

1 **Draft**

2 **The Morpho-Somatic and Chromosomal Changes in Colchicine Polyploidy Induction**
3 **Colocasia esculenta Var. Antiquorum**

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9 **Abstract**

10 Increased productivity of *Colocasia esculenta* var. *Antiquorum* is very necessary to increase
11 the mass of tubers. The polyploid mutation method using colchicine can increase tuber size by
12 increasing the number of chromosomes. Therefore, the study aimed to determine the effect of
13 colchicine on the morphology and polyploid changes of *Colocasia esculenta* var. *Antiquorum*
14 at various concentrations and duration of immersion in vitro. The research was carried out as
15 a completely randomized factorial design. The first factor is colchicine's concentration,
16 consisting of a concentration of 0.0%, 0.05%, 0.075%, and 0.1%. The second factor is the
17 immersion time which consists of 8 hours of immersion and 16 hours of immersion. The
18 results showed that the best combination of concentration and immersion time on somatic
19 morphology was 0.075% colchicine with 8 hours and 16 hours of immersion. Meanwhile, the
20 best these combination to chromosomal changes was 0.05% with 16 hours of immersion.
21 Therefore, these results indicate that the treatment of 0.05% colchicine concentration with a
22 soaking time of 16 hours can be recommended for the induction of polyploid mutations in
23 *Colocasia esculenta*.

24 Keywords: *Colocasia esculenta*, Polyploidy mutation, Colchicine, Diploid, Tetraploid, In vitro

27

28 **Introduction**

29 Colocasia esculenta var. Antiquorum is a type of taro with a small tuber size (small
30 corm taro), also known as Japanese taro (Maretta et al. 2016). Colocasia esculenta var.
31 Antiquorum tends to be more resistant to weather changes where this plant can live again,
32 although with a different season character than before (Rosnadelly et al. 2018). Based on data
33 from the Food Security Service of South Sulawesi Province, the productivity of Colocasia
34 esculenta var. Antiquorum in 2020 was 7.214 tons. However, it resulted in exports of
35 Colocasia esculenta L. tubers only amounting to 300 tons/ha due to the low productivity of
36 Colocasia esculenta L. (Nagano et al. 2016; Suminarti 2011).

37 Increasing the productivity of Colocasia esculenta var. Antiquorum can be done through
38 plant breeding. One method of plant breeding is through the induction of polyploidy
39 mutations. Polyploidy induction aims to increase crop productivity because polyploid
40 (tetraploid) plants are known to have a more significant figure, fruit size, tuber, or flower than
41 diploid plants (Suryo 2007). Another advantage of tetraploid plants is their resistance to
42 specific environmental stresses (Deng et al. 2012).

43 Polyploidy can be done by giving chemical mutagens such as colchicine to plant
44 meristem tissue. Colchicine can change the number of plant chromosomes and cause gene
45 mutations on the scale of seeds and vegetatively propagated plants. In addition, it induces
46 polyploidy by inhibiting the formation of spindle fibers during cell division. In contrast, the
47 number of chromosomes increases, but cell division does not occur, resulting in the
48 production of polyploid cells with a doubled number of chromosomes (Manzoor et al. 2018;
49 Ade et al. 2010).

50 Colchicine is an important mutagen that prevents microtubule formation and doubles the
51 number of chromosomes. It is commonly used to develop polyploid plants and functions as a

52 mitotic toxin, producing multiple mutagenic effects in plants (El-Nashar et al., 2015).
53 Colchicine not only helps in doubling chromosomes but also causes plant mutations. Plants
54 that have mutated through colchicine are known as Colchis-mutants (Ari et al. 2015). The
55 concentration of colchicine for seed treatment is usually in the range of 0.1% - 0.8%, but high
56 doses cause malformations and reduce the production of tetraploid plants. So, it is
57 recommended to use colchicine with the lowest possible concentration (Pirkoohi et al. 2011).

58 The success of polyploidy induction depends on the explant, plant species, colchicine
59 concentration, and duration of exposure. Concentrations that are too high cause problems in
60 the form of abnormalities in developing seedlings (Manzoor et al. 2018). So this needs to be
61 done very carefully by a plant breeder. Several studies on polyploid induction using
62 colchicine have been successful with different concentrations, such as *Papaver bracteatum* L.
63 (Esfahani et al. 2020), *Allium Sativum* L. (Ayu et al. 2019), *Colchicum chalcedonicum* Azn.
64 (Karlik et al. 2020), *Neolamarckia cadamba* (Eng et al. 2021), *Dionaea muscipula* Ellis (Jala,
65 2014), *Nigella sativa* (Gupta et al. 2021), and *Rhododendron fortunei* L. (Mo et al. 2020).
66 Therefore, polyploid induction can be used to create or select better offspring for further use.

