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In Vitro Enhancement of Bacoside in Brahmi (*Bacopa monnieri*) Using Colchicine

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Abstract

Present investigation on *in vitro* effects of two levels of colchicine (0.1% and 0.2%) for 1, 2, 3, 4 and 5 hours treatment on leaf explants of Brahmi (*Bacopa monnieri*) collected from Paithan (Aurangabad) was conducted with a view to induce somaclonal variations and enhancement in the bacoside content in regenerated plants. Bramhi plants regenerated through previously treated leaf explants with colchicine showed morphological variations in leaf shape, number of leaves per node, leaf arrangement, etc. and enhance the bacoside content. 0.2% colchicine treatment for 5 hours showed maximum variations in number of leaves per node under *in vitro* conditions; however, these variations were not continued after subculture. While the 0.1% colchicine treatment for 2 hours significantly increase the bacoside content up to 0.72% i.e., more than four folds to the *in vitro* regenerated Brahmi plants without colchicine treatments.

Keywords: Bacoside; Colchicines; Morphology; Phyllotaxy; Somaclonal variation; *Bacopa monnieri*

Introduction

Bacopa monnieri, a small herb, commonly called as Brahmi belongs to the family Scrophulariaceae. It grows in the humid climate, mainly distributed in damp and marshy tracts in the subtropical region of the Indian subcontinent. It requires a well-drained, moist, sandy loam soil, rich in organic matter and grows well at a temperature from 30°C to 40°C [1]. Brahmi is an important Ayurvedic medicinal herb used for the improvement of intelligence, memory and revitalization of sensory organs [2]. The major chemical entity shown to be responsible for neuropharmacological effects and the nootropic action of *B. monnieri* is triterpenoid saponin [3]. The medicinal properties of *Bacopa monnieri* responsible for improving memory related function have been attributed to the presence of different types of saponins such as Bacosides (A, B, C and D) called the "memory chemicals" i.e., important secondary metabolite.

Brahmi has a good market demand due to its medicinal properties. Estimated consumption of this drug in India is 1000 tons per year [4]. More than 90% of plant species used by the industry are however collected from the wild source of which 70% involves unorganized harvesting. This factor poses a serious threat to the genetic stock and the biodiversity of medicinal plant. The natural regeneration is also hampered by death of plant at two leaf stage and specific habitat requirement. The report published by National Medicinal Plant Board (NMPB), Government of India and Technology Information Forecasting and Assessment Council (TIFAC) in 2007 recommended immediate attention to few medicinal plants, among which Bacopa monnieri prominently features, which makes this plant in the category of highly endangered plants in India [5]. Moreover, because of the heavy demand and short supply, it is the most adulterated species in Ayurvedic formulations. So, there is need to find the alternatives to enhance the production of Bacoside content of Brahmi. In present scenario plant cell culture is an attractive alternative approach to produce bioactive secondary metabolites.

According to Leva et al. [6] the somaclonal variations have been observed because of the changes in chromosome number and structure, in which polyploidy is the most frequent. However, the somaclonal variations have a great potential for the crop-improvement [7]. These variations have been evaluated for morphological traits like pigment production, biochemical characters like nicotine synthesis, chromosome number and structure [8]. Somaclonal variations can be artificially induced by *in vitro* mutagenesis using various physical as well as chemical agents and by the polyploidation.

Colchicine is one of the chemical known to induce somaclonal variations by polyploidation in cultured plant cells. It is a highly poisonous alkaloid, originally extracted from *Colchicum autumnale* which is used in the medicine, especially for the treatment of gout. In plants colchicine binds to the tubulin, one of the main constituents of microtubules and therefore to nuclear spindle and thus the development of spindle fiber is hampered which results in inhibition of mitotic division. This further leads to the induction of polyploidy. Colchicine is used to induce polyploids, which increases secondary metabolite production potential and has been used for many years to produce valuable compounds in plants [9]. Dhawan and Lavania [10] reviewed that the induced polyploidy can confer enhanced production and/or qualitative improvement in the biochemical profile of secondary metabolites. However, there are very few reports on the enhancement of Bacoside production in *Bacopa monnieri*. In this context, the aim of this study was to discover the optimum level of colchicine and the treatment duration for the enhancement of bacoside synthesis in *B. monnieri*.

