Improvement of growth and bacoside production in *Bacopa monnieri* through induced autotetraploidy with colchicine

7 Phithak Inthima[1,2], Kawee Sujipuli[2,3]

9 [1] Plant Tissue Culture Research Unit, Department of Biology, Faculty of Science, Naresuan
10 University, Phitsanulok, Thailand
11 [2] Center of Agricultural Biotechnology, Naresuan University, Phitsanulok, Thailand
12 [3] Department of Agricultural Science, Faculty of Agriculture Natural Resources and
13 Environment, Naresuan University, Phitsanulok, Thailand

15 The authors contributed equally to this work.

17 Corresponding Author:
18 Phithak Inthima[1,2]
19 Plant Tissue Culture Research Unit, Department of Biology, Faculty of Science, Naresuan
20 University, Phitsanulok, Thailand
21 Email address: phithaki@nu.ac.th

23 Kawee Sujipuli[2,3]
24 Department of Agricultural Science, Faculty of Agriculture Natural Resources and Environment,
25 Naresuan University, Phitsanulok 65000, Thailand
26 Email address: kawees@nu.ac.th

27

28

Abstract

30 *Bacopa monnieri* is a medicinal herb that is increasing in demand in Thailand. However, the lack
31 of high-bacoside cultivars has limited pharmaceutical utilization and production. Here,
32 chromosome doubling in *B. monnieri* was attempted to improve biomass and bacoside content in
33 its seedling. Nodal segments were treated with colchicine (0, 0.025, 0.05, 0.075, 0.1, and 0.5%
34 w/v) for 24 or 48 hours before transferring to multiple shoot induction medium (½MS medium
35 supplemented with 0.2 mg L^{-1} BAP). Of 326 tested clones, 18 and 84 were mixoploids and
36 autotetraploids, respectively. The highest autotetraploid-induction percentage (14.6%) was found
37 after treated with 0.5% (w/v) colchicine, and 48 hours exposure. From 28 selected autotetraploid
38 clones, 21 and 13 have significantly higher fresh and dry weight compared to the diploid clone,

39 respectively. The maximum fresh and dry weight of autotetraploid plants was 2.8 and 2.0-time
40 higher than diploid plants, respectively. Moreover, the maximum total bacoside content (1.55 mg
41 plant^{-1}) was obtained from an autotetraploid plant, which was 2.3-fold higher than the level in
42 diploid plants. These novel autotetraploids have the potential to be developed as resources for
43 value-added improvements in the medicinal and pharmaceutical industries.
44
45

46 **Introduction**
47 *Bacopa monnieri* (commonly known as waterhyssop or brahmi in Ayurvedic medicine) is a
48 small succulent herb that grows in marshy and moist tropical regions (Tripathi et al., 2012). It
49 has been extensively utilized as a traditional Ayurvedic medicine to enhance memory and brain
50 rejuvenation, promote longevity and improve intellectual functions (Gubbannavar et al., 2012).
51 Various pharmacological effects of brahmi have been reported including anti-parkinson's
52 (Jansen et al., 2014), antidepressant (Rauf et al., 2014), anti-anxiety (Pandareesh et al., 2014),
53 and as an Alzheimer's disease treatment (Uabundit et al., 2010). The bioactive compounds of
54 brahmi are mainly associated with triterpenoid saponins as complex mixtures (Deepak et al.,
55 2005). Of these, bacoside A is the core active compound which acts as a memory booster
56 (Ramasamy et al., 2015). Biosynthetic pathway of triterpenoid saponins, like sterols, had been
57 hypothesized through both the mevalonic acid (MVA) and the methylerythritol phosphate (MEP)
58 pathways (Augustin et al., 2011), resulting in the formation of intermediate substance as the
59 farnesyl pyrophosphate (*FPP*) by two enzyme actives of dimethylallyl diphosphate
60 (*DMAPP*), and geranyl pyrophosphate (*GPP*) (Acharya et al., 2009). This substance converts
61 into a C$_{30}$ molecule squalene by squalene synthase (*SQS*), and its molecule subsequently
62 cyclizes into triterpene skeletons by 2, 3-oxidosqualene (*OSCs*) (Niua et al., 2014). Finally,
63 their skeletons are catalyzed by cytochrome P450-dependent monooxygenases (*CYP450*) and
64 glycosyltransferase (*GT*) to produce triterpenoid saponins (Abbassi et al., 2015). Since the
65 triterpenoid saponin pathway involved with many genes, so the improvement of a new variety
66 for increasing saponin by pollination breeding is time consuming and extremely difficult
67 (Samaddar et al., 2012).
68
69 Many products containing brahmi are widely available today. To satisfy the high commercial
70 demand, 1,000 tons of raw plant material were used by herbal industries in 2011 (Muthiah et al.,
71 2013), with most of this collected from the natural resources (Tewari, 2000) and this led to the
72 rapid depletion of wild natural plants. However, cultivation of brahmi is restricted to certain
73 agroclimatic conditions in wetlands and muddy shores, and natural regeneration through seeds or
74 cuttings is slow and requires good irrigation facilities. Moreover, improvement of new varieties
75 of brahmi by conventional breeding is time consuming and difficult since the plant has a small
76 flower and a high number of chromosomes, 2n=2*x*=64 (Samaddar et al., 2012). Therefore, new
77 techniques have been developed to improve brahmi production. One of the most popular
78 techniques is elicitation with elicitors (physical agent, biological or chemical substances)

79 treatments (Sharma et al., 2013) to increase bacoside content. However, since there are few
80 reports concerning biosynthetic pathways in brahmi, the effects and functions of elicitation are
81 difficult to predict. One technique to solve this limitation involves creating new sustainable
82 varieties of brahmi through polyploidization using colchicine treatment because it is a rapid,
83 cost-effective, efficient and safe method (Sattler et al., 2016). Moreover, previous studies have
84 reported that tetraploid plants exhibit increased heterosis (hybrid vigor) such as increased oil
85 content in *Ocimum basilicum* (Omidbaigi et al., 2010), wider and thicker leaves in *Fagopyrum*
86 *tataricum* (Wang et al., 2017), and increased root size in *Echinacea purpurea* (Chen et al., 2016).
87 Previous studies have shown that the brahmi polyploids have been improved by enlarging the
88 flower size (Escandón et al., 2006) and the shoot (Sangeetha et al., 2011), this is possible that the
89 biomass and bacoside yield could be improved by polyploidization. In this study, chromosome
90 doubling in *B. monnieri* was attempted to improve biomass and bacoside content in its seedling.
91 The results may be beneficial for further genetic improvements to increase the medicinal value of
92 brahmi and raw drug production.
93
94

