Scientific Research Publishing

American Journal of Plant Sciences, 2021, 12, 1347-1360
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

In Vitro Propagation and Cytological Analysis of *Boerhaavia diffusa* L—An Important Medicinal Plant Species of Bangladesh

Sweety Majumder, Animesh Biswas, Mohammad Mahbubur Rahman

Department of Botany, University of Chittagong, Chittagong, Bangladesh
Email: majumdersweety01@cu.ac.bd

How to cite this paper: Majumder, S., Biswas, A. and Rahman, M.M. (2021) *In Vitro* Propagation and Cytological Analysis of *Boerhaavia diffusa* L—An Important Medicinal Plant Species of Bangladesh. *American Journal of Plant Sciences*, **12**, 1347-1360.
https://doi.org/10.4236/ajps.2021.129095

Received: July 19, 2021
Accepted: September 12, 2021
Published: September 15, 2021

Abstract

An effective and consistent *in vitro* propagation technique for *Boerhaavia diffusa* L. was developed using different types of explants and media compositions. Shoot apex and nodal explants of field grown plants were aseptically cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of auxins (IAA and NAA) and cytokinins (BAP and Kn). The maximum number of multiple shoot buds (6.65 ± 0.09) obtained from nodal explants on MS medium containing 2.0 mg/l BAP + 0.5 mg/l IAA. Multiple shoot buds underwent rapid elongation (4.24 ± 0.06 cm) on MS media fortified with 3.0 mg/l BAP + 0.5 mg/l NAA. Half strength MS media supplemented with 1.0 mg/l IBA + 0.5 mg/l IAA was better for induction and proliferation (6.42 ± 0.07) of roots and 75% of plantlets were successfully acclimatized to *ex vitro* condition, exhibiting a normal development. Somatic chromosome number of *in vivo* and *in vitro* grown plants were confirmed to be 2n = 26. Chromosome length ranged from 1.40 to 2.43 μm in the mother plants and 1.34 to 2.31 μm for *in vitro* grown plants. The total form percent (TF%) of mother and *in vitro* grown plants was 42.91% and 41.16%, respectively. The karyotype formula of *in vivo* grown plants was 2n = 26 = $4L^{sm} + 6M^{sm} + 2M^{m} + 14S^{m}$, whereas that of the *in vitro* grown plants was 2n = 26 = $8L^{sm} + 4M^{sm} + 2M^{m} + 12S^{m}$. The frequency of the chromosome having arm more than 2:1 was 0.08 for *in vivo* grown plants and 0.15 for *in vitro* raised plants. Thus, according to Stebbins classification (1971) the karyotype of both plants falls into 2A symmetrical type.

Keywords

Boerhaavia diffusa, Explant, Micropropagation, Chromosome, Karyotype

1. Introduction

Boerhaavia diffusa L., generally known as Punarnava, is a perennial creeping, diffusely branched herb belonging to the Nyctaginaceae family. Punarnava leaves are usually 1.25 - 5 cm long, thick, broadly ovate or suborbicular, rounded at the apex, usually white beneath. The flowers are small in size and pink in color, 4 - 10 together, in small umbels, arranged in very slender, axillary and terminal panicles [1]. The *Boerhaavia diffusa* plant contains many bioactive compounds like tannins, flavonoids, alkaloids (punarnavine), glycosides, steroids, terpenoids, phenolic compounds, rotenoids (boeravinones A-O) etc. [2] [3]. *B. diffusa* has various types of pharmacological properties like antilymphoproliferative, antiurethritis, antiasthmatic, antibacterial, anti-inflammatory, antileprosy, antidiabetic, immune-modulation and anti-metastatic [4]. In Bangladesh, there is virtually no cultivation of medicinal plants on any significant scale [5] for commercial purpose. Therefore, it is essential to take urgent steps for large scale propagation of this medicinal plant species, to conserve the plant and also meet the demand of ayurvedic industries.

Seeds of *B. diffusa* show a very low germination rate and vegetative propagation is limited by lower number of individuals. Micropropagation is the only rapid process for the mass propagation of *B. diffusa* and there have been few reports of tissue culture of *B. diffusa*. The tissue culture is also a powerful tool for accelerating the genetic breeding. The application of biotechnology especially *in vitro* culture provides an important tool to propagate the selected genotypes [6]. *In vitro* technique may be directed to development of different systems depending on the practical needs. Conventional cultivation methods are both slow and inadequate to meet the demand. The benefits of this technique are that it can make available a continuous, dependable source of natural products. Karyotype analysis has been used for resolving the taxonomic and evolutionary tribulations in many plant species [7] [8]. There was no detailed report on *in vitro* propagation and karyotypic analysis of *Boerhaavia diffusa* available in Bangladesh. In this article *in vitro* propagation and karyotype analysis of *B. diffusa* is described in detail.

2. Material and Method

The nodal segments and shoot apices from juvenile twigs of field grown plants of *B. diffusa* were collected and thoroughly washed under running tap water. The materials were then separated into small pieces, surface sterilized with 5% savlon and liquid soap (Tween 20) for 5 - 10 minutes with constant shaking. Then the explants were washed with distilled water 3 - 4 times for complete removal of detergent and taken under laminar airflow cabinet then transferred to 500 ml sterilized conical flask. After rinsing with 70% ethanol for less than 60 seconds, they were immersed in 0.1 ml $HgCl_2$ for 10 minutes and washed with sterile distilled water 4 - 5 times and then disinfected explants were cut into