67 Based on the description above, it is necessary to research the Induction of Polyploidy
68 Mutations in Plants *Colocasia esculenta* var *Antiquorum*. The aim is to determine the effect of
69 colchicine on morphology and chromosome polyploidy of *Colocasia esculenta* var
70 *Antiquorum* plantlets on the interaction of concentration and immersion time in vitro. This
71 study's novelty is the first study in the induction of polyploidy mutations in *Colocasia*
72 *esculenta* var. *Antiquorum* plant.

73 **Materials and Methods**

74

75 **Experimental design**

76 The study was performed at the Tissue Culture Laboratory, Department of
77 Agricultural Cultivation, Faculty of Agriculture, Hasanuddin University, Makassar, South
78 Sulawesi, 90245, Indonesia. This study was conducted as a 2-factor factorial (F2F)
79 experiment with a Completely Randomized Design (CRD) as the environmental design with
80 three replications in each of the eight treatments. The combination of treatment between
81 colchicine concentration and immersion time is presented in Table 1. In one replication, there
82 were three shoots. In this study, there were 82 individual shoots, as the experimental unit
83 observed.

84 **Experimental conditional**

85 The plantlets used as material for polyploidy induction were eight weeks old *Colocasia*
86 *esculenta* var *Antiquorum* shoots which were then used as explants. The in vitro shoots were
87 removed, and the petioles were cut to a size of 1-3 cm. The immersion is carried out in
88 laminar, and the equipment used is sterile. Immersion was done by immersion of the shoots in
89 colchicine solution at various concentrations of 0% (sterile distilled water) (c0), 0.05% (c1),
90 0.075% (c2), and 0.1% (c3). The shoots were immersed in a culture bottle containing 15 ml of
91 colchicine solution and shaken at 100 rpm on a shaker for 8 hours (c1) and 16 hours (c2).
92 After immersion, the explants were washed with sterile distilled water 3-4 times (sterile) and
93 then planted into shoot propagation media. The shoot propagation medium was MS media
94 with 2 mg/l BAP and 1 mg/l IBA. The medium contains sugar (30 g/l), and the pH of the
95 media is adjusted to 5.8 and compacted with 7 g/l agar. The shoots were kept in an incubation
96 room at a temperature of 25-26°C with 16 hours/day irradiation and an irradiation intensity of
97 $\pm 1,000-2,000$ lux (Modification of Dwiyani, 2015). Shoots were incubated for 16 WAP
98 (Week After Planting). Observations were made every week from outside the culture bottle
99 for 16 WAP.

100 **Observation of parameters**

101 Several parameters observed from shoot explants were the percentage of living shoots
102 (%), the number of living leaves, the number of roots and the number of compound shoots.

103 Percentage of living explants = $\frac{\sum \text{explants planted} - \text{explants dead}}{\sum \text{explants planted}} \times 100\%$

104 **Chromosome analysis with flow cytometry**

105 Polyploidy level analysis was performed using flow cytometry. Samples were taken
106 from the treated plants' leaves of $\pm 25 \text{ mm}^2$. The sample was then ground using a razor blade
107 in a petri dish with 0.2 ml of extraction buffer (solution A from Partec Kit), then incubated for
108 10 minutes at room temperature. After that, the supernatants from the sample were filtered
109 using nylon mesh measuring 30 μm and placed in a cuvette which was then added to 1 ml of
110 DAPI solution. The sample was incubated again for 1 minute. The sample is then inserted into
111 the Flow cytometry tool. The tool will read the light intensity of the measured sample to show
112 the number of ploidy changes that occur, which will be shown through the graph displayed in
113 the tool (Qalby et al. 2020).

114 **Data analysis**

115 The F test analyzed data to determine the interaction between colchicine concentration
116 and immersion time. If the variance obtained has a significant effect, perform the DMRT
117 (Duncan's Multiple Range Test) tests at a 5% significance level to determine the effect of
118 differences between treatments. Data processing used the STAR (Statistical Tool for
119 Agricultural Research) program and Microsoft Office Excel 2019. Data were analyzed by
120 correlation analysis to see the relationship between the parameters.