Materials & Methods

95
96 **Plant material and colchicine treatment**
97 Nodal segments (1 cm in length) of diploid *B. monnieri* were cut from one month in vitro culture
98 plantlets. These segments were treated with ½MS (Murashige & Skoog, 1962) liquid medium
99 supplemented with one of six different colchicine concentrations (0.025, 0.05, 0.075, 0.1 or 0.5%
100 w/v) in parallel with nodal segments in ½MS only as the control. All samples were incubated
101 with shaking at 100 rpm for 24 or 48 hours under the condition of 25 ± 2 °C in a 10-hour
102 photoperiod with the light intensity of 20 µmol m^{-2} s^{-1} provided by warm white LED lamps.
103 After treatment, all colchicine treated explants were rinsed with sterilized distilled water five
104 times and then transferred to solidified ½MS medium supplemented with 0.2 mg L^{-1} 6-
105 Benzylaminopurine (BAP, Sharma et al., 2010). The cultures were kept for 8 weeks under the
106 same standard condition as mentioned above, but without shaking.
107

108 ***In vitro* regenerant screening and flow cytometry analysis**
109 Shoots of eight-week-old treated regenerants were excised and transferred to fresh solidified
110 ½MS medium without hormone, and cultured under the same standard conditions for 6 weeks.
111 The putative polyploid plants were screened using the criteria of bigger and thicker leaves and
112 stems, as compared to diploid plantlets (Escandón et al., 2006). These plants were further
113 confirmed for polyploidy level by a flow cytometry assay. A leaf sample (100 mg) was chopped
114 with a razor blade in a petri dish containing 1 mL nuclei extraction buffer (200 mM Tris, 4 mM
115 $MgCl_2.6H_2O$, 0.5% (v/v) Triton X-100, pH 7.5) according to the method of Pfosser et al. (1995).
116 The nuclei suspension was filtered through a 40 µm nylon net (Merck Millipore Ltd., Germany)
117 and stained using the Muse™ Cell Cycle Kit (Merck KGaA, Germany). The polyploidy level

118   was analyzed on a Guava® easyCyte Flow Cytometer with InCyte™ software version 2.7 (Merck

119   KGaA, Germany).

120

121   **Morphological observations in autotetraploid plants**

122   The autotetraploid clones were *in vitro* proliferated on ½MS for 8 weeks and the plantlets with

123   the same height were transplanted into pots containing ½Hoagland solution (HS, Hoagland &

124   Arnon, 1950) and kept under greenhouse condition for 30 days. At least five plants per clone

125   were grown and observed for biomass, height, leaf thickness, stem diameter and additionally

126   quantifies for bacoside content. For measurement of leaf thickness and stem diameter, the 5th leaf

127   and internode were cross section with a razor blade. The samples were observed and measured

128   under light microscope. The measurements were undertaken with 5 measuring points per leaf or

129   stem of five individual plants.

130

131   **Quantification of bacoside content**

132   Bacoside content was quantified using the modified method of Bansal et al. (2016). Briefly,

133   aerial parts of 30-day-old hydroponically grown plants were dried at 40 °C for 2 days and then

134   ground to a fine powder. The powder (0.1 g) was then extracted with 3 mL methanol by

135   incubated at room temperature for 1 hour, sonicated for 15 minutes and then incubated in the

136   dark at 4 °C for 5 minutes. After centrifugation at 8,000 rpm for 5 minutes, the supernatant was

137   filtered through a 0.45-µm nylon filter (Tianjin Fuji Science and Technology Co., Ltd., China).

138   Bacoside content in the filtered extracts was analyzed on a HPLC system (Shimadzu, Japan)

139   equipped with Purospher® STAR-RP-18 endcapped (5 µm) LiChroCART® 250-4.6 column

140   (Merck, Germany). The chromatographic condition was as follows: (i) the mobile phase was

141   0.2% (v/v) aqueous phosphoric acid, pH 0.3 and acetonitrile (65:35 v/v), (ii) the flow rate was 1

142   mL min^{-1}, and (iii) bacoside was detected at 205 nm using a SPD-10A VP UV-Vis detector

143   (Shimadzu, Japan). The bacoside content in the plant extracts was calculated by a linearity

144   equation of peak areas against known concentrations bacosides (Sigma-Aldrich, US).

145

146   **Statistical analysis**

147   The experiment was conducted using a complete randomized design. The differences in fresh

148   weight, dry weight and height of autotetraploid and diploid plants were compared by *t*-test. Other

149   data were analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range

150   test (DMRT).

151

152

153   ## Results and Discussion

154   **Effect of colchicine on growth and phenotypic variation of regenerated brahmi**

155   Eight weeks after transfer of the colchicine-treated nodal segments to new ½MS, the highest

156   percentage of shoot multiplication (22.0 regenerants per explant) was found in the segments

157   treated with 0.025% colchicine for 24 hours (Table 1). These shoots were found to be healthier

158 than the control and other treatments (Fig. 1A). Shoot multiplication and shoot length
159 significantly decreased with increasing concentrations and exposure times of colchicine as
160 compared to untreated explants (Table 1, Fig. 1A). Increasing concentrations and exposure times
161 of colchicine have been shown to inhibit growth and shoot regeneration of explants (Javadian et
162 al., 2017; Wang et al., 2017). Moreover, higher concentrations of colchicine with longer
163 exposure times can be toxic to plants leading to tissue abnormalities (Megbo, 2010) and dead
164 (Mondin et al., 2018)
165
166 Some regenerants of colchicine-treated explants showed distinct morphological feature changes
167 in leaf and stem such as albinoism (Fig. 1B), a spiral stem (Fig. 1C), variegated leaves (Fig. 1D),
168 whorls of three (Fig. 1E) and four (Fig. 1F) leaves, and a fasciated stem (Fig. 1G). These results
169 indicated that colchicine treatment induced phenotypic changes in the regenerated brahmi.
170 Several published studies have reported that colchicine-induced wide morphological variations
171 such as leaf arrangement and size in *Platycodon grandiflorus* (Wu et al., 2011) and *Hebe*
172 (Gallone et al., 2014), stomatal size in *Pogostemon cablin* (Yan et al., 2016) and cassava
173 (Mondin et al., 2018), and leaf, flower, and seed size in *Fagopyrum tataricum* (Wang et al.,
174 2017).
175
176 **Flow cytometry analysis for determining polyploidy level in colchicine-treated plantlets**
177 From a total of 1,262 regenerants, 326 putative autotetraploid clones were selected based on
178 criteria of bigger and thicker leaves and stems as compared to diploid plantlets. Polyploidy level
179 was analyzed by flow cytometry. The DNA content peak of a diploid control plant ($2x$; 2n=64)
180 was set at channel 100 (Fig. 2A) and the peak of an autotetraploid plant ($4x$; 2n=128) was
181 measured at channel 200, which was twofold intensity compared to that of diploid plantlets (Fig.
182 2B). Of 326 clones tested, only 18 and 84 clones were mixoploid (a plant composed of both $2x$
183 and $4x$ cell, Fig. 2C) and autotetraploid, respectively (Table 1). Among the twelve colchicine
184 treatment combinations, the mixoploid- and autotetraploid-induction efficiencies (%) ranged
185 between 0.0-5.1% and 0-14.6%, respectively (Table 1). The highest autotetraploid-induction
186 efficiency (14.6%) was found in the combination of treated with 0.5% (w/v) colchicine and
187 exposure for 48 hours (Table 1). Moreover, the highest mixoploid induction (5.1%) was found
188 after treatment with 0.1% (w/v) colchicine and exposure of 48 hours (Table 1). In contrast,
189 treatment of explants with 0.025% colchicine for 24 hours did not induce autotetraploid in any
190 regenerated plantlets (Table 1). In this study, nodal segments of brahmi treated with 0.5%
191 colchicine solution for 48 hours was the most useful condition to generate the highest number of
192 autotetraploid plantlets. Because colchicine plays an important role in inhibiting microfilament
193 and microtubule formation and disturbs mitosis at the anaphase stage, chromosomes do not
194 migrate to the opposite ends of the cell during cell division, resulting in chromosome doubling in
195 the cell (Pour et al., 2012). Furthermore, a recent study reported that colchicine affected
196 numerous genes related to the spindle, the chromosomal kinetochore, vesicles, cellulose and the
197 processes of cytoplasm movement, chromatid segregation, as well as membrane and cell wall