minute pieces (0.5 - 1.0 cm) with a sterilized surgical blade and then inoculated onto the culture media. Murashige and Skoog (MS) medium containing 8% (w/v) agar supplemented with various concentrations and combinations of cytokinins (BAP and Kn) and auxins (NAA and IAA) was used for organogenesis. In order to induce differentiation, the regenerated plantlets were subcultured on medium supplemented with various concentration and combination of auxins (NAA and IAA) and cytokinins (BAP and Kn) and these were then cultured on elongation media. After that induced shoot buds were carefully removed from the medium and further washed with double distilled water appropriately to avoid any trace of medium on roots. *In vitro* regenerated shoot buds (5 - 6 cm long) were transferred onto the half strength MS medium supplemented with the auxins (NAA, IBA and IAA) for induction of root. The pH of the medium was adjusted to 5.8 prior to the addition of agar before autoclaving at 121˚C for 30 minutes under a pressure of 1.1 kg/cm^2. All the culture vessels with inoculated explants were then taken to the culture room for inoculation. The culture room was maintained at a regular cycle of 14 hours light and 16 hours dark with a light intensity of 2000 - 3000 lux. The temperature of culture room was maintained at 25˚C ± 2˚C. After proper root formation, the well rooted plantlets were removed from culture vessels and rinsed with running tap water for complete removal of medium which was attached to the roots; the well developed plantlets with roots were placed to pots containing a mixture of soil and compost (1:1). The hardened plants were transferred to a greenhouse (28˚C ± 20˚C, RH 90%) before transferring them in to the field. 15 explants were used for each treatment and all experiments data were statistically analyzed and expressed as mean ± standard deviation.

The roots of *in vivo* and *in vitro* grown plants were collected when they become 1 - 1.5 cm in size at 9.30 to 10.30 a.m. First growing healthy roots were collected and pretreated with saturated solution of Para-dichlorobenzene (PDB) for 3 h at room temperature (28˚C - 30˚C). The roots were then preserved in 70% (v/v) alcohol for long time preservation at 4˚C in refrigerator. The pretreated Roots were hydrolyzed in a mixture of 1 N HCl for 10 seconds at 60˚C. After gradually washing with distilled water, roots were deep into 2% (w/v) aqueous solution of iron alum for 5 - 10 minutes. Then the roots were further washed with distilled water for 3 - 4 times. Roots were stained in 0.5% (w/v) aqueous solution of haematoxylin for 15 - 20 minutes, and then squashed in a drop of 0.5% (w/v) acetocarmine. After that the hydrolyzed root tips were soaked using a filter paper and placed on a glass slide. After that the meristematic region of roots was cut with a fine blade. A clean cover glass was placed on the material then the prepared slides were then examined under an Optica Vison Pro microscope at a magnification of 1000×. Chromosomes were measured based on well separated metaphase cells. Classification of chromosomes was done on the basis of the nomenclature of Levan [9]. Several karyomorphological parameters were estimate by software (Karyotype XY).

3. Results and Discussion

3.1. *In Vitro* Propagation of *B. diffusa*

In order to establish a suitable protocol for *in vitro* mass scale propagation of *Boerhaavia diffusa* L., shoot apices and nodal segments of field grown plants were collected and aseptically cultured on MS medium supplemented with different concentrations of cytokinin (BAP and Kn) and auxin (NAA, IAA and IBA) in different combinations. Multiple shoot buds produced directly from nodal segments and shoot apices in different PGR containing media. Different concentrations of cytokinin BAP (0.5 - 3.0 mg/l) or Kn (0.5 - 3.0 mg/l) alone and in combination with different concentrations of NAA or IAA were used in MS medium for induction and development of MSBs in nodal segments and shoot apices of *B. diffusa*. Data were recorded after 30 days of culture and are presented in Table 1 and Table 2. Though nodal segments gave response in all the concentrations of BAP individually and BAP in combination with NAA or IAA within 12 - 20 days, the per cent of explant showing proliferation varied from 38 to 90 %. The highest percentage (90%) of MSBs induction form nodal segments was found in the medium containing 2.0 mg/l BAP + 0.5 mg/l IAA and this medium also produced highest number of MSBs (6.65 ± 0.09) per explant (Figure 1(a)). The nodal segments and shoot apices directly differentiated into multiple shoot buds when cultured on MS medium supplemented with a broad spectrum of PGR's combinations. Such direct organogenesis was reported to happen in many other medicinal plants including, *Passiflora foetida* [10], *Ocimum sanctum* [11], *Nilgirianthus ciliates* [12]

For elongation, the induced multiple shoot buds from nodal segments and shoot apices were individually cultured in aseptic condition on elongation media. Elongation media were prepared with different concentrations of cytokinins and auxin in MS medium. The results obtained from this experiment are presented in Table 3. The highest elongation (4.24 ± 0.06 cm) was observed in the medium fortified with 3.0 mg/l BAP + 0.5 mg/l NAA (Figure 1(c) & Figure 1(d)) after 30 days of inoculation. The effect of BAP in combination with NAA has been noted in many other medicinal plant species *viz Plectranthus amboinicus* [13], *Orthosiphon stamineus* [14] and *Viola serpens* [15].

In terms of root induction, Five to seven cm long *in vitro* grown shoots were separated and transferred to rooting media. Half strength MS medium fortified with different concentrations and combinations of auxins (IBA, IAA and NAA) were used for rooting experiment. The results of these experiment presented in Table 4. Among the three types of auxins, IBA was superior to that of IAA and NAA in terms of induction of roots per shoot. The maximum number of roots (6.42 ± 0.07) per shoot was formed in half strength MS medium supplemented with 1.0 mg/l IBA + 1.0 mg/l IAA and highest elongation (4.37 ± 0.09 cm) was also observed in the same medium (Figure 2(a) & Figure 2(b)). Half strength MS medium with combination of two auxins was found effective for rooting of many other medicinal plants such as *Caralluma diffusa* [16] and *Clausena heptaphylla* [17].

Table 1. Effects of BAP individually and in combination with NAA or IAA on induction of MSBs in nodal segments and Shoot Apices of *B. diffusa*.