121 **Results**

122

123 **Percentage of Living Shoots of *Colocasia esculenta* var *Antiquorum* In Vitro**

124 Based on Fig. 1, it can be seen that the concentration of colchicine treatment with
125 different immersion times caused varying effects on the percentage of living shoots of

126 *Colocasia esculenta* var. *Antiquorum*. In the control treatment (colchicine 0%) with 8 hours
127 and 16 hours of immersion, there was no shoot death from the first week to the sixteenth week
128 after treatment. While the colchicine treatment of 0.05%, 0.075%, and 0.1% with 8 hours and
129 16 hours of immersion, shoot death began to occur from the first week until the sixteenth
130 week after treatment. The 0% colchicine treatment (control) with 8 hours and 16 hours of
131 immersion showed that the percentage of shoot life was 100%. Colchicine treatments of
132 0.05% and 0.075% with 8 hours immersion showed a percentage of shoot life of 44.44% and
133 16 hours immersion of 27.78%. The 0.1% colchicine treatment with 8 hours immersion
134 showed a percentage of shoot life of 33.33% and 16 hours immersion time of 22.22%. The
135 0.01% colchicine treatment caused the highest shoot mortality of all other treatments. Path
136 analysis could partition correlation values into direct and indirect effects (Manjunatha et al.,
137 2017). The direct influence could be defined as the standard deviation given by a character to
138 the total standard deviation of the primarily intended characters (Singh and Chaudhary,
139 2007). Path analysis was based on characters significantly correlated to phenotypic and
140 genetic correlations. However, the correlation value used as a basis of path analysis was only
141 its genetic correlation. The genetic correlation was free from environmental effects, so
142 determining supporting characters would be more effective and efficient. The use of genetic
143 correlation as the basis of path analysis in identifying yield-supporting characters was also
144 reported by Krishnamurthy et al. (2013) in rice. Based on Table 2, the best character showing
145 the most significant direct influence was productive tillers, with a value of 0.43. This result
146 was also by previous studies by Anshori et al. (2018), who reported that the number of tillers
147 direct influences character to yield and could predict productivity. Therefore, productive
148 tillers can be used as supporting characters in the adaptability selection index to salinity
149 stress.

150 The lethal concentration of LC100 was found in plant regeneration without colchicine
151 treatment, while LC50 if there was a 50% decrease in the regeneration of control plants. In
152 looking at the relationship between the percentage of shoot explants that live with colchicine
153 concentration treatment and soaking time, a linear regression equation can be used, namely Y
154 $= 7.2751x + 82.378$. Based on Fig. 2, the relationship between the percentage of surviving
155 shoot explants was inversely proportional to the concentration and duration of colchicine
156 immersion. The higher the concentration and the longer the immersion of colchicine, the
157 lower the number of explants that survived. The coefficient of determination (R^2) is 0.3131.
158 LC50 occurred at 0.1% colchicine concentration with 8 hours of immersion. Morphology of
159 *Colocasia esculenta* var. *Antiquorum* result of in vitro induction of polyploidy. The effect of
160 concentration level and duration of immersion colchicine on *Colocasia esculenta* var
161 morphological changes. *Antiquorum* in vitro can be seen in Fig. 2.

162 The analysis results of the mean square of variance in the polyploid mutation induction
163 were shown in Table 2. The analysis showed that the treatment duration of colchicine
164 immersion in *Colocasia esculenta* var *Antiquorum* significantly affected all observation
165 parameters. The colchicine concentration level also showed a significant effect for all
166 observation parameters. The interaction effect between concentration level (C) and immersion
167 time (T) also significantly affected the number of leaves, roots, and shoots. In addition to
168 these parameters, it is also seen in the coefficient of diversity in the medium category.

169 **Number of living leaves**

170 The results of the DMRT test ($\alpha = 0.05$) Fig. 3 showed the number of living leaves of
171 *Colocasia esculenta* var. *Antiquorum* in the best treatment, c0t2 (23.00 leaves), was
172 significantly different from c2t2 (20.33 leaves), c1t2 (6.67 leaves), and c3t2 (5.33 leaves).
173 The lowest mean number of living leaves of *Colocasia esculenta* var *Antiquorum* in the

174 treatment c3t1 (2.00 leaves) was significantly different from c2t1 (4.00 leaves), c1t1 (5.67
175 leaves), c0t1 (18.67 leaves).

176

177

178 **Number of roots**

179 The results of the DMRT test ($\alpha = 0.05$) Fig. 4 showed the number of roots of the best
180 *Colocasia esculenta* var *Antiquorum* in the treatment c0t2 (13.00 roots) was significantly
181 different from c2t2 (4.00 roots), c3t2 (2.67 roots), c1t2 (2.33 leaves). The lowest mean
182 number of roots of *Colocasia esculenta* var *Antiquorum* in the treatment c3t1 (1.00 roots) was
183 significantly different from c0t1 (5.00 roots) but not significantly different from c1t1 (2.33
184 roots), c2t1 (1.67 roots).