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Materials and Methods

Brahmi (*Bacopa monnieri*) plantlets were collected from Paithan (19°28′33.9852′′ N and 75°22′45.0948′′ E), Tal. Paithan, Dist. Aurangabad, Maharashtra, India and planted in nursery. The healthy, disease free and young leaf explants were selected for experiment [11]. The leaf samples were surface sterilized. For the induction of polyploidy, colchicine was prepared by dissolving in 1% Dimethyl Sulfoxide (DMSO) solution and sterilized by autoclaving. The sterilized leaf explants were treated with 0.1 and 0.2% colchicine for 1, 2, 3, 4 and 5 hrs. These experiments were performed by factorial arrangement based on complete randomized design with three replications. Treated explants were inoculated on Murashige and Skoog (MS) media supplemented with 6-benzylaminopurine (BA) (1.1 µM) and Indole-3-butyricacid (IBA) (0.30 µM) with 3% sucrose. The pH of culture media was maintained in between 5.6-5.8. The inoculated culture tubes were incubated in culture room at 25 ± 2°C for 16/8 hrs photoperiod.

After 4 weeks of inoculation the regenerated multiple shoots were removed from culture tubes and washed with water to remove debris of media. The morphological observations were recorded and biomass was oven dried at 60°C for 12 hrs. Dried explants were crushed with mortar and pestle. Uniform sample was used for extraction. The powdered samples were soaked in 3 ml absolute ethanol and kept for 24 hrs. The extracts were then filtered by Whatman No. 42 filter papers [12]. The ethanol extract was used for quantitative detection of Bacoside content in Brahmi. Ethanolic extract (40 µl) was taken from 3 ml and final volume was made up to 4 ml by using ethanol. The analysis and detection was carried out by using UV Spectrophotometer at 278 nm. Standard graph of bacoside was prepared by using bacoside mixture purchased from Sigma Aldrich. The reading of test sample was compared with the standard bacoside [13].

The data obtained was analyzed by "Analysis of Variance" method [14]. The variance due to the treatments was compared with the error variance to find out "df" values and ultimately results were inferred at 5% and 1% probability level of significance. The standard error for factors based on error variance was calculated. Whenever, the result was found to be significant, critical difference (CD) is calculated for comparison of treatments mean at 5% and 1% of significance (CD at 5% and 1%).

Results and Discussion

Micropropagation of Brahmi (*Bacopa monnieri*)

The colchicine treated leaves of Brahmi (*Bacopa monnieri*) were regenerated under *in vitro* condition. The leaf curling was observed a week after the inoculations followed by the callus formation at wounded sites and shoot initiation simultaneously after the second week of inoculation (Figure 5a). Shoot initiation was due to the synergistic effect of BA (1.1 µM) and IBA (0.30 µM). These results agree with those reported previously by Escandón et al. [15] with regards to shoot multiplication in nodal explants of Brahmi with the BA (0.25 mg/L). Similarly, Nagahatenna and Peiris [16] studied *Hemidesmus indicus* (L.) R. Br. (Iramusu) increase in shoot number in nodal explants after treatment of colchicine under *in vitro* condition. Artichart [17] also observed increase in shoot proliferation in *Dendrobium chrysotoxum* under *in vitro* condition.