PGRs supplement in the media (mg/l)		Explants	% of explants gave response	Time (d) require for induction of MSBs	Average* no. of MSBs/explant ($\bar{x}$ ± SE)
BAP	0.5	NS	58	14 - 18	1.15 ± 0.04^{m}
		SA	35	14 - 20	2.15 ± 0.08^{k}
	1.0	NS	65	14 - 18	1.11 ± 0.09^{n}
		SA	54	14 - 20	2.21 ± 0.06^{j}
	2.0	NS	68	12 - 16	2.13 ± 0.07^{l}
		SA	61	12 - 18	2.89 ± 0.10^{e}
	3.0	NS	42	15 - 20	1.05 ± 0.08^{o}
		SA	39	15 - 22	2.11 ± 0.10^{l}
BAP + NAA	2.0 + 0.2	NS	66	15 - 20	2.69 ± 0.09^{i}
		SA	61	12 - 16	2.66 ± 0.08^{g}
	2.0 + 0.5	NS	70	15 - 17	3.11 ± 0.07^{f}
		SA	82	10 - 12	6.15 ± 0.09^{a}
	2.0 + 1.0	NS	77	11 - 14	2.63 ± 0.06^{j}
		SA	75	10 - 14	4.79 ± 0.06^{b}
	3.0 + 0.2	NS	68	12 - 15	2.84 ± 0.08^{g}
		SA	64	11 - 13	2.8 ± 0.12^{f}
	3.0 + 0.5	NS	61	14 - 18	2.68 ± 0.05^{i}
		SA	64	11 - 13	2.8 ± 0.12^{f}
	3.0 + 1.0	NS	56	12 - 15	2.24 ± 0.06^{k}
		SA	60	12 - 14	3.86 ± 0.12^{c}
BAP + IAA	2.0 + 0.2	NS	73	13 - 20	4.03 ± 0.01^{d}
		SA	56	12 - 15	2.80 ± 0.09^{f}
	2.0 + 0.5	NS	90	12 - 15	6.65 ± 0.09^{a}
		SA	72	11 - 14	3.02 ± 0.06^{d}
	2.0 + 1.0	NS	81	14 - 16	6.15 ± 0.12^{b}
		SA	66	10 - 14	2.49 ± 0.07^{i}
	3.0 + 0.2	NS	48	13 - 17	4.26 ± 0.08^{c}
		SA	57	12 - 15	2.89 ± 0.10^{e}
	3.0 + 0.5	NS	43	13 - 16	3.81 ± 0.09^{e}
		SA	46	13 - 16	2.53 ± 0.06^{h}
	3.0 + 1.0	NS	38	12 - 18	2.80 ± 0.08^{h}
		SA	32	14 - 20	2.07 ± 0.05^{m}

d = days, MSBs = Multiple shoot buds, NS = Nodal Segment, SA = Shoot apices *values are the means of three replicates with 15 explants. Figures in the column having the same letter(s) within a location did not differ significantly according to DMRT ≤ 0.05.

Table 2. Effects of Kn individually and in combination with NAA or IAA on induction of MSBs in nodal segments and Shoot Apices of *B. diffusa*.

PGRs supplement in the media (mg/l)		Explants	% of explants gave response	Time (d) require for induction of MSBs	Average* no. of MSBs/explant ($\bar{x}$ ± SE)
Kn	0.5	NS	35	10 - 16	2.21 ± 0.10^{k}
		SA	35	10 - 16	1.17 ± 0.06^{j}
	1.0	NS	45	11 - 14	2.14 ± 0.06^{l}
		SA	45	11 - 14	1.12 ± 0.08^{j}
	2.0	NS	56	11 - 15	2.52 ± 0.05^{h}
		SA	56	11 - 15	1.13 ± 0.12^{j}
	3.0	NS	31	10 - 15	1.20 ± 0.10^{k}
		SA	31	10 - 15	0.51 ± 0.04^{ij}
Kn + NAA	2.0 + 0.2	NS	37	11 - 14	2.48 ± 0.03^{i}
		SA	54	12 - 16	2.36 ± 0.05^{f}
	2.0 + 0.5	NS	48	11 - 14	3.27 ± 0.07^{d}
		SA	65	12 - 18	2.88 ± 0.07^{b}
	2.0 + 1.0	NS	54	10 - 16	2.51 ± 0.06^{f}
		SA	78	10 - 14	3.87 ± 0.05^{a}
	3.0 + 0.2	NS	47	11 - 13	2.63 ± 0.08^{g}
		SA	51	11 - 14	2.86 ± 0.09^{c}
	3.0 + 0.5	NS	35	12 - 15	2.36 ± 0.07^{j}
		SA	42	12 - 16	2.37 ± 0.06^{f}
	3.0 + 1.0	NS	28	13 - 18	2.10 ± 0.08^{m}
		SA	36	10 - 14	2.10 ± 0.08^{g}
Kn + IAA	2.0 + 0.2	NS	54	12 - 16	3.67 ± 0.05^{c}
		SA	37	11 - 14	1.66 ± 0.07^{h}
	2.0 + 0.5	NS	65	12 - 18	3.76 ± 0.52^{b}
		SA	48	11 - 14	2.84 ± 0.08^{d}
	2.0 + 1.0	NS	78	10 - 14	4.21 ± 0.06^{a}
		SA	54	10 - 16	1.67 ± 0.07^{h}
	3.0 + 0.2	NS	51	11 - 14	3.67 ± 0.05^{c}
		SA	47	11 - 13	2.44 ± 0.08^{e}
	3.0 + 0.5	NS	42	12 - 16	2.79 ± 0.04^{f}
		SA	35	12 - 15	1.66 ± 0.09^{h}
	3.0 + 1.0	NS	36	10 - 14	2.88 ± 0.09^{e}
		SA	25	13 - 16	1.08 ± 0.09^{k}

d = days, MSBs = Multiple shoot buds, NS = Nodal Segment, SA = Shoot apices. *values are the means of three replicates with 15 explants. Figures in the column having the same letter(s) within a location did not differ significantly according to DMRT ≤ 0.05.