198 development (Zhou et al., 2017). For this reason, colchicine is a highly potent antimitotic agent
199 which has been successfully used for inducing polyploidy in many plant species (Pour et al.,
200 2012; Wang et al., 2017; Mondin et al., 2018).
201

Agricultural characters in autotetraploid plantlets
203 Of the 84 autotetraploids, 28 clones were selected (based on in vitro growth performances) and
204 transplanted into ½HS under greenhouse conditions for 30 days. The results showed that 21 and
205 13 examined autotetraploids clones had significantly higher fresh and dry weight compared to
206 diploid clone, respectively (Figs. 3A and 3B). The highest fresh (1.50 g plant^{-1}) and dry (0.072 g
207 plant^{-1}) weight was found in autotetraploid-plant clone 4x-103 (Fig. 4A), which was around 2.8
208 and 2.0-time higher than diploid plants, respectively (Figs. 3A and 3B). This was probably due to
209 the autotetraploid having thicker leaves (Figs. 4B and 4D) and larger stem diameter (Figs. 4C
210 and 4E) than its diploid progenitor. In contrast, shoot height in 6 autotetraploid clones slightly
211 increased (16.0 to 18.8 cm) more than diploid plant (14.2 cm), and almost half (16 of 28) of the
212 tetraploid clones were not significantly different from the diploid plant controls (Fig. 3C).
213 The results clearly showed that most of the selected autotetraploid plants had significantly
214 greater fresh weight, dry weight, and shoot height than diploid plants. The reasons of this were
215 possible that colchicine-side and double-gene-dosage effects, that might either increase or
216 decrease biomass and metabolite production of polyploids (Javadian et al., 2017; Salma et al.,
217 2018). In some cases, polyploids were much more effective in superior vegetative-tissue shapes
218 and sizes than diploids, which may be the reason for an increase in biomass yields (Wang et al.,
219 2017). Another reason for this was that colchicine might have induced morphological variations
220 such as leaf and stem thickness, causing enhanced fresh weight and dry weight (Niu et al., 2016).
221 Moreover, chromosome doubling might have induced the differential gene expression between
222 diploid and synthesized tetraploid, therefore affecting the various phenotypic traits (Liqin et al.,
223 2019). The previous study has shown differences in gene expression between diploid and triploid
224 of *Populus*, involved in carbohydrate and lipid metabolism in triploid, reflected a more
225 significant increase in carbon metabolism and utilization efficiency than diploids, which may be
226 responsible for the fast-growing trait observed in triploids (Cheng et al., 2015). The organ sizes
227 (plant height, leaf area, and petiole length) were significantly superior in polyploids than diploids
228 *Populus* (Liqin et al., 2019), since polyploids might be up-regulated the growth-regulating factor
229 (GRF) gene family, played vital roles in the control of plant growth development (Wu et al.,
230 2014).
231

Accumulation of bacoside content in autotetraploid explant
233 Of the 28 autotetraploid clones, 14 representatives were selected using the criteria of higher fresh
234 weight and/or dry weight than diploid plants. Total bacoside content of 14 representatives was
235 quantified using HPLC as shown in Table 2. Only one autotetraploid clone (4x-59, Fig. 4A) had
236 a significantly ($p \leq 0.05$) higher total bacoside contents than the diploid clones. However, two
237 autotetraploid clones (4x-59 and 4x-103) had maximum amounts of bacoside at 1.55 and 1.10 mg

238 plant^{-1} respectively, as compared to diploid clone (0.68 mg plant^{-1}) and significantly ($p \leq 0.05$)
239 higher than diploid clone by 2.3 and 1.6 folds, respectively. This suggested that their polyploidy
240 may be associated with improving secondary metabolite content in brahmi.
241 Since the biosynthetic pathways of secondary metabolites involve many steps and require several
242 enzymes, it was hypothesized that doubling chromosome number in autopolyploid plants may
243 increase the number of gene copies. This may lead to increased enzymatic synthesis and
244 metabolic activity, which may provide additional substrates for enhancing secondary plant
245 metabolites (Salma et al., 2017). Previous publications have reported more abundant proteins
246 related to the biosynthesis of secondary metabolites in autotetraploid than diploid *Paulownia*
247 *australis* (Wang et al., 2017). Similarly, the polyploidy-mediated change in phenotypic traits
248 may appear as an effect of secondary metabolite biosynthesis. For example, enhancement of the
249 leaf area and leaf thickness in autotetraploid *Stevia rebaudiana* were positively affected
250 glycoside biosynthesis as a result of increased photosynthesis (Hegde et al., 2015). Similarly,
251 polyploidization enhances the content of triterpenoid in *Jatropha curcas* (Niu et al., 2016),
252 patchouli alcohol in *Pogostemon cablin* (Yan et al., 2016), and flavonoid in *Fagopyrum*
253 *tataricum* (Wang et al., 2017) as compared to diploid plants. Additionally, it has been reported
254 that the level of artemisinin in tetraploids of *Artemisia annua* was higher than diploid due to a
255 significant increase of artemisinin metabolite-specific genes (Lin et al., 2011). Likewise,
256 podophyllotoxin production in tetraploid *Linum album* was increased over diploid due to higher
257 expression of genes related to its biosynthesis (Javadian et al., 2017). So, the enhancement of
258 secondary metabolite production of tetraploid compared to diploid might be that the expression
259 of crucial biosynthesis genes in tetraploid was increased.
260
261

Conclusions

263 This present study demonstrated that colchicine-mediated technique was successfully used in *in*
264 *vitro* induction of autotetraploidy in *Bacopa monnieri*. Most autotetraploid plants were superior
265 in agricultural characteristics (such as fresh weight, dry weight and total bacoside content) than
266 the original parental diploid plants. This suggests that the novel autopolyploids developed here
267 may be of use in improving the quality and quantity of medicinal plant raw material, to the
268 benefit of the medical and pharmaceutical industries.
269
270

Acknowledgements

272 The authors are appreciative for the comments and English language editing of Prof. Duncan
273 Richard Smith, Ph.D., Institute of Molecular Biosciences, Mahidol University, Thailand.