Effect colchicine treatments on morphology of regenerated plants

In the present investigation colchicine treatment found to be affecting the phenotypic characteristics of the regenerated plants under *in vitro* condition. Data presented in Table 1 and depicted in Figure 5d and 5e showed that the colchicine concentration at 0.2% for 5 hrs incubation period showed the substantial variation in phyllotaxy (Figure 5c) and number of leaves produced, i.e., 3, 4, 5 and 6 leaves per node in contrast to control where two leaves per node were observed, Whereas, the other treatments also showed the increase in the number of leaves per node. These variations were not consistently observed in all the replications. Hence, there is no basis for statistical analysis. Also, there is abnormal pigmentation observed in the leaves showing yellowish, pale yellow and colourless leaves (Figure 5c and 5d) and the variations in the leaf shape and size (Figure 5b-5e) as compare to the normal plant regenerated without colchicine treatment. Leva et al. [6] suggested that the morphological characters are used for the detection of somaclonal variation. However, these variations were not continued, when recultured on the same regeneration media, hence these variations are the epigenetic somaclonal variations [18]. Colchicine treatment affects the chromosomal number and expression of genes which further results in cytological abnormalities [19,20], qualitative and quantitative phenotypic mutation [21], sequence change, gene activation and silencing [22]. Epigenetic activation of DNA elements further suggests that epigenetic changes may also be involved in cytogenetic instability through modification of heterochromatin, and as a basis of phenotypic variation through the modulation of gene function [23]. It was also observed that DNA methylation patterns are highly variable among regenerated plants and their progeny provides evidence that DNA modifications are less stable in culture than in seed-grown plants [18]. However, there were no previous evidences of such type of phenotypic variations in *B. monnieri* reported by the earlier research workers. Similar type of somaclonal variations were recorded by Israeli et al. [24] in banana cultivars under *in vitro* condition. According to Lee and Phillips [25] and Hwang and Ko [26] such results might be due to the increase in chromosome numbers [27]. Similar results were observed by the Escandon et al. [15], they reported the change of leaf and flower size of the Brahmi after treatment with Colchicine. Babil et al. [28], Leva et al. [6], Mujib [29] and Omidbaigi et al. [30] also reported the effects of the colchicines on the leaf size of various plants. Many other researchers have reported that the increase of ploidy often causes anatomical and structural changes in the plants [10]. Skirvin et al. [31] reported that the causes of somaclonal variation are not well understood and have not been elucidated. However, among the heritable types of variation, single base-pair changes, chromosome deletions, translocations, and changes in ploidy have been encountered [32].

0.1 and 0.2% Cholchicine Treatments for 1, 2, 3, 4 and 5 hrs incubation period	Morphological variations			
	3 leaves per node	4 leaves per node	5 leaves per node	6 leaves per node
C_1I_1	-	-	-	-
C_1I_2	+	-	-	-
C_1I_3	+	+	-	-
C_1I_4	-	+	-	-
C_1I_5	+	+	-	-
C_2I_1	-	-	-	-
C_2I_2	-	-	-	-
C_2I_3	+	+	-	-
C_2I_4	-	-	-	-
C_2I_5	+	+	+	+

*__Note:__ "+" the treatments produces more than two leaves per node and "-" the treatments produces normal two leaves per node. C_1- 0.1% and C_2- 0.2% Colchicine. I_1- 1 hr, I_2- 2 hr, I_3- 3 hr, I_4- 4 hr, I_5- 5 hr incubation period.

Table 1: Variation in No. of Leaves per Node.

Effect of colchicine on shoot multiplication, fresh weight, dry weight and bacoside content

Data presented in Table 2 (Figures 1-4) revealed that the shoot number of Brahmi was influenced significantly due to different concentration of Colchicine. Colchicine concentration at the rate of 0.1% recorded highest number of shoots (45.00 shoots per explant) which was found significantly superior over the concentration of 0.2% with regard to number of shoots per explant. Similar type of reduction in number of shoots with increase in colchicine concentration was observed by Nagahatenna and Peiris [16] in *Hemidesmus indicus*. The fresh weight and dry weight of Brahmi was found non-significantly influenced by the colchicine concentrations. The highest concentration of bacoside, i.e., 0.362% dry weight (DW) was found in colchicine 0.1% which was significantly superior over rest of the treatments. Xing et al. [33] and Butt et al. [34] observed enhancement in secondary metabolite production after colchicine treatment in *Catharanthus roseus* and Rose varieties respectively.