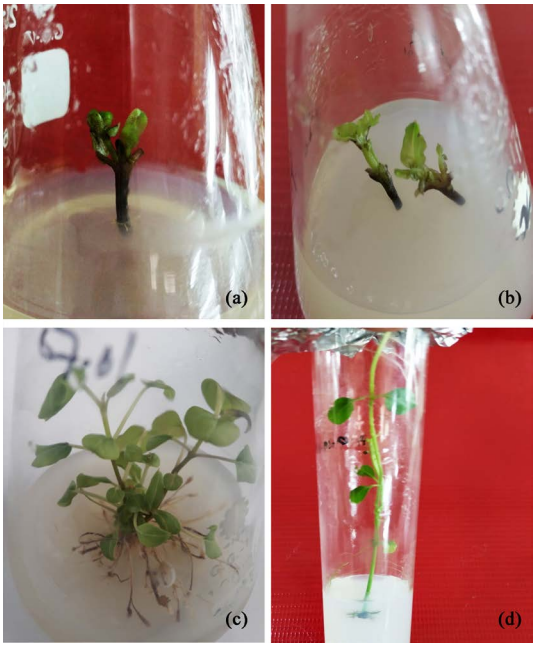

Figure 1. Induction and development of MSBs in nodal segments and shoot apices of *B. diffusa* through direct organogenesis. a) Initiation of MSBs in shoot apices after 12 days of culture; b) Induction of MSBs in nodal segments; c - d) Proliferation and elongation of MSBs on MS medium containing MS + 2.0 mg/l BAP + 0.5 mg/l IAA.

Table 3. Effects of different concentrations and combinations of plant growth regulators (PGRs) on the elongation of multiple shoot buds of *B. diffusa.*

PGRs supplement in the medium (mg/l)		Initial* length (cm) of individual shoot buds ($\bar{x}$ ± SE)	Length* (cm) of individual shoot buds after 30 d of culture ($\bar{x}$ ± SE)
BAP + NAA	2.0 + 0.5	0.90 ± 0.05^{k}	2.15 ± 0.06^{w}
	2.0 + 1.0	1.37 ± 0.07^{f}	3.66 ± 0.11^{q}
	3.0 + 0.5	2.03 ± 0.08^{a}	4.24 ± 0.06^{n}
	3.0 + 1.0	1.81 ± 0.08^{b}	3.82 ± 0.09^{o}
BAP + IAA	2.0 + 0.5	1.51 ± 0.11^{d}	3.70 ± 0.08^{p}
	2.0 + 1.0	1.39 ± 0.10^{e}	3.49 ± 0.06^{r}
	3.0 + 0.5	1.37 ± 0.10^{f}	3.22 ± 0.07^{s}
	3.0 + 1.0	1.62 ± 0.07^{c}	3.20 ± 0.02^{s}
Kn + NAA	2.0 + 0.5	1.06 ± 0.05^{i}	1.35 ± 0.07^{z}
	2.0 + 1.0	1.26 ± 0.07^{g}	2.91 ± 0.05^{t}
	3.0 + 0.5	1.08 ± 0.09^{h}	3.20 ± 0.07^{s}
	3.0 + 1.0	0.98 ± 0.12^{j}	1.87 ± 0.06^{x}
Kn + IAA	2.0 + 0.5	0.90 ± 0.11^{k}	2.18 ± 0.03^{v}
	2.0 + 1.0	1.07 ± 0.08^{h}	2.47 ± 0.10^{u}
	3.0 + 0.5	0.77 ± 0.11^{l}	2.59 ± 0.09^{t}
	3.0 + 1.0	0.54 ± 0.06^{m}	1.58 ± 0.08^{y}

d = days, MSBs = Multiple shoot buds. *values are the means of three replicates with 15 explants. Figures in the column having the same letter(s) within a location did not differ significantly according to DMRT ≤ 0.05.

Table 4. Effects of different concentrations and combinations of plant growth regulators (PGRs) on the development of roots in elongated shoot buds of *B. diffusa.*

PGRs supplements in the media (mg/l)		Days to root induction	% of micro shoot produced roots	Average* number of roots/micro shoot ($\bar{x}$ ± SE)	Average* length (cm) roots after 30 d of culture ($\bar{x}$ ± SE)
1/2 MS	0	18 - 25	33	2.23 ± 0.03^{m}	1.23 ± 0.11^{z}
1/2 MS + IBA	0.5	18 - 25	50	3.65 ± 0.03^{g}	2.76 ± 0.04^{u}
	1.0	16 - 20	65	5.45 ± 0.09^{b}	3.15 ± 0.04^{s}
	2.0	17 - 24	69	4.49 ± 0.08^{e}	2.91 ± 0.10^{t}
	3.0	17 - 22	73	3.03 ± 0.01^{i}	2.00 ± 0.15^{x}
1/2 MS + IAA	0.5	14 - 18	67	4.12 ± 0.09^{f}	2.12 ± 0.03^{w}
	1.0	15 - 17	76	4.91 ± 0.06^{d}	2.50 ± 0.04^{u}
	2.0	13 - 17	73	3.49 ± 0.05^{h}	3.18 ± 0.06^{r}
	3.0	14 - 16	58	3.39 ± 0.12^{h}	2.02 ± 0.04^{u}
1/2 MS + NAA	0.5	20 - 25	45	2.88 ± 0.05^{k}	2.02 ± 0.11^{y}
	1.0	22 - 25	57	3.01 ± 0.16^{j}	3.30 ± 0.02^{p}
	2.0	20 - 22	65	2.32 ± 0.07^{l}	2.53 ± 0.16^{v}
	3.0	18 - 20	38	3.39 ± 0.12^{h}	2.02 ± 0.24^{x}
1/2MS + IBA + IAA	1.0 + 0.5	12 - 16	87	5.43 ± 0.08^{b}	3.19 ± 0.05^{q}
	1.0 + 1.0	14 - 17	94	6.42 ± 0.07^{a}	4.37 ± 0.09^{n}
1/2 MS + IBA + NAA	1.0 + 0.5	13 - 18	61	5.21 ± 0.05^{c}	3.90 ± 0.11^{o}
	1.0 + 1.0	15 - 22	55	4.76 ± 0.12^{d}	2.56 ± 0.03^{v}

d = days, MSBs = Multiple shoot buds. *values are the means of three replicates with 15 explants. Figures in the column having the same letter(s) within a location did not differ significantly according to DMRT ≤ 0.05.