185 **Number of compound shoots**

186 The results of the DMRT test ($\alpha = 0.05$) Fig. 5 showed the number of the best
187 *Colocasia esculenta* var. *Antiquorum* compound shoots in the treatment c0t2 (32.00 shoots)
188 were significantly different from c3t2 (17.67 shoots), c2t2 (16.33 shoots), c1t2 (9.00 shoots),
189 but c3t2 (17.67 shoots) was not significantly different from c2t2 (16.33 shoots). The lowest
190 average number of shoots of *Colocasia esculenta* var. *Antiquorum* in the treatment c3t1 (5.67
191 shoots) was significantly different from c1t1 (14.00 shoots), c2t1 (19.33 shoots), c0t1 (29.67
192 shoots).

193 **Ploidy analysis of plants *Colocasia esculenta* var. *Antiquorum***

194 The ploidy analysis using flow cytometry on *Colocasia esculenta* var plant
195 samples. *Antiquorum* in Table 3 shows that the difference in concentration and duration of
196 colchicine immersion can affect plant chromosomes. Chromosomes increased in the treatment
197 c0t1, c1t1, c2t1, c3t1, c0t2, c1t2, and c3t1b showed that the chromosomes detected were 2n-

198 4n= 24 (diploid) to 48 (tetraploid). In the c2t2 treatment (0.075% colchicine, immersion 16
199 hours) showed, chromosomes detected 2n-3n-4n= 24 (diploid), 36 (triploid) to 48 (tetraploid).

200 Based on the percentage of gated chromosomes, it was found that in the treatment c0t1
201 and c0t2, the dominant chromosome was 2n (diploid) with a percentage of 67.70% and very
202 much different from the 4n chromosome (tetraploid).) with a percentage of 4.17%, so it is
203 diploid. In the c1t1 treatment (0.05% colchicine, immersion 8 hours), the dominant
204 chromosome was 4n (tetraploid) with a percentage of 36.97% but not much different from
205 chromosome 2n (diploid) with a percentage of 28.35% so that it was putative-tetraploid
206 because it allowed ploidy selection to occur. In the treatment c2t1 and c3t1, the dominant
207 chromosomes were 2n (diploid) with a percentage of 52.94% and 55.26%, different from the
208 chromosome 4n (tetraploid) (with a percentage of 24.99% and 16.25%, so it is mixoploid. In
209 the treatment c1t2 and c3t2, the dominant chromosome was 4n (tetraploid) with a percentage
210 of 54.10% and 44.36%, very much different from the chromosome 2n (diploid) with a
211 percentage of 20.27% and 28.24% which do not allow ploidy selection to occur so that they
212 are tetraploid. In treatment c2t2 (0.075% colchicine, immersion 16 hours) showed the
213 dominant chromosome is 4n (tetraploid) with a percentage of 35.50% but not much different
214 from chromosome 2n (diploid) with a percentage of 29.94% and 3n (triploid) with a
215 percentage of 15.39% so that it is putative -tetraploid because it allows ploidy selection.

216 The results of the flow cytometry analysis in Fig. 6 show the chromosome peaks in *Colocasia*
217 *esculenta* var. *Antiquorum* plants were seen in all treatments interpreted as standards for
218 diploid (2n) and tetraploid (4n) cells. Apart from diploid (2n) and tetraploid (4n) cells, triploid
219 cells (3n) were also seen. Although the seven treatments found diploid (2n), triploid (3n), and
220 tetraploid (4n) cells, the peak height of each cell was different in each treatment.

221 **Correlation analysis**

222 The results of the correlation analysis in Table 4 show the relationship between the
223 number of leaf parameters in the in vitro polyploidy of *Colocasia esculenta* var. *Antiquorum*.
224 The number of leaves parameter is a parameter that can be evaluated in the polyploidy of
225 *Colocasia esculenta* var. *Antiquorum* in vitro. The number of leaves was positively correlated
226 with the number of roots (0.80) and positively correlated with the number of compound
227 shoots (0.75).

228

229 **Discussion**

230

231 Polyploidy is a plant breeding technique that is carried out to get plants that have a
232 chromosome number of more than $2n$ (diploid). Polyploidy can be done using mutagens, one
233 of which is colchicine. Colchicine is one of the chemicals known to induce somaclonal
234 variation in polyploidization in cultured plant cells. In plants, colchicine binds to tubulin, one
235 of the main constituents of microtubules, thereby inhibiting the development of spindle fibers
236 and inhibiting further mitotic division leading to the induction of polyploidy (Kharde et al.
237 2017). Colchicine is used to induce polyploidy, which increases the potential for metabolite
238 production and has been used for many years to produce valuable compounds in plants
239 (Bernard et al. 2012).