274
275

References

277

278 Abbassi SJ, Vishwakarma RK, Patel P, Kumari U, Khan BM. 2015. *Bacopa monniera*
279 recombinant mevalonate diphosphate decarboxylase: Biochemical characterization.
280 *International Journal of Biological Macromolecules* 79:661–668

281

282 Acharya DAC, Mitaine-Offer AC, Kaushik N, Miyamoto T, Paululat T, Mirjolet JF, Duchamp
283 O, Lacaille-Dubois MA. 2009. Cytotoxic spirostane-type saponins from the roots of *Chloro*
284 *phytum borivilianum*. *Journal of Natural Products* 72:177–181.

285

286 Augustin JM, Kuzina V, Andersen SB, Bak S. 2011. Molecular activities, biosynthesis and
287 evolution of triterpenoid saponins. *Phytochemistry* 72: 435–457

288

289 Bansal M, Reddy MS, Kumar A. 2016. Seasonal variations in harvest index and bacoside A
290 contents amongst accessions of *Bacopa monnieri* (L.) Wettst. collected from wild populations.
291 *Physiology and Molecular Biology of Plants* 22:407–413

292

293 Chen R, Yang Y, Wu H. 2016. A comparative study on rooting of in vitro regenerated shoots in
294 haploid, diploid and tetraploid purple coneflower (*Echinacea purpurea* L.). *Biotechnology &*
295 *Biotechnological Equipment* 30:44–48

296

297 Cheng S, Yang J, Liao T, Zhu X, Suo Y, Zhang P, Wang J, Kang X. 2015. Transcriptomic
298 changes following synthesis of a *Populus* full-sib diploid and allotriploid population with
299 different heterozygosities driven by three types of 2n female gamete. *Plant Molecular Biology*
300 89:493–510

301

302 Deepak M, Sangli GK, Arun PC, Amit A. 2005. Quantitative determination of the major saponin
303 mixture bacoside A in *Bacopa monnieri* by HPLC. *Phytochemical Analysis* 16:24–29

304

305 Escandón AS, Hagiwara JC, Alderete LM. 2006. A new variety of *Bacopa monnieri* obtained by
306 *in vitro* polyploidization. *Electronic Journal of Biotechnology* 9:181–186

307

308 Gallone A, Hunter A, Douglas GC. 2014. Polyploid induction in vitro using colchicine and
309 oryzalin on *Hebe* 'Oratia Beauty': Production and characterization of the vegetative traits.
310 *Scientia Horticulturae* 179:59–66

311

312 Gubbannavar JS, Harisha CR, Chandola HM. 2012. A comparative study of roots of *Bacopa*
313 *monnieri* (L.) Pennel and *Bacopa floribunda* (R.Br.) wettst. *International Journal of*
314 *Pharmacognosy and Phytochemical Research* 4:8–11

315

316 Hegde SN, Rameshsing CN, Vasundhara M. 2015. Characterization of *Stevia rebaudiana*
317 Bertoni polyploids for growth and quality. *Medicinal Plants* 7:188–195
318
319 Hoagland DR, Arnon DI. 1950. The water-culture method for growing plants without soil,
320 Circular 347, California Agricultural Experiment Station, University of California, California
321
322 Jansen R, Brogan B, Whitworth A Okello E. 2014. Effects of five ayurvedic herbs on locomotor
323 behaviour in a *Drosophila melanogaster* parkinson's disease model. *Phytotherapy Research*
324 28:1789–1795
325
326 Javadian N, Karimzadeh G, Sharifi M, Moieni A, Behmanesh M. 2017. In vitro polyploidy
327 induction, changes in morphology, podophyllotoxin biosynthesis, and expression of the related
328 genes in *Linum album* (Linaceae). *Planta* 245:1165–1178
329
330 Javadian N, Karimzadeh G, Sharifi M, Moieni A, Behmanesh M. 2017. In vitro polyploidy
331 induction: changes in morphology, podophyllotoxin biosynthesis, and expression of the related
332 genes in *Linum album* (Linaceae). *Planta* 245:1165–1178
333
334 Lin X, Zhou Y, Zhang J, Lu X, Zhang F, Shen Q, Wu S, Chen Y, Wang T, Tang K. 2011.
335 Enhancement of artemisinin content in tetraploid *Artemisia annua* plants by modulating the
336 expression of genes in artemisinin biosynthetic pathway. *Biotechnology and Applied*
337 *Biochemistry* 58:50–57
338
339 Liqin G, Jianguo Z, Xiaoxia L, Guodong R. 2019. Polyploidy-related differential gene
340 expression between diploid and synthesized allotriploid and allotetraploid hybrids of *Populus*.
341 *Molecular Breeding* 39:69
342
343 Megbo BC. 2010. The physiological effects of colchicine in Okra, *Hibiscus esculentus* L, plant
344 growth and development. *International Journal of Scientific & Engineering Research* 1:29–33
345
346 Mondin M, de Mello e Silva PAKX, Latado RR, Mourão Filho FAA. 2018. In vitro induction
347 and regeneration of tetraploids and mixoploids of two cassava cultivars. *Crop Breeding and*
348 *Applied Biotechnology* 18:176–183
349
350 Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco
351 tissue cultures. Physiologia Plantarum 15:437–497
352
353 Muthiah JVL, Shunmugiah KP, Manikadan R. 2013. Genetic fidelity assessment of encapsulated
354 in vitro tissues of *Bacopa monnieri* after 6 months of storage by using ISSR and RAPD markers.
355 Turkish Journal of Botany 37:1–10

356

357   Niu L, Tao YB, Chen MS, Fu Q, Dong Y, He H, Xu ZF. 2016. Identification and
358   characterization of tetraploid and octoploid *Jatropha curcas* induced by colchicine. *Caryologia*
359   69:58–66

360

361   Niua Y, Luoa H, Suna C, Yangb TJ, Donga L, Huanga L, Chen S. 2014. Expression profiling
362   of the triterpene saponin biosynthesis genes FPS, SS, SE, and DS in the medicinal plant *Panax*
363   *notoginseng*. *Gene* 533:295–303

364

365   Omidbaigi R, Mirzaeea M, Hassanib ME, Moghadamc MS. 2010. Induction and identification of
366   polyploidy in basil (*Ocimum basilicum* L.) medicinal plant by colchicine treatment. International
367   Journal of Plant Production 4:87–98

368

369   Pandareesh MD, Anand T, Bhat PV. 2014. Cytoprotective propensity of *Bacopa monnieri*
370   against hydrogen peroxide induced oxidative damage in neuronal and lung epithelial cells.
371   *Cytotechnology* 68:157–172

372

373   Pfosser M, Amon A, Lelley T, Heberle-Bors E. 1995. Evaluation of sensitivity of flow cytometry
374   in detecting aneuploidy in wheat using disomic and ditelosomic wheat-rye addition lines.
375   *Cytometry Part A* 21:387–393

376

377   Pour MP, Moieni A, Ebrahimi A, Javidfar F. 2012. Doubled haploid plants following colchicine
378   treatment of microspore-derived embryos of oilseed rape (*Brassica napus* L.). *Plant Cell, Tissue*
379   *and Organ Culture* 108:251–256