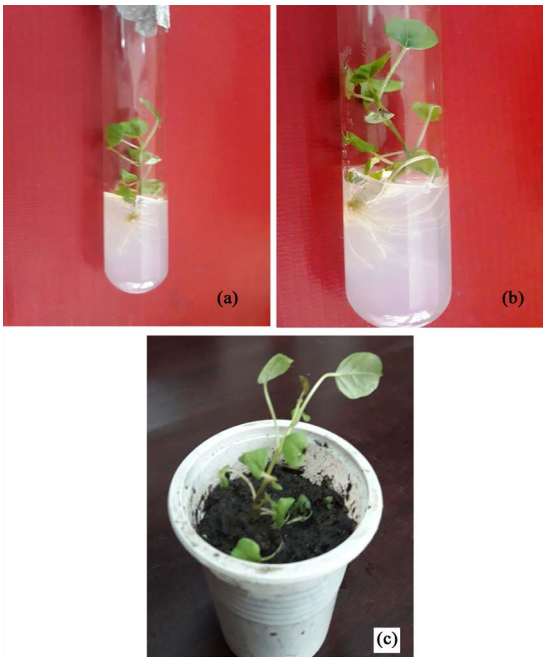

Figure 2. Rooting of shoot buds of *B. diffusa* and their establishment in pots. a - b) Induction and proliferation of roots in 1/2 MS medium containing 1.0 mg/l IBA + 1.0 mg/l IAA; c) *In vitro* developed complete plantlets after transplantation in plastic pot.

In order to establish the *in vitro* developed complete seedlings in the outside environment, the culture vessels with well profuse rooted plantlets were taken out of the culture room and follow several steps for acclimatization. Then the plantlets were planted in small plastic pots (Figure 2(c)) containing garden soil and compost in the ratio of 1:1 and kept in room temperature for 3 - 5 days and about 75%. Regenerated plantlets were morphologically uniform having normal phenotype.

3.2. Karyomorphological Studies of *Boerhaavia diffusa*

3.2.1. Karyotype of *In Vivo* Grown *B. diffusa*

Chromosomal analysis of *in vivo* plants revealed the presence of 2n = 26 chromosomes in the somatic cells. The individual chromosome length ranged from 1.40 to 2.43 µm (Table 5). In the basic set there were five submetacentric and eight metacentric chromosomes. The centromeric formula was, therefore, 5sm + 8 m (Figure 3(a)). Of the five submetacentric chromosomes two were long and three were medium. Among the eight metcentric chromosomes one was medium and seven were small (Figure 3(b) & Figure 3(c)). The appropriate karyotypic formula was $2L^{sm} + 3M^{sm} + 1M^{m} + 7S^{m}$. The total length of haploid complement was 23.13 µm and TF% was 42.91. The total length of the long arms was 13.20 µm and that of the short arms was 9.93 µm. The proportion between the longest and the shortest chromosome was less than 2:1 and the frequency of chromosomes having arm ratio more than 2:1 was 0.08. Thus the karyotype fell in 2A symmetrical type. Similar types of observations were noted in some other plants by few scientists such as *Liriope spicata* [18] and *Impatiens balsamina* [19].

Table 5. Length, Arm ratio, Relative length (RL), Centromeric index (CI), Centromeric type (CT), Chromosome type, TF% and Centromeric formula of mitotic metaphase chromosome of *in vivo* grown plants of *B. diffusa.*

Chromosome pair	Long arm (l) µm	Short arm (s) µm	Total length (T) µm	Arm ratio (l/s)	RL (%)	CI (%)	CT	Chromosome type	TF%	Centromeric formula
1	1.56	0.87	2.43	1.79	10.51	35.80	sm	L		
2	1.56	0.82	2.38	1.90	10.29	34.44	sm	L		
3	1.31	0.75	2.06	1.76	8.91	36.28	sm	M		
4	1.35	0.67	2.02	2.02	8.73	33.10	sm	M		
5	1.26	0.66	1.92	1.91	8.30	34.39	sm	M		
6	0.91	0.91	1.82	1.00	7.87	50.00	m	M		
7	0.80	0.8	1.60	1.00	6.92	50.00	m	S	42.91	5 sm + 8 m
8	0.76	0.76	1.52	1.00	6.57	50.00	m	S		
9	0.76	0.76	1.52	1.00	6.57	50.00	m	S		
10	0.75	0.75	1.50	1.00	6.49	50.00	m	S		
11	0.75	0.75	1.50	1.00	6.49	50.00	m	S		
12	0.73	0.73	1.46	1.00	6.31	50.00	m	S		
13	0.70	0.70	1.40	1.00	6.05	50.00	m	S		