Effect of incubation period on shoot multiplication, fresh weight, dry weight and bacoside content

Incubation period significantly influenced the number of shoots in *B. monnieri*. Data presented in Table 2 (Figures 1-4) indicated that the colchicine treatment for 5, 1 and 4 hrs were at par and recorded significantly higher number of shoots (48.50, 47.33 and 44.34 respectively) over 2 and 3 hrs incubation period. The incubation period of 2 hrs also proved significantly effective over incubation period for 3 hrs. The effect of different incubation periods on fresh weight per explants was found significant. Colchicine treatment of 5 hrs was significantly superior over rest of the treatments and produced maximum fresh weight (1.04 gm per explant). Significantly higher dry weight (0.082 gm per explants) of *B. monnieri* was obtained with the incubation period of 5 hrs. However, least dry weight (0.029 gm per explant) was obtained at incubation period of 2 hrs. Lindayani et al. [35] and Jala [36] observed significant variations in morphological characters and biomass production. Highly significant concentration of bacoside (0.527% DW) was found in 2 hrs colchicine treatment and least (0.133% DW) in 5 hrs treatment.

Interaction effect of colchicine concentration and incubation period on shoot multiplication, fresh weight, dry weight and bacoside content

Data presented in Table 3 (Figures 1-4) revealed that the number of shoots per explant was influenced significantly due to interactions of colchicine concentration and incubation period. The highest number of shoots per explant (61.33) of *B. monnieri* was found in colchicine 0.1% for 5 hrs incubation period while the least number of shoots per explant (22.33) of *B. monnieri* was found in colchicine 0.2% for 3 hrs. The treatments of colchicine concentration 0.1% for 5 hrs, 1 hr and 0.2% for 5 hrs were at par with each other for the enhancement of fresh weight (1.10, 1.00 and 0.99 gm per explants respectively) and found superior over rest of the treatments presented in Table 4 (Figure 2). The data presented in Table 5 (Figure 3) showed that the 0.08 gm dry weight was obtained from the explants after the treatments of colchicine concentration 0.1% for 4, 5 hrs and 0.2% for 5 hrs were at par with each other and superior over rest of the treatments. Data given in Table 6 (Figure 4) indicated that the highest concentration of bacoside (0.717% DW) was produced by the colchicine 0.1% for 2 hrs incubation period and significantly superior over the rest of the treatments. It was found more than four folds to the *in vitro* regenerated Brahmi plants in the

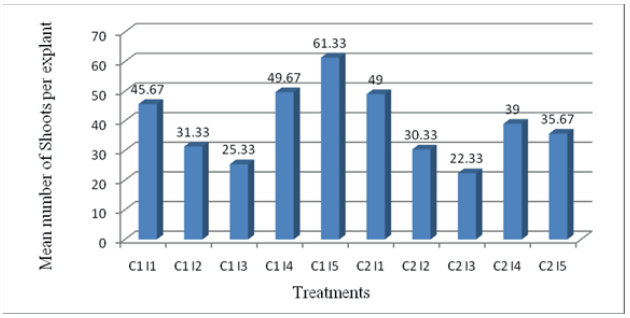

Figure 1: Effect of the colchicines concentration and incubation period on Mean number of shoots per explants in *B. monnieri.*