380

381   Ramasamy S, Chin SP, Sukumaran SD, Buckle MJC, Kiew LV, Chung LY. 2015. In silico and
382   in vitro analysis of bacoside A aglycones and its derivatives as the constituents responsible for
383   the cognitive effects of *Bacopa monnieri*. *PLOS ONE* 10:e0126565

384

385   Rauf K, Subhan F, Abbas M, Ali SM, Ali G, Ashfaq M, Abbas G. 2014. Inhibitory effect of
386   bacopasides on spontaneous morphine withdrawal induced depression in mice. *Phytotherapy*
387   *Research* 28:937–939

388

389   Salma U, Kundu S, Hazra AK, Ali MN, Mandal N. 2018. Augmentation of wedelolactone
390   through in vitro tetraploid induction in *Eclipta alba* (L.) Hassk. *Plant Cell, Tissue and Organ*
391   *Culture* 133:289–298

392

393   Salma U, Kundu S, Mandal N. 2017. Artificial polyploidy in medicinal plants, Advancement in
394   the last two decades and impending prospects. *Journal of Crop Science and Biotechnology* 20:9–
395   19

396

397 Samaddar T, Nath S, Halder M, Sil B, Roychowdhury D, Sen S, Jha S. 2012. Karyotype analysis
398 of three important traditional Indian medicinal plants, *Bacopa monnieri*, *Tylophora indica* and
399 *Withania somnifera*. *The Nucleus* 55:17–20

400

401 Sangeetha N, Ganesh D. 2011. Optimization of protocol for in vitro polyploidization in genetic
402 improvement of *Bacopa monnieri* L. *International Journal of Biological Technology* 2:28–34

403

404 Sattler MC, Carvalho CR, Clarindo WR. 2016. The polyploidy and its key role in plant breeding.
405 *Planta* 243:281–296

406

407 Sharma P, Yadav S, Srivastava A, Shrivastava N. 2013. Methyl jasmonate mediates upregulation
408 of bacoside A production in shoot cultures of *Bacopa monnieri*. *Biotechnology Letters* 35:121–
409 1125

410

411 Sharma S, Kamal B, Rathi N, Chauhan S, Jadon V, Vats N, Gehlot A, Arya S. 2010. In vitro
412 rapid and mass multiplication of highly valuable medicinal plant *Bacopa monnieri* (L.) Wettst.
413 *African Journal of Biotechnology* 9:8318–8322

414

415 Tewari DN. 2000. Report of the task force on conservation and sustainable use of medicinal
416 plants. Government of India, Planning Commission, New Delhi

417

418 Tripathi N, Chouhan DS, Saini N, Tiwari S. 2012. Assessment of genetic variations among
419 highly endangered medicinal plant *Bacopa monnieri* (L.) from central India using RAPD and
420 ISSR analysis. *3 Biotech* 2:327–336

421

422 Uabundit N, Wattanathorn J, Mucimapura S, Ingkaninan K. 2010. Cognitive enhancement and
423 neuroprotective effects of *Bacopa monnieri* in Alzheimer's disease model. *Journal of*
424 *Ethnopharmacology* 127:26–31

425

426 Wang LJ, Sheng MY, Wen PC, Du JY. 2017. Morphological, physiological, cytological and
427 phytochemical studies in diploid and colchicine-induced tetraploid plants of *Fagopyrum*
428 *tataricum* (L.) Gaertn. *Botanical Studies* 58:2

429

430 Wu L, Zhang D, Xue M, Qian J, He Y, Wang S. 2014. Overexpression of the maize *GRF10*, an
431 endogenous truncated growth-regulating factor protein, leads to reduction in leaf size and plant
432 height. *Journal of Integrative Plant Biology* 56:1053–1063

433

434 Wu YX, Yang FH, Zhao XM, Yang WD. 2011. Identification of tetraploid mutants of
435 *Platycodon grandiflorus* by colchicine induction. *Caryologia* 64:343–349

436

437   Yan HJ, Xiong Y, Zhang HY, He ML. 2016. In vitro induction and morphological characteristics

438   of octoploid plants in *Pogostemon cablin*. *Breeding Science* 66:169–174

439

440   Zhou K, Fleet P, Nevo E, Zhang X, Sun G. 2017. Transcriptome analysis reveals plant response

441   to colchicine treatment during on chromosome doubling. *Scientific Reports* 7:8503

Effect of concentration and treatment duration of colchicine on polyploidy induction in nodal segments of *Bacopa monnieri*.

[a] Average value represents as mean ± SE of a number of regenerants per nodal segment, calculated from eight nodal segments at 8 weeks after culture. Different letters within the same column indicate significant differences, analyzed by Duncan's Multiple Range Test (DMRT) at $p \leq 0.01$.

[b] Percentage value was calculated from number of mixoploid or tetraploid per total number of regenerants in each treatment.

[c] Data were summarized by all regenerants from colchicine treatment combinations.

1 **Table 1** Effect of concentration and treatment duration of colchicine on polyploidy induction in nodal segments of *Bacopa monnieri*.

| Time of treatment (h) | Colchicine (% w/v) | No. of regenerant | | Flow cytometry analysis | | | | |
| | | | | No. of tested regenerants | Mixoploid | | Tetraploid | |
		Total	Average[a]		Number	%[b]	Number	%[b]
24	0	97	12.1 ± 3.4 b	27	0	0	0	0
	0.025	176	22.0 ± 9.4 a	25	1	0.6	0	0
	0.05	112	14.0 ± 4.4 ab	29	1	0.9	3	2.7
	0.075	149	18.6 ± 5.8 ab	34	2	1.3	14	9.4
	0.1	120	15.0 ± 4.6 ab	35	5	4.2	9	7.5
	0.5	115	14.4 ± 8.8 ab	33	1	0.9	7	6.1
	Total[c]	*672*		*156*	*10*	*1.4*	*33*	*4.9*
48	0	81	10.1 ± 5.6 b	26	0	0	0	0
	0.025	151	18.9 ± 2.9 ab	34	1	0.7	2	1.3
	0.05	143	17.9 ± 10.5 ab	35	0	0	15	10.5
	0.075	108	13.5 ± 5.0 ab	33	0	0	11	10.2
	0.1	99	12.4 ± 2.9 b	36	5	5.1	10	10.1
	0.5	89	11.1 ± 4.5 b	32	2	2.2	13	14.6
	Total[c]	*590*		*170*	*8*	*1.4*	*51*	*8.6*
Total[c]		**1,262**		**326**	**18**	**1.4**	**84**	**6.7**

2 [a] Average value represents as mean ± SE of a number of regenerants per nodal segment, calculated from eight nodal segments at 8

3 weeks after culture. Different letters within the same column indicate significant differences, analyzed by Duncan's Multiple Range

4 Test (DMRT) at $p \leq 0.01$.

5 [b] Percentage value was calculated from number of mixoploid or tetraploid per total number of regenerants in each treatment.

6 [c] Data were summarized by all regenerants from colchicine treatment combinations.