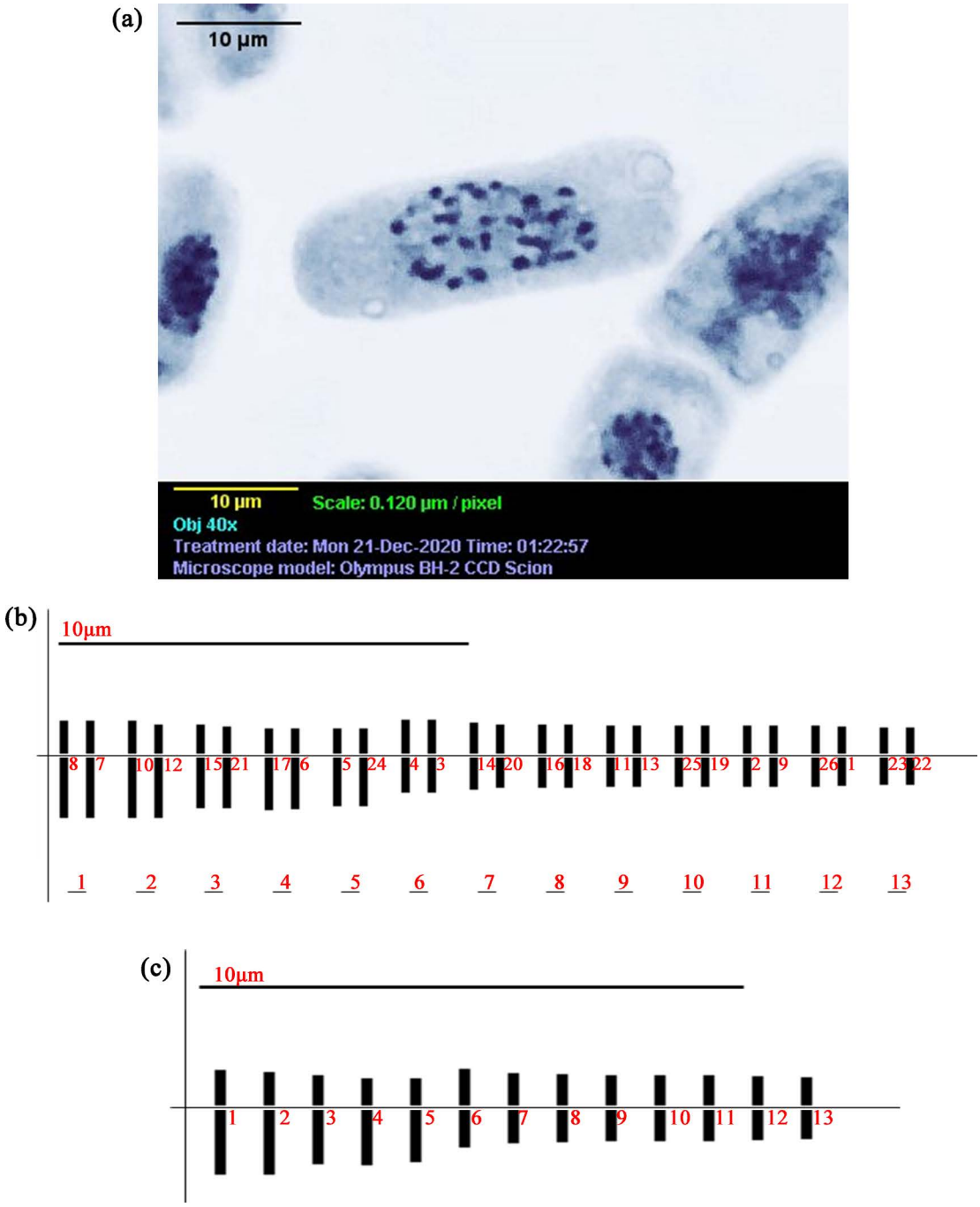

Figure 3. (a) Microscopic photograph of somatic metaphase chromosomes of mother plant of *B. diffusa*; ((b), (c)) Ideogram of mother plant, Scale bar = 10 µm; (b) Diploid complement, 2n = 26; (c) Haploid complement, n = 13.

3.2.2. Karyotype of *In Vitro* Grown *B. diffusa*

There were 26 chromosomes in each somatic of *in vitro* grown plants (Figure 4(a)) and the range of individual chromosome length was 1.34 to 2.31 µm (Table 6). Among the 13 basic chromosomes, six were submetacentric and seven were metacentric chromosomes (centromeric formula = 6 sm + 7 m). Four of the six submetacentric chromosomes were long and two were medium (Figure 4(b) &