240 Flow cytometry analysis is a modern method for determining the ploidy level in plants.
241 Flow cytometry has proven to be a fast and efficient method for estimating ploidy levels in
242 almost all plant tissue regeneration and is particularly suitable for studies involving large
243 sample sizes (De et al. 2010; Eeckhaut et al. 2005). For example, Jones et al. (2008) and De et
244 al. (2004) used flow cytometry to determine the ploidy level and genome size of 200 diverse
245 species and *Rhododendron* cultivars which are now a valuable database for breeders. Flow

246 cytometry also determined the ploidy level of induced polyploid adventitious shoots from
247 three *Rhododendron* hybrids.

248 Based on the results of flow cytometry analysis in this study (Table 3 and Fig. 7), the
249 number of chromosomes in the plant cell *Colocasia esculenta* var. *Antiquorum* doubled to
250 tetraploid (4n). It indicates that *Colocasia esculenta* var. *Antiquorum* can be induced by
251 polyploidy by treating with colchicine concentration and immersion time. In addition, the
252 multiplication of chromosomes that occurred varied in each treatment of colchicine
253 concentration and immersion time. Visually, the plantlets (Fig. 3) can confirm the doubling of
254 chromosomes in the *Colocasia esculenta* var. *Antiquorum* plant. The increase in leaf size and
255 petiole in *Colocasia esculenta* var. *Antiquorum* was seen in the c1t2 treatment (0.05%
256 colchicine, immersion 16 hours). In addition, an increase also occurred in the size of shoots
257 formed in the colchicine treatment. So it is by the statement of Mahyuni et al. (2015) stated
258 that diploid plants have a smaller morphological size than polyploid plants.

259 In this study, it was shown that the doubling of the chromosomes formed occurred
260 randomly, giving a non-uniform effect on each cell in an individual. The best chromosomal
261 doubling occurred at low concentrations, namely 0.05% colchicine concentration with 16
262 hours immersion time of 54.10% higher than 44.36% colchicine 0.1% concentration and
263 0.075% colchicine concentration of 35.50%. At 8 hours of immersion, the same thing
264 happened; more tetraploid chromosomes were formed at a low concentration of 0.05% by
265 36.97%. This proves that mutations in plants are different even in the same plant. This follows
266 the results of the polyploidization study of Heo et al. (2016), who concluded that the most
267 prolonged immersion with low colchicine concentrations induced the highest percentage of
268 polyploidy production efficiency among other treatments on *L. leichtlinii* plants. In orchid
269 plants, higher concentrations of colchicine resulted in shorter pods and some dry and dead

270 pods due to colchicine toxicity at high concentrations (1000 and 2000 mg/L) (Sudirman et al.
271 2021).

272 Table 3 and Fig. 7 show that the doubling of the chromosomes formed is diploid,
273 mixoploid, and tetraploid. Mixoploid plants have diploid and tetraploid nuclei, which are
274 assessed based on the relative number of nuclei (Cimen 2020; Koutoulis et al. 2005). In
275 polyploidy induction, a high percentage of mixoploid yield is generally known as a procedure
276 drawback because the unstable polyploidy state often returns wholly or partially to the diploid
277 state after successive cell division cycles (Esfahani et al., 2020). In addition, developing two
278 different cells in a mixoploid bud causes competition during its growth. According to Kainth
279 and Grosser (2010), diploid cells have a faster division rate than autotetraploid cells, allowing
280 the resulting mixoploid shoots to become diploid again.

281 A tetraploid is a polyploid containing four sets of chromosomes. Compared to diploids,
282 tetraploids produce more secondary metabolites and biomass, and tetraploids are drought-
283 tolerant and disease-resistant (Sattler et al. 2016; Xu et al. 2014). Tetraploids can be
284 artificially induced using mitotic spindle inhibitors such as colchicine (Wang et al. 2016).
285 Tetraploid plants have giant leaf and shoot sizes than diploid plants (Kurtz et al. 2020). The
286 study of Wulandari et al. (2015) concluded that 0.1% colchicine treatment for 1 hour was the
287 most efficient in producing tetraploid Pumelo Nambangan citrus plants.