Accumulation of bacoside contents in autotetraploid plants of *Bacopa monnieri* after growth in ½HS for 30 days, analyzed using HPLC technique.

The data represent the mean ± SE of three biological replicates. Different letters within the same column indicate significant differences analyzed by DMRT at $p \leq 0.05$.

[a] The yield of bacoside was calculated on the basis of dry weight of individual plant.

1 **Table 2** Accumulation of bacoside contents in autotetraploid plants of *Bacopa monnieri* after growth in ½HS for 30 days, analyzed

2 using HPLC technique.

Clone no.	Content (%DW)					Yield (mg plant^{-1})[a]				
	Bacoside A	Bacopaside II	Bacopaside X	Bacopasaponin C	Total bacoside	Bacoside A	Bacopaside II	Bacopaside X	Bacopasaponin C	Total bacoside
2*x*	0.22 ± 0.03 ab	0.66 ± 0.12 a-c	0.17 ± 0.05 b	0.72 ± 0.12 bc	1.77 ± 0.31 ab	0.08 ± 0.01 b-d	0.25 ± 0.05 c-e	0.06 ± 0.02 bc	0.27 ± 0.05 b-d	0.68 ± 0.13 b-d
4*x*-9	0.12 ± 0.02 c-g	0.54 ± 0.12 bc	0.57 ± 0.11 a	0.25 ± 0.06 d-f	1.48 ± 0.31 b	0.05 ± 0.01 c-f	0.23 ± 0.05 de	0.25 ± 0.04 a	0.11 ± 0.02 d	0.64 ± 0.13 b-d
4*x*-19	0.14 ± 0.03 b-g	0.67 ± 0.13 a-c	0.00 ± 0.00 b	0.62 ± 0.13 b-e	1.43 ± 0.29 b	0.06 ± 0.01 b-f	0.30 ± 0.06 c-e	0.00 ± 0.00 c	0.28 ± 0.06 b-d	0.64 ± 0.13 b-d
4*x*-20	0.05 ± 0.01 g	0.33 ± 0.05 c	0.37 ± 0.07 a	0.27 ± 0.06 d-f	1.01 ± 0.19 b	0.02 ± 0.00 f	0.14 ± 0.02 e	0.16 ± 0.02 ab	0.11 ± 0.02 d	0.43 ± 0.07 d
4*x*-23	0.20 ± 0.02 a-c	0.85 ± 0.14 ab	0.00 ± 0.00 b	0.84 ± 0.12 b	1.89 ± 0.28 ab	0.11 ± 0.01 ab	0.46 ± 0.05 a-c	0.00 ± 0.00 c	0.47 ± 0.03 b	1.04 ± 0.08 bc
4*x*-40	0.07 ± 0.01 e-g	0.34 ± 0.04 c	0.38 ± 0.05 a	0.15 ± 0.03 f	0.94 ± 0.12 b	0.05 ± 0.01 c-f	0.23 ± 0.05 de	0.26 ± 0.05 a	0.11 ± 0.03 d	0.64 ± 0.14 b-d
4*x*-56	0.10 ± 0.02 d-g	0.48 ± 0.10 bc	0.54 ± 0.11 a	0.26 ± 0.05 d-f	1.37 ± 0.29 b	0.04 ± 0.01 d-f	0.16 ± 0.04 e	0.19 ± 0.04 a	0.09 ± 0.02 d	0.47 ± 0.11 d
4*x*-59	0.25 ± 0.05 a	1.05 ± 0.24 a	0.12 ± 0.01 b	1.30 ± 0.29 a	2.73 ± 0.60 a	0.15 ± 0.03 a	0.60 ± 0.13 a	0.07 ± 0.01 bc	0.74 ± 0.15 a	1.55 ± 0.31 a
4*x*-62	0.17 ± 0.04 a-d	0.75 ± 0.15 a-c	0.00 ± 0.00 b	0.68 ± 0.16 b-d	1.59 ± 0.34 b	0.09 ± 0.02 bc	0.40 ± 0.07 a-d	0.00 ± 0.00 c	0.36 ± 0.07 bc	0.84 ± 0.16 b-d
4*x*-88	0.09 ± 0.01 d-g	0.40 ± 0.02 bc	0.39 ± 0.02 a	0.19 ± 0.02 ef	1.07 ± 0.06 b	0.05 ± 0.00 c-f	0.19 ± 0.01 de	0.19 ± 0.01 a	0.09 ± 0.00 d	0.53 ± 0.02 cd
4*x*-94	0.15 ± 0.04 b-e	0.77 ± 0.19 a-c	0.00 ± 0.00 b	0.81 ± 0.22 b	1.74 ± 0.44 ab	0.07 ± 0.01 b-e	0.35 ± 0.08 b-e	0.00 ± 0.00 c	0.37 ± 0.09 bc	0.80 ± 0.19 b-d
4*x*-98	0.06 ± 0.02 e-g	0.42 ± 0.12 bc	0.40 ± 0.12 a	0.20 ± 0.06 ef	1.08 ± 0.32 b	0.04 ± 0.01 d-f	0.24 ± 0.07 de	0.23 ± 0.07 a	0.11 ± 0.04 d	0.62 ± 0.20 b-d
4*x*-99	0.15 ± 0.03 b-f	0.70 ± 0.15 a-c	0.00 ± 0.00 b	0.38 ± 0.09 c-f	1.23 ± 0.27 b	0.06 ± 0.02 c-f	0.31 ± 0.08 c-e	0.00 ± 0.00 c	0.17 ± 0.05 cd	0.54 ± 0.14 cd
4*x*-103	0.13 ± 0.03 b-g	0.68 ± 0.14 a-c	0.00 ± 0.00 b	0.61 ± 0.14 b-e	1.42 ± 0.31 b	0.10 ± 0.02 b	0.53 ± 0.10 ab	0.00 ± 0.00 c	0.47 ± 0.10 b	1.10 ± 0.22 ab
4*x*-115	0.06 ± 0.03 fg	0.42 ± 0.10 bc	0.53 ± 0.14 a	0.38 ± 0.10 c-f	1.39 ± 0.36 b	0.03 ± 0.01 ef	0.20 ± 0.04 de	0.26 ± 0.06 a	0.19 ± 0.04 cd	0.68 ± 0.16 b-d

3 The data represent the mean ± SE of three biological replicates. Different letters within the same column indicate significant

4 differences analyzed by DMRT at $p \leq 0.05$.

5 [a] The yield of bacoside was calculated on the basis of dry weight of individual plant.

Figure 1

Effect of concentrations and treatment duration of colchicine on shoot regeneration in *Bacopa monnieri* at 8 weeks after transferred to ½MS (A).