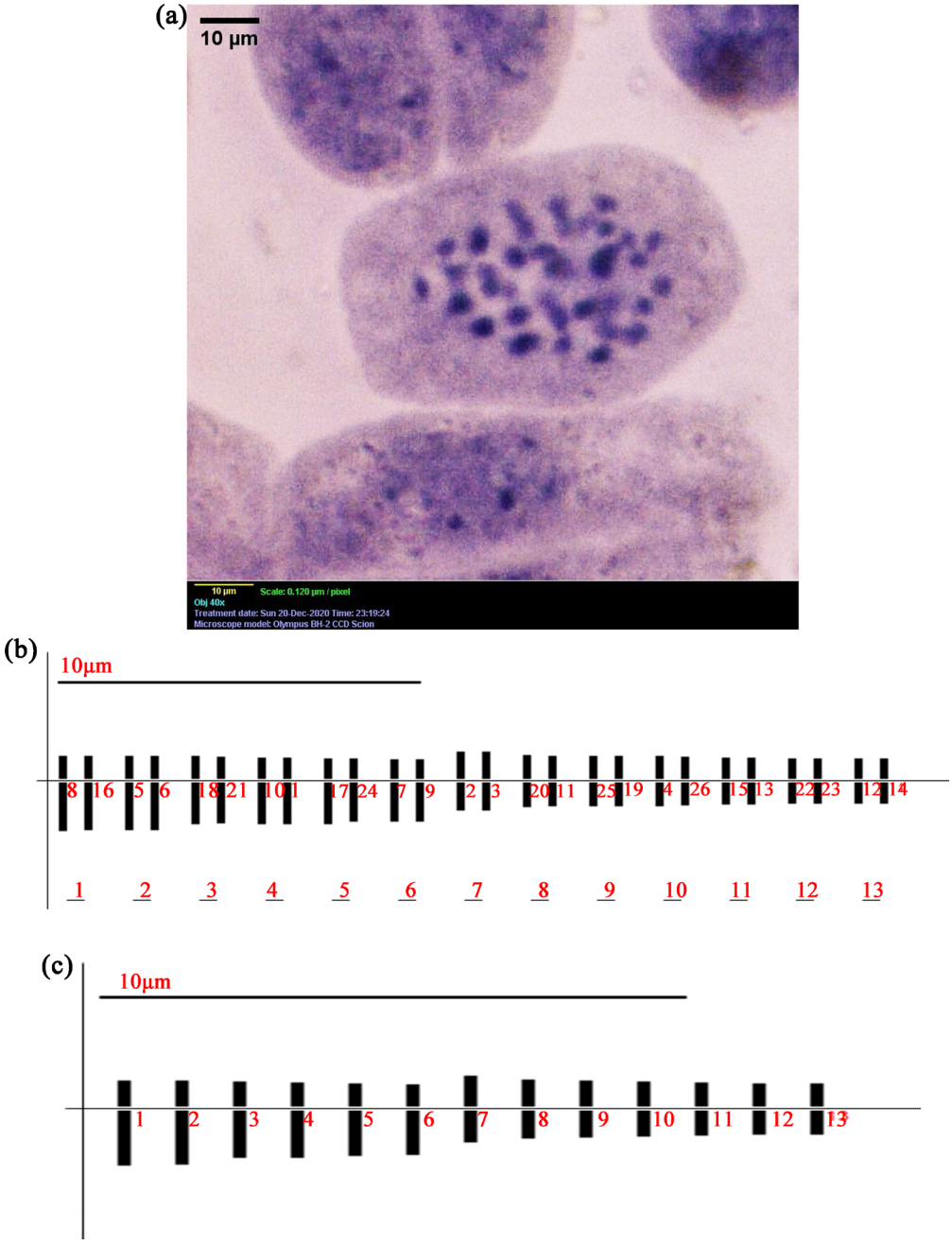

Figure 4. (a) Microscopic photograph of somatic metaphase chromosomes of *in vitro* grown plant *B. diffusa*; ((b), (c)) Ideogram of *in vitro* grown plant, Scale bar = 10 µm; (b) Diploid complement, 2n = 26; (c) Haploid complement, n = 13.

Figure 4(c)). Of the seven metacentric chromosomes, one was medium and six were small. The karyotypic formula was $4L^{sm} + 2M^{sm} + 1M^{m} + 6S^{m}$. The total length of haploid complement was 23.07 µm and TF% was 41.16. The length of the total long arms was 13.57 µm and that of the short arms was 9.50 µm. The ratio of the longest and the shortest chromosomes was less than 2:1 and the frequency of chromosomes with the arm ratio more than 2:1 was 0.15. This karyotype fell in 2A symmetric type.

Table 6. Length, Arm ratio, Relative length (RL), Centromeric index (CI), Centromeric type (CT), Chromosome type, TF% and Centromeric formula of mitotic metaphase chromosome of *in vitro* grown plants of *B. diffusa.*

Chromosome pair	Long arm (l) µm	Short arm (s) µm	Total length (T) µm	Arm ratio (l/s)	RL (%)	CI (%)	CT	Chromosome type	TF%	Centromeric formula
1	1.55	0.76	2.31	2.05	10.01	32.74	sm	L		
2	1.55	0.75	2.30	2.06	9.97	32.62	sm	L		
3	1.33	0.75	2.08	1.78	9.02	35.98	sm	L		
4	1.36	0.71	2.07	1.92	8.97	34.28	sm	L		
5	1.31	0.66	1.97	1.98	8.54	33.52	sm	M		
6	1.25	0.65	1.90	1.92	8.24	34.23	sm	M		
7	0.90	0.90	1.80	1.00	7.8	50.00	m	M	41.16	6 sm + 7 m
8	0.77	0.77	1.54	1.00	6.68	50.00	m	S		
9	0.75	0.75	1.50	1.00	6.50	50.00	m	S		
10	0.75	0.75	1.50	1.00	6.50	50.00	m	S		
11	0.71	0.71	1.42	1.00	6.16	50.00	m	S		
12	0.67	0.67	1.34	1.00	5.81	50.00	m	S		
13	0.67	0.67	1.34	1.00	5.81	50.00	m	S		

4. Conclusion

The result of this research work revealed that large scale *in vitro* propagation of *B. diffusa* is possible through direct organogenesis from nodal segments and shoot apices. MS medium containing 2.0 mg/l BAP + 0.5 mg/l IAA is the best for induction of multiple shoot buds and half MS medium fortified with 1.0 mg/l IBA + 1.0 mg/l IAA is the best root induction and root elongation. The survival percentage of the well rooted plantlets to the outside environment was 75%. The results of karyomorphological analysis indicate that basal media, plant growth regulators, culture time and subcultures did not affect the *in vitro* grown plants. There generated plants were morphologically similar to the mother plants of the selected medicinal plant species. There were very minute karyotypic variations between selected *in vivo* and *in vitro* plant species. Karyomorphological study of present investigation will be helpful to understand the number, morphology of chromosomes which are useful in cytotaxonomy and also beneficial for further research in cytogenetics.

Acknowledgements

The financial support from the Research &Publication cell in the University of Chittagong, Bangladesh for conducting this research work is gratefully acknowledged.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Yusuf, M., Begum, J., Hoque, M.N. and Chowdhury, J.U. (2009) Medicinal Plants of Bangladesh (Revised and Enlarged). Bangladesh Council of Scientific and Industrial Research (BCSIR) Laboratories, Chittagong, 794.

