carefully separated and transferred to fresh medium and subsequent subculture was done every three weeks. This procedure enhanced the life of *B. diffusa* callus cultures to almost 30-36 weeks.

The HPTLC result of *B. diffusa* callus indicates the presence of flavonoids such as kaempferol, quercetin and myrecetin. The earlier reports also shows the presence of flavonoids and other compounds in the *in vitro* cultures of *Hypericum* [KARTNIG & al. 1996; BERNARDI & al. 2007; SHILPASHRE & RAI, 2009], *Mormodica charantia* [AGARWAL & KAMAL, 2004], and *B. diffusa* [CHRISTIAN & al. 2006]. The present study is only a preliminary work towards identification of *in vitro* biosynthesis of flavonoids.

Conflict of interest

The authors have not declared any conflict of interest.

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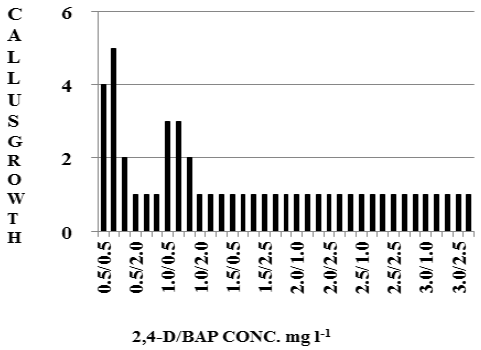

Fig 1. Effect of 2,4-D and BAP concentrations on growth of *B. diffusa* callus. On the horizontal axis concentration above the bar indicate 2,4-D and that below indicate BAP. The callus growth has been measured on a scale of 1 to 5 taking into consideration size of callus at the end of growing period, morphological quality of callus and subculturable or not.

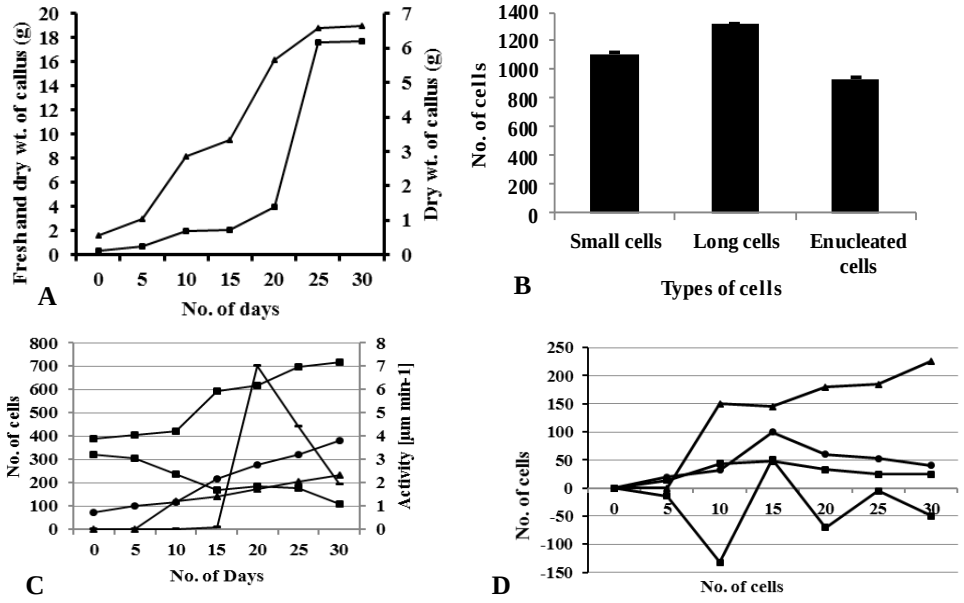

Fig 2 A. *B. diffusa* callus growth, elucidated by fresh weight (▲—▲) and dry weight (■ — ■) changes over a period of 30 days; **Fig 2 B.** Numbers of different types of cells at the end of a growth period of 30 days in the callus cultures of *B. diffusa*; **Fig 2 C.** Variation in small (■-■), long nucleated (●-●), long but enucleated (▲-▲), total Cells (**x-x**) and peroxidase (— - —) over a period of 30 days in the callus cultures of *B. diffusa*. This graph clearly represents that as the numbers of small nucleated cells turns to enucleated cells there is simultaneously increases of peroxidise activity. The small isodiametric cells were used to initiate callus; **Fig 2 D.** Is a balance sheet, small cells (■-■), was mostly negative throughout culture period except on day 15 when a slight increment of cells. long and nucleated cells (●-●), long but enucleated cells (▲-▲), and total cells (**x-x**) at five day intervals in callus cultures of *B. diffusa*. Total culture period 30 days.