288 Asif (2001) and Tesfaye (2005) later reported that chromosomes were doubled in *Musa*
289 *balbisiana*, various *Musa acuminata* subspecies, and *Ensete ventricosum* species using seeds,
290 respectively. In polyploid induction, colchicine is very efficient inducing (Ganga, 2002).
291 Chromosomal induction was doubled depending on the variable amount of media, antimetabolic
292 agent, type of explant, exposure time, and concentration. Flow cytometry is the pre-eminent
293 method for induced evaluation. Polyploidization. Alternative confirmation methods, such as
294 chromosome number and morphological observations, were also used.

295 Urwin (2014) in his study reported that a concentration of 0.1% and 6 hours of
296 immersion with the immersion method was able to duplicate chromosomes in Lavandins
297 plants (*Lavandula × intermedia* cvs. Grosso and Seal). Sadhukhan et al. (2014) reported that
298 colchicine at a concentration of 0.0005% with 6 hours of immersion could duplicate
299 chromosomes in African marigold (*Tagetes erecta*) plants. The method is carried out by
300 placing the entire plant with roots in a colchicine solution and applying it to the apical shoots
301 on cotton soaked in colchicine.

302 In this study, different concentrations of colchicine treatment and immersion time were
303 found to affect the morphological characteristics of regenerated plants under in vitro
304 conditions. This follows a study conducted by Jala (2014), concluding that the effect of
305 colchicine concentration and duration of time for immersion in *D. muscipula* can be observed
306 in changes in morphology and growth rate, survival rate, and variations that occur at high
307 concentrations of colchicine and immersion time. Longer than the control.

308 Based on the results of the DMRT analysis, there were differences in the number of
309 living leaves (Fig. 4), the number of roots (Fig. 5), and the number of compound shoots (Fig.
310 6). Based on the graph, it shows that the highest data was obtained in the c0t2 treatment (0.0%
311 colchicine, immersion 16 hours) and the lowest data was obtained in the c3t1 treatment (0.1%
312 colchicine, immersion 8 hours). This indicates that adding a small concentration of colchicine
313 and increasing immersion time will significantly affect the morphology of *Colocasia*
314 *esculenta* var. *Antiquorum*. In addition, the increase in concentration and duration of
315 immersion will also cause more exposed cells to be damaged or fail in the growth process.
316 This is the opinion of Allum et al. (2007), who states that if the concentration is correct, there
317 will be a doubling of chromosomes in the cell.

318 In this study, a concentration of 0.0% showed the best morphology. It happened
319 because, in the colchicine treatment, there was stress on the plant, affecting its morphology.

320 However, in subsequent subcultures, other differences can be seen between the concentration
321 of 0.0% and the concentration of colchicine (0.05%, 0.075%, and 0.1%) because the plants
322 were no longer under stress due to colchicine administration. The nature of the colchicine
323 mutagen influenced the morphological differences in each treatment. Colchicine is highly
324 toxic to plants. Therefore low doses with a long exposure period are considered reliable to
325 reduce its toxic effect and increase the rate of polyploid production (Sajjad et al. 2013). The
326 optimal amount of colchicine used in polyploid production varies widely, with concentrations
327 ranging from 0.01% (Thao et al. 2003) to 1.0% (Demtsu et al. 2013).

328 In the correlation analysis in Table 4, the researcher measures the closeness between the
329 variables without paying attention to the affected variables. These variables influence or the
330 magnitude of the influence of one variable on other variables. In this study, the main character
331 is the number of leaves because it can be used as a character to identify tetraploid plants. The
332 results of the correlation analysis showed that four characters were positively correlated,
333 including a very significant positive correlation on the number of roots (0.80) and a
334 significantly positive correlation on the parameters of the number of shoots (0.75), speed of
335 shoots (0.69) and speed of rooting (0.66). Correlation proves the extent to which there is a
336 relationship or close relationship between one or more variables (Astuti, 2017). Correlation
337 with strong character conditions can be used as an indirect selection tool for the main
338 character (Rohaeni 2012).

339 In conclusion, the colchicine had the best effect on the formation of polyploids of Safira
340 taro was the interaction between 0.05% colchicine concentration and 16 hours immersion
341 time (c1t2) which resulted in tetraploid (4n) of 54.10% and colchicine concentration of 0.1%
342 with 16 hours of immersion (c3t2) which yielding a tetraploid (4n) of 44.36%. Colchicine
343 which gives the best effect on morphological parameters, is the interaction between colchicine
344 concentration of 0.075% with 16 hours of immersion (c2t2) which produces several leaves as

345 much as 20.33 leaves, colchicine concentration of 0.075% with 16 hours of immersion (c2t2)
346 which produces a total number of 4.00 roots and colchicine concentration of 0.075% with 8
347 hours of immersion (c2t1) which resulted in the number of shoots as many as 19.33 shoots.