[2] Parmar, D., Jain, N.K. and Tomar, V. (2018) Anti-Arthritic Evaluation of Different Extracts of *Boerhaavia diffusa* Linn. in FCA Induced Arthritis in Rats. *Journal of Drug Delivery and Therapeutics*, **8**, 388-393. https://doi.org/10.22270/jddt.v8i5-s.1922

[3] Krishnamoorthy, P.K.P., Muthukumaran, S., Maheswaran, A. and Sukumaran, P. (2017) Isolation, Purification and Characterization of Boeravinone B from *Boerhaavia diffusa* Linn. *International Research Journal of Pharmacy*, **8**, 140-144. https://doi.org/10.7897/2230-8407.0811233

[4] Mehrotra, S., Singh, V.K., Agarwal, S.S., Maurya, R. and Srimal, R.C. (2002) Anti-Lympho Proliferative Activity of Ethanolic Extract of *Boerhaavia diffusa* Roots. *Experimental and Molecular Pathology*, **72**, 236-242. https://doi.org/10.1006/exmp.2002.2427

[5] Begum, F. (2002) *Ex Situ* Conservation of Medicinal Plants for Primary Health Care in Laxmipur District in Bangladesh. *Workshop on Wise Practices and Experimental Learning in the Conservation and Management of Himalayan Medicinal Plants*, Kathmandu, 15-20 December 2002.

[6] Campbell, M.M., Brunner, M.A., Jones, M.H. and Strauss, H.S. (2003) Forestry's Fertile Crescent: The Application of Biotechnology to Forest Trees. *Plant Biotechnology Journal*, **1**, 141-154. https://doi.org/10.1046/j.1467-7652.2003.00020.x

[7] Samadder, T., Nath, S., Halder, M., Sil, B., Roychowdhury, D., Sen, S., *et al.* (2012) Karyotype Analysis of Three Important Traditional Indian Medicinal Plants *Bacopa monnieri*, *Tylophora indica*, and *Withania somnifera*. *Nucleus*, **55**, 17-20. https://doi.org/10.1007/s13237-012-0048-2

[8] Young, H.A., Sarath, G. and Tobias, C.M. (2012) Karyotype Variation Is Indicative of Subgenomic and Ecotypic Differentiation in Switchgrass. *BMC Plant Biology*, **12**, 117. https://doi.org/10.1186/1471-2229-12-117

[9] Levan, A., Fredga, K. and Sandberg, A.A. (1964) Nomenclature for Centromeric Position on Chromosomes. *Hereditas*, **52**, 201-220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

[10] Shekhawat, M.S., Kannan, N., Manokari, M. and Ravindran, C.P. (2015) *In Vitro* Regeneration of Shoots and *ex Vitro* Rooting of an Important Medicinal Plant *Passiflora foetida* L. through Nodal Segment Cultures. *Journal of Genetic Engineering and Biotechnology*, **13**, 209-214. https://doi.org/10.1016/j.jgeb.2015.08.002

[11] Jamal, M., Sharif, I.H., Shakil, M., Rahman, A., Banu, N.A., Islam, M.R. and Nazmuzzaman, M. (2016) *In Vitro* Regeneration of a Common Medicinal Plant, *Ocimum sanctum* L. for Mass Propagation. *African Journal of Biotechnology*, **15**, 1269-1275. https://doi.org/10.5897/AJB2015.15200

[12] Rameshkumar, R., Largia, M.J.V., Satish, L., Shilpa, J. and Ramesh, M. (2017) *In Vitro* Mass Propagation & Conservation of *Nilgirianthus ciliates* through Nodal Explants: A Globally Endangered, High Trade Medicinal Plant of Western Ghats. *Plant Biosystems—An International Journal Dealing with All Aspects of Plant Biology*, **151**, 204-211. https://doi.org/10.1080/11263504.2016.1149120

[13] Rahman, Z.A., Noor, E.S.M., Ali, M.S.M., Mirad, R. and Othman, A.N. (2015) *In Vitro* Micropropagation of a Valuable Medicinal Plant, *Plectranthus amboinicus*.

American Journal of Plant Sciences, **6**, 1091-1097.
https://doi.org/10.4236/ajps.2015.68113

[14] Elangomathavan, R., Kalaivanan, P., Hariharan, P.S. and Beaulah, N. (2017) High Efficient Protocol for Callus Induction and Regeneration of a Medicinal Plant *Orthosiphon stamineus*. *International Journal of Advanced Research in Biological Sciences*, **4**, 113-122. https://doi.org/10.22192/ijarbs.2017.04.01.012

[15] Jha, R.S., Naz, R., Asif, A., Okla, K.M., Soufan, W., Al-Ghamdi, A.A. and Ahmad, A.A. (2020) Development of an *in Vitro* Propagation Protocol and a Sequence Characterized Amplified Region (SCAR) Marker of *Viola serpens* Wall. ex Ging. *Plants* (*Basel*), **9**, 246. https://doi.org/10.3390/plants9020246

[16] Prabu, M.K., Samydurai, P., Subbaiyan, B., Thangapandian, V. and Binu, T. (2013) *In Vitro* Propagation of a Rare Succulent Medicinal Plant *Caralluma diffusa* (Wight) N.E.Br. *Research in Plant Biology*, **3**, 8-17.

[17] Majumder, S. and Rahman, M.M. (2016) Effect of Different Plant Growth Regulators on *in Vitro* Propagation of *Clausena heptaphylla* (Roxb.)—An Aromatic and Medicinal Shrub. *Journal of Pharmacognosy and Phytochemistry*, **5**, 58-63.

[18] Zhou, Q., Zhou, J., Chen, J. and Wang, X. (2009) Karyotype Analysis of Medicinal Plant *Liriope spicata var. prolifera* (Liliaceae). *Biologia*, **64**, 680-683.
https://doi.org/10.2478/s11756-009-0069-8

[19] Momtaz, N.S.U., Kabir, G., Uddeen, M.M. and Yasmin, N. (2007) Karyotypic Study of Seven Types of *Impatiens balsamina* L. *Journal of Biological Sciences*, **15**, 147-152.
https://doi.org/10.3329/jbs.v15i0.2155