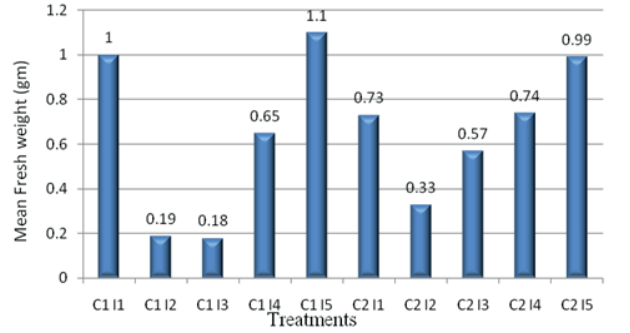

Figure 2: Effect of the colchicines concentration and incubation period on Mean fresh weight (gm) per explants in *B. monnieri.*

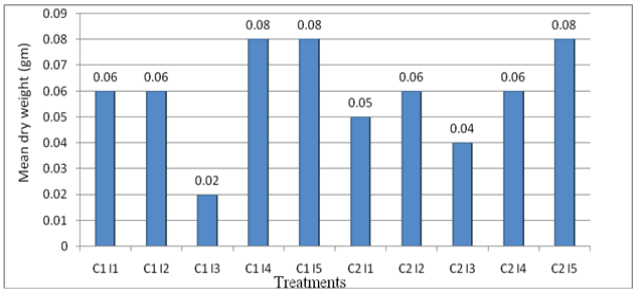

Figure 3: Effect of the colchicines concentration and incubation period on Mean dry weight (gm) per explants in *B. monnieri.*

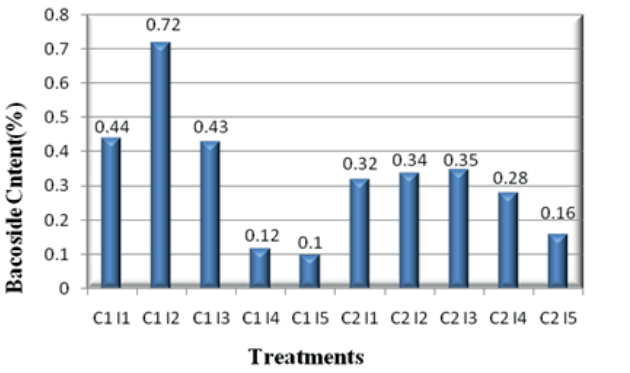

Figure 4: Effect of the colchicines concentration and incubation period on Mean Bacoside content (% DW) in *B. monnieri*.

Colchicine level	Mean No. of Shoot	Mean Fresh weight (gm)	Mean Dry Weight (gm)	Mean Bacoside content (% DW)
C_1-0.1%	45	0.62	0.06	0.362
C_2-0.2%	35.26	0.65	0.06	0.291
SE	1.85	0.03	0.0028	0.0016
CD	5.5	N.S.	N.S.	0.005
Incubation Period				
I_1	47.33	0.86	0.058	0.38
I_2	23.83	0.37	0.029	0.527
I_3	30.38	0.25	0.061	0.388
I_4	44.34	0.69	0.07	0.203
I_5	48.5	1.04	0.082	0.133
SE	1.17	0.019	0.0017	0.0026
CD	3.47	0.057	0.0052	0.0080
Interaction effect				
SE	2.48	0.04	0.0039	0.003
CD	7.33	0.12	0.118	0.011

Where C_1-0.1% and C_2-0.2% Colchicine and I_1-1 hr, I_2-2 hr, I_3-3 hr, I_4-4 hr, I_5-5 hr incubation period. DW=Dry weight.

Table 2: Effect of colchicine level and incubation period on No. of Shoot, fresh wt., dry wt. and bacoside content of Brahmi (*Bacopa monnieri*).

F_2 / F_1	I_1	I_2	I_3	I_4	I_5
C_1	45.7	31.3	25.3	49.7	61.3
C_2	49	30.3	22.3	39	35.7

SE ± 2.48, CD=7.33 Where F_1=Colchicine concentration and F_2=Incubation period

Table 3: Interaction effect of colchicine and incubation period on number of shoots per explants of Brahmi (*Bacopa monnieri*).