348 **Reference**

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493 Tetraploid *Echinacea purpurea* (L.) Moench. *Plant Cell Tissue Organ Cult.* 116: 323-
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496 Table 1. *Combination of Treatment Between Colchicine Concentration and Immersion Time*

497

Concentration	Immersion Time	
	t1	t2
c0	c0t1	c0t2
c1	c1t1	c1t2
c2	c2t1	c2t2
c3	c3t1	c3t2

498 Notes: C Concentration of colchicine (c0 = 0%; c1 = 0.05%; c2 = 0.075%; c3 = 0.1%); T Immersion Time (t1 =
499 8 hours; t2 = 16 hours).

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501 Table 2. *Middle square analysis of variance on several observation parameters in in vitro*

502 *polyploid induction experiments of colocasia esculenta var. Antiquorum plants*

SD	DF	Middle Square in <i>Annalysis of Variance</i> (ANNOVA)		
		Number of Living Leaves	Number of Roots	Number of Compound Shoots
Time Immersion (T)	1	234.375**	54.000**	15.042 ^{ns}
Colchicine Concentration (C)	3	349.708**	67.667**	494.153**
Interaction of T x C	3	70.708**	18.111**	86.708**
Error	16	4.333	0.542	12.458
CV		16.82%	19.44%	18.40%

503 Note: **: Very Significant ($P \leq 0.01$), *: Significant ($P \leq 0.05$), ns : No Significant, SD: Source of Diversity,

504 DB: Degree of Free, CV: Coefficient of Variance

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512 Table 3. *Results of polyploidy analysis with flow cytometry in colocasia esculenta var.*
 513 *Antiquorum plants in vitro induction of colchicine.*

Treatment	Chromosomes Detected	% Gated	Dominant Chromosomes	Explanation ^(*)
Control (c0t1, c0t2)	2n-4n	67.70 + 4.17	2n	Diploid
C1t1	2n-4n	28.35 + 36.97	4n	Putatif-Tetraploid
c2t1	2n-4n	52.94 + 24.99	2n	Mixoploid
c3t1	2n-4n	55.26 + 16.25	2n	Mixoploid
c1t2	2n-4n	20.27 + 54.10	4n	Tetraploid
c2t2	2n-3n-4n	29.94 + 15.39 + 35.50	4n	Putatif-Tetraploid
c3t2	2n-4n	28.24 + 44.36	4n	Tetraploid

514 Note: 2n (Diploid); 3n (Triploid); 4n (Tetraploid); c0t1 (0% Colchicine, Immersion 8 hours); c1t1 (Colchicine
 515 0.05%, Immersion 8 hours); c2t1 (Colchicine 0.075%, Immersion 8 hours); c3t1(Colchicine 0.1%, Immersion 8
 516 hours); c0t2 (0% Colchicine, Immersion 16 hours); c1t2 (Colchicine 0.05%, Immersion 16 hours); c2t2
 517 (Colchicine 0.075%, Immersion 16 hours); c3t2 (Colchicine 0.1%, Immersion 16 hours). (*) refers to Shahriari-
 518 Ahmadi et al. 2008 and based on the difference between diploid and tetraploid ≤ 1.50)

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520 Table 4. *Results of correlation analysis on all observation parameters of colocasia esculenta*
 521 *var. Antiquorum plants at various concentrations and duration of in vitro immersion of*
 522 *colchicine.*

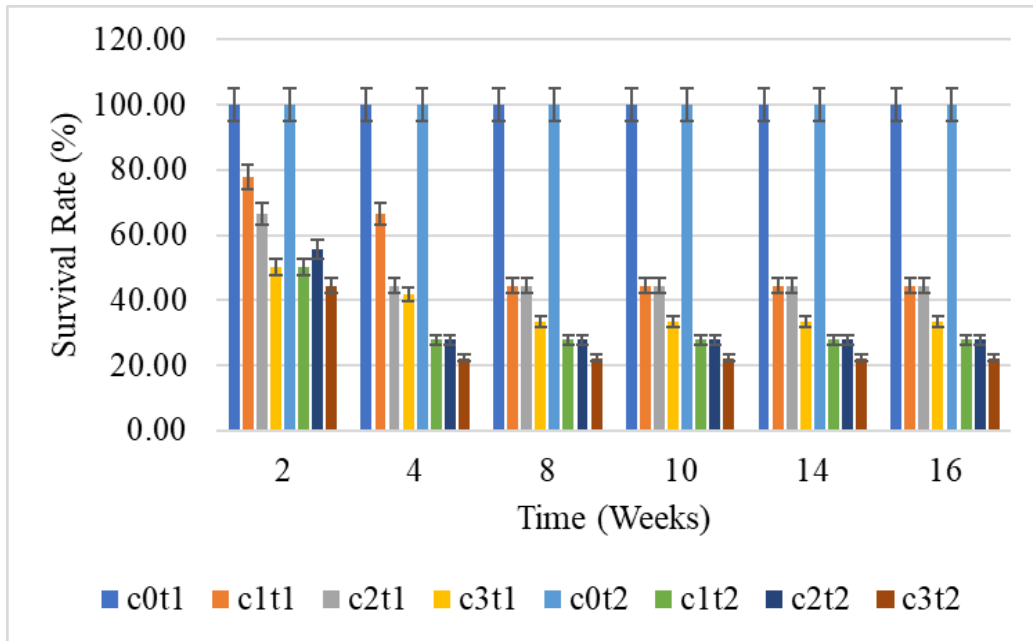
	Number of Compound Shoots	Number of Roots	Number of Living Leaves
Number of Compound Shoots	1.00	0.79**	0.75*
Number of Roots		1.00	0.80**
Number of Leaves			1.00