Some morphological variations were observed in regenerated *Bacopa monnieri* after colchicine treatment such as albino leaf and stems (B), spiral stem (C), variegated leaves (D), whorl of three leaves (E), whorl of four leaves (F), and a fasciated stem (arrow) with two apical shoots (G). Bars indicates 1 cm.
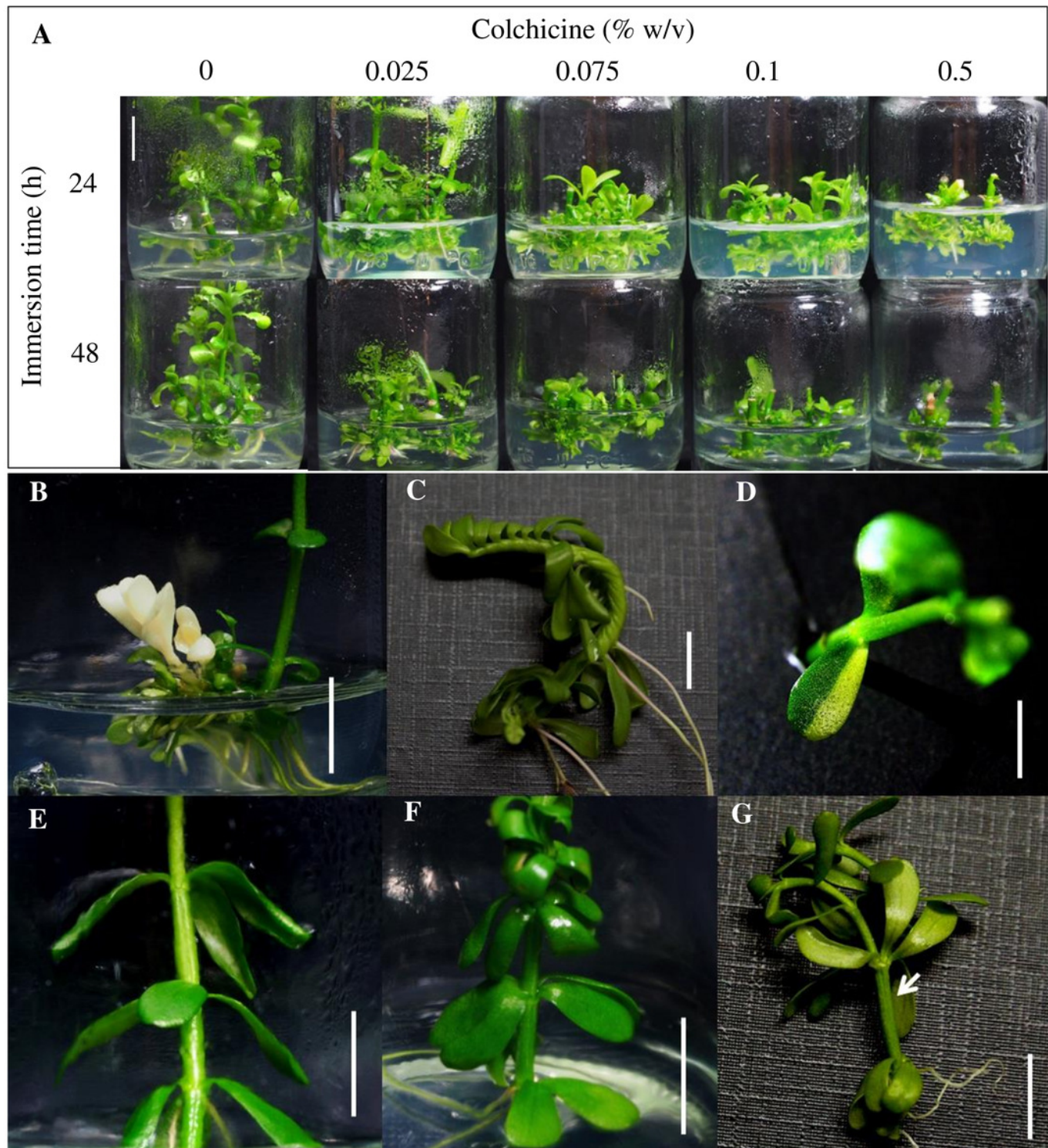
Figure 2

Identification of polyploid *Bacopa monnieri* using flow cytometry. Histograms of diploid (A), autotetraploid (B) and mixoploid (C) are shown.
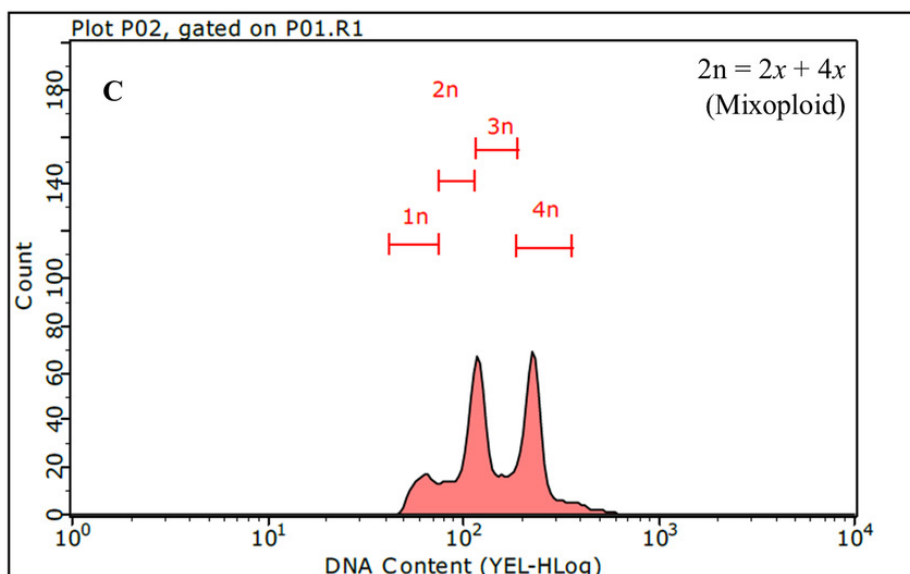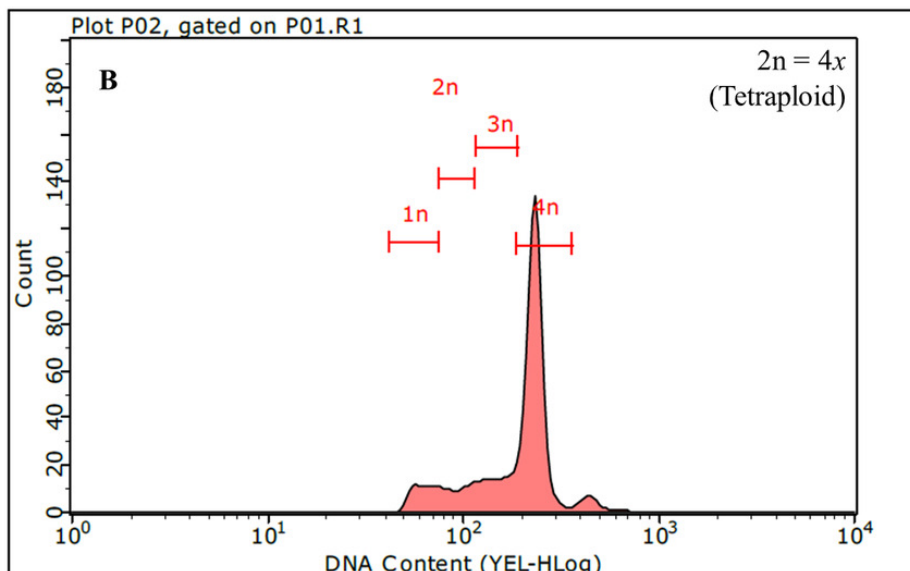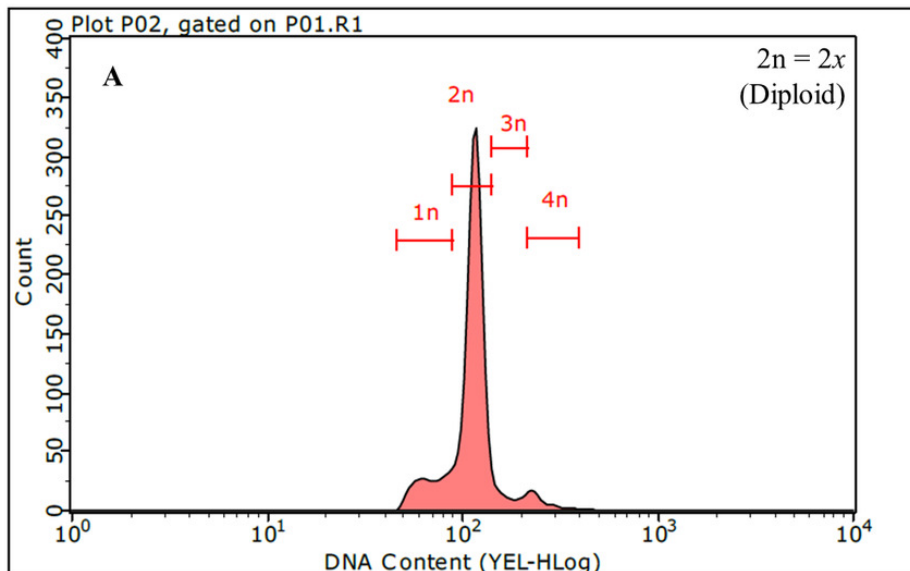
Figure 3