F_2 / F_1	I_1	I_2	I_3	I_4	I_5
C_1	1.00	0.19	0.18	0.65	1.10
C_2	0.73	0.33	0.57	0.74	0.99

SE ± 0.04, CD=0.12, Where F_1=Colchicine concentration and F_2=Incubation period

Table 4: Interaction effect of colchicine and incubation period on fresh weight of Brahmi (*Bacopa monnieri*).

F_2 / F_1	I_1	I_2	I_3	I_4	I_5
C_1	0.06	0.06	0.02	0.08	0.08
C_2	0.05	0.06	0.04	0.06	0.08

SE ± 0.0039, CD=0.0118, Where F_1=Colchicine concentration and F_2=Incubation period

Table 5: Interaction effect of colchicine and incubation period on Dry weight of Brahmi (*Bacopa monnieri*).

F_2 / F_1	I_1	I_2	I_3	I_4	I_5
C_1	0.437	0.717	0.430	0.123	0.103
C_2	0.323	0.337	0.347	0.283	0.163

SE ± 0.0036, CD=0.0110, Where F_1=Colchicine concentration and F_2=Incubation period

Table 6: Interaction effect of colchicine and incubation period on bacocide content of Brahmi (*Bacopa monnieri*).

same media without colchicine treatment. The lowest concentration of bacoside (0.103% DW) was produced in colchicine 0.1% for 5 hrs incubation.

As we observed in the present investigation, there are variations in number of shoots, fresh weight, dry weight in colchicine treated plants. The number of shoots observed in 0.1% colchicine treatment was more as compare to 0.2% colchicine treatment. But there was change in their size in the regenerated shoots from 0.1% colchicine treatment were stunted and thinner compare to 0.2% colchicine treatment, that's why, there biomass was less than the 0.2% colchicine treatment. Escondon et al. [15] observed morphological variations in colchicine treated *in vitro* regenerated *B. monnieri* plants. Also, Lindayani et al. [35], Jala [36] and Vijayalakshmi and Singh [37] observed that the variations in concentration of colchicine and incubation period for treatment, the resulting plants showed varied characteristics. Colchicine treatment changes the regenerated plants characteristics in different ways. It is observed that there is lack of either increasing or decreasing trend which is commonly observed in case of growth hormones studies. It is interesting to note that the increase in concentration of colchicine and treatment time changing regenerated plant characteristics in very bizarre fashion as presented in Table 2. There is no correlation among shoot number, fresh weight, dry weight and colchicine treatments (Figure 6).

The results obtained from this research work clearly indicates that colchicine treatments for various time periods induces change in number of Shoots, fresh weight, dry weight and bacoside content of Brahmi (*Bacopa monnieri*). Colchicine is an antimitotic substances used for the polyploidy induction in the plants. Vijayalakshmi and Singh [37], Ganga and Chezhiyan [38], Amiri et al. [39] and Ahmed et al. [40] used colchicine for the polyploidation in plants. It binds to cell protein tubulin and arrests mitosis in metaphase due to failure of spindle formation. It causes depolymerisation and disappearance of the fibrillar microtubules in granulocytes and other motile cells, inhibiting their migration as well as metabolic and phagocytic activity [17]. In many plant species colchicine causes side effects such as sterility, abnormal growth and morphology, chromosome losses or rearrangements and gene mutation [18].