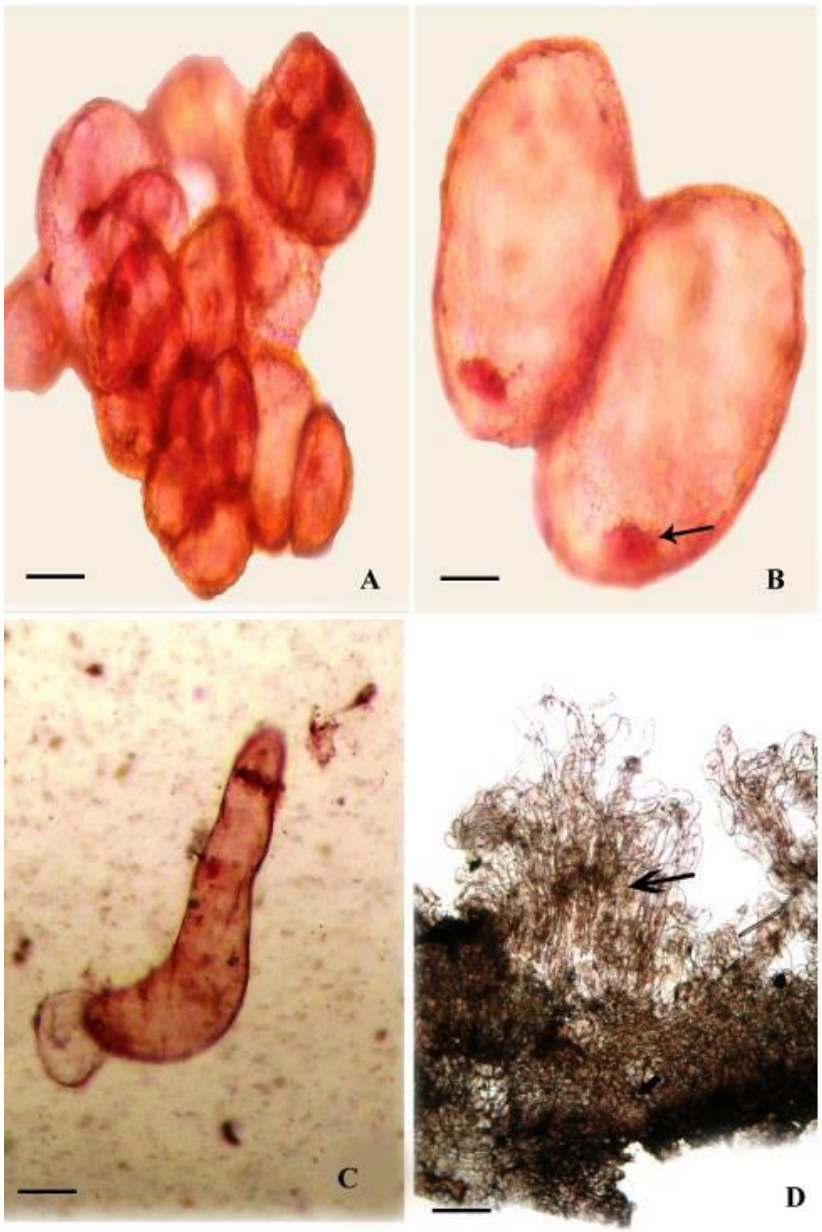

Fig 3. A-F: Types of cells in the callus of *B. diffusa*, A. small isodiametric cells with centrally placed nucleus, B. elongated cells with peripheral nucleus, C. enucleated cells, in which nucleus did not stained with acetocarmine, D. vertical sections of 15-day old callus. Note the small isodiametric cells (small, thick arrow), and the elongated cells (long, thin arrow); Cw = cell wall; dCyt = dense cytoplasm; dp = depositions; N = nucleus. (Bar: Fig A 13.5 µm; Fig B 13 µm; Fig C 40 µm; Fig D 41 µm; Fig E 19 µm; Fig F15.83 µm)