Fresh weight (A), dry weight (B) and shoot height (C) in selected autotetraploid plants of *Bacopa monnieri* after growth in ½HS for 30 days.

Data are the mean of five plants and the error bar represents SE. The single asterisk (*), and double asterisk (**) indicate significant difference ($p \leq 0.05$) and highly significant difference ($p \leq 0.01$) of autotetraploid plants ($4x$) compared to diploid plant ($2x$) by t-test, respectively.
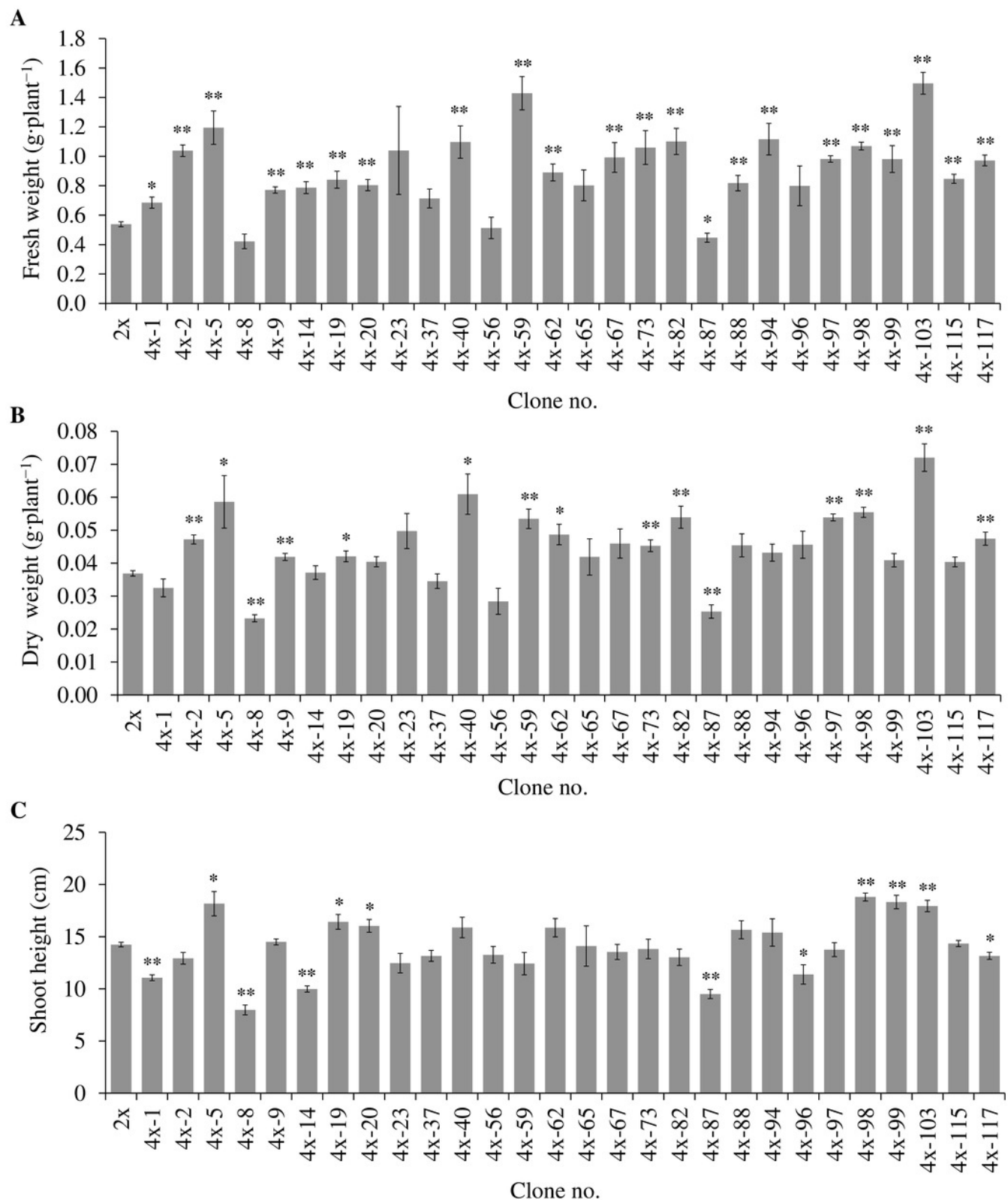
Figure 4

Morphological characters in diploid (A) and five representative autotetraploid (B-E) plants of Bacopa monnieri after growth in ½HS for 30 days; bar indicates 5 cm.

Cross sections represent leaf thickness (F-G) and stem diameter (H-I) of diploid (F and H) and one representative sample of tetraploid (G and I) Bacopa monnieri, bars indicate 250 μm. Measurement of leaf thickness (J) and stem diameter (K) were undertaken with 5 measuring points per leaf or stem of five individual plants after growth in ½HS for 30 days. The different letters above the bars indicate significant difference at p ≤ 0.01 according to DMRT.