523 Note: Numbers followed by a sign mean that they are significantly different from the table $r 0.05 = 0.62$ (*); r
 524 $0.01 = 0.79$ (**).

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529 Figure 1. Graph of Living Shooting Percentage of *Colocasia esculenta* var. *Antiquorum* from First Week to
 530 Sixteenth Week After In Vitro Induction. c0t1 (0% Colchicine, Immersion 8 hours); c1t1 (Colchicine 0.05%,
 531 Immersion 8 hours); c2t1 (Colchicine 0.075%, Immersion 8 hours); c3t1(Colchicine 0.1%, Immersion 8 hours);
 532 c0t2 (0% Colchicine, Immersion 16 hours); c1t2 (Colchicine 0.05%, Immersion 16 hours); c2t2 (Colchicine
 533 0.075%, Immersion 16 hours); c3t2 (Colchicine 0.1%, Immersion 16 hours).

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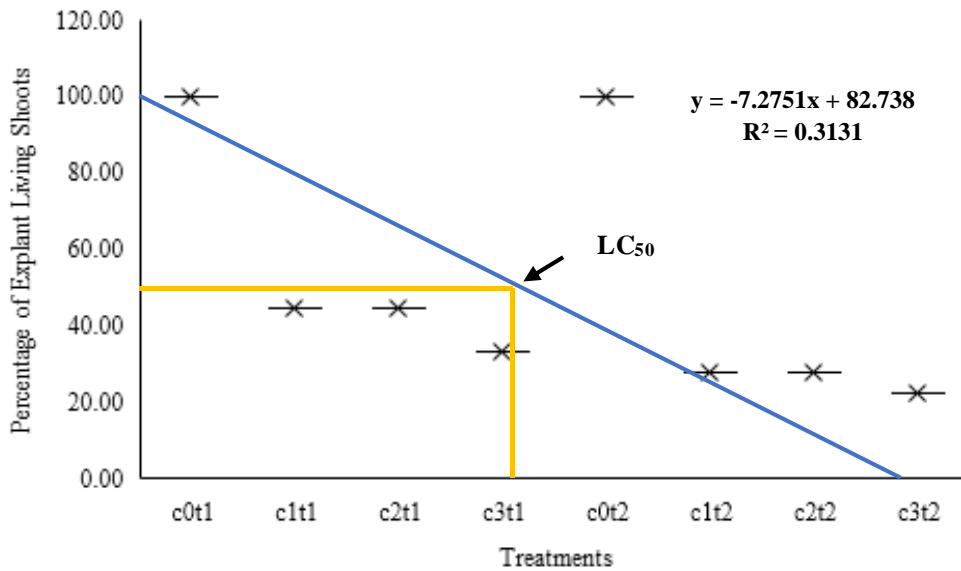
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547 Figure. 2. Graph Letal Concentrasion of Living Shooting Percentage of *Colocasia esculenta* var. *Antiquorum*
 548 from First Week to Sixteenth Week After In Vitro Induction. c0t1 (0% Colchicine, Immersion 8 hours); c1t1
 549 (Colchicine 0.05%, Immersion 8 hours); c2t1 (Colchicine 0.075%, Immersion 8 hours); c3t1(Colchicine 0.1%,
 550 Immersion 8 hours); c0t2 (0% Colchicine, Immersion 16 hours); c1t2 (Colchicine 0.05%, Immersion 16 hours);
 551 c2t2 (Colchicine 0.075%, Immersion 16 hours); c3t2 (Colchicine 0.1%, Immersion 16 hours).

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