Likewise, the changes observed in the fresh weight and dry weight of Brahmi due to the colchicine treatments. Belabbassi et al. [41] stated that the secondary metabolite induction may cause decreased cell mass and increased metabolite production. The aim of biotechnological process is to increase the target molecule content in medicinal plant species by improving biomass and secondary metabolite content associated with polyploidy [42]. It has been reported by Shahriari et al. [43] that tetraploid hairy roots of *Hyoscyamus muticus* showed an increase of 17% in dry weight compared with diploid hairy roots as well as increase of hyoscyamine content up to two folds in tetraploids obtained through colchicine treatments in *Hyoscyamus muticus*

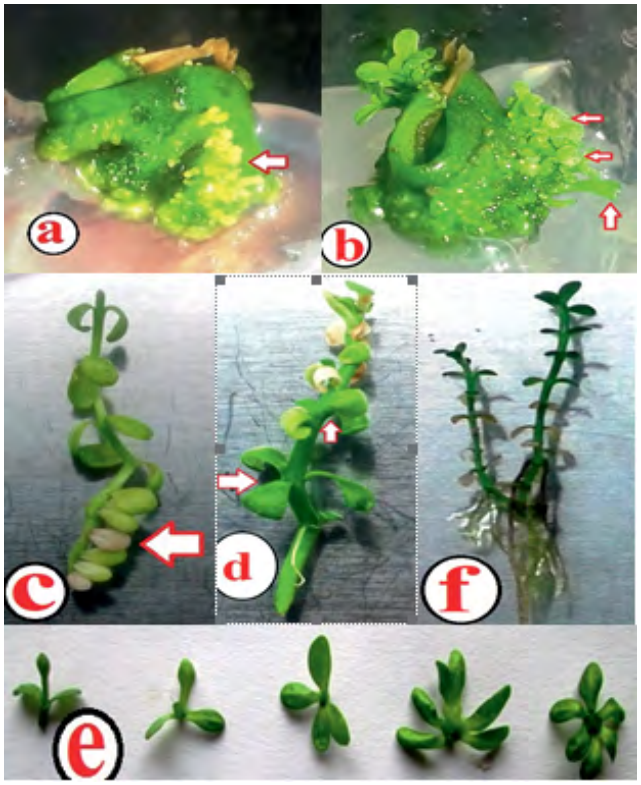

Figure 5: Morphological variations observed in regenerated Brahmi after Colchicine Treatment (Arrow). (a) Initiation of shoots from colchicine treated Leaf explants, (b) Variant Leaf regeneration from Colchicine treated leaf explants, (c) Variations in Leaf arrangements, (d) Variations in no. of leaves per node, (e) Variations in No. of Leaves per Node (Normal two to six leaves per Node), (f) *In vitro* regenerated normal whole Brahmi Plant.

Figure 6: Micropropagated plants after colchicine treatments: (a) Colchicine 0.1% for 1, 2, 3, 4 and 5 hours, (b) Colchicine 0.2% for 1, 2, 3, 4 and 5 hours.

L. Goluch and Skomra [44] recorded significant changes in levels of various types of terpens found in *Humulus lupulus* L. by using colchicine under *in vitro* conditions, whereas, significant increase in the proportion of humulene and limonene and reduced content of myrcene was observed. The currently undertaken investigation is the first report on the enhancement of bacoside production in Brahmi using colchicine. Similar type of work was done by the various researchers in other plants and found the similar trend of increase in the production of secondary metabolite by the colchicines treatment. Caruso et al. [45] observed the increase in the phenylpropanoid content in wild *Solanum commersonii*. Roopdarshani and Gayatri [46] reported significant biochemical traits in *Curcumma longa* (turmeric). Belabbassi et al. [41] recorded 206% increase in Hyoscyamine in *Datura stramonium*. Kaensaksiri et al. [47] demonstrated that the tetraploid plants showed a 1.37-fold increase in Asiaticoside content over the diploid plants.

Conclusions

This investigation was carried out for enhancement of bacoside content of Brahmi using various treatments of colchicine for different time periods and it showed that 0.1% colchicine treatment for 2 hrs is best suitable treatment for enhancing the bacoside content compared to rest other treatments. The maximum variation was observed more in case 0.2% colchicine treatments for 5 hrs compared to others. It clearly indicates that there is no relation between induced somaclonal variations and bacoside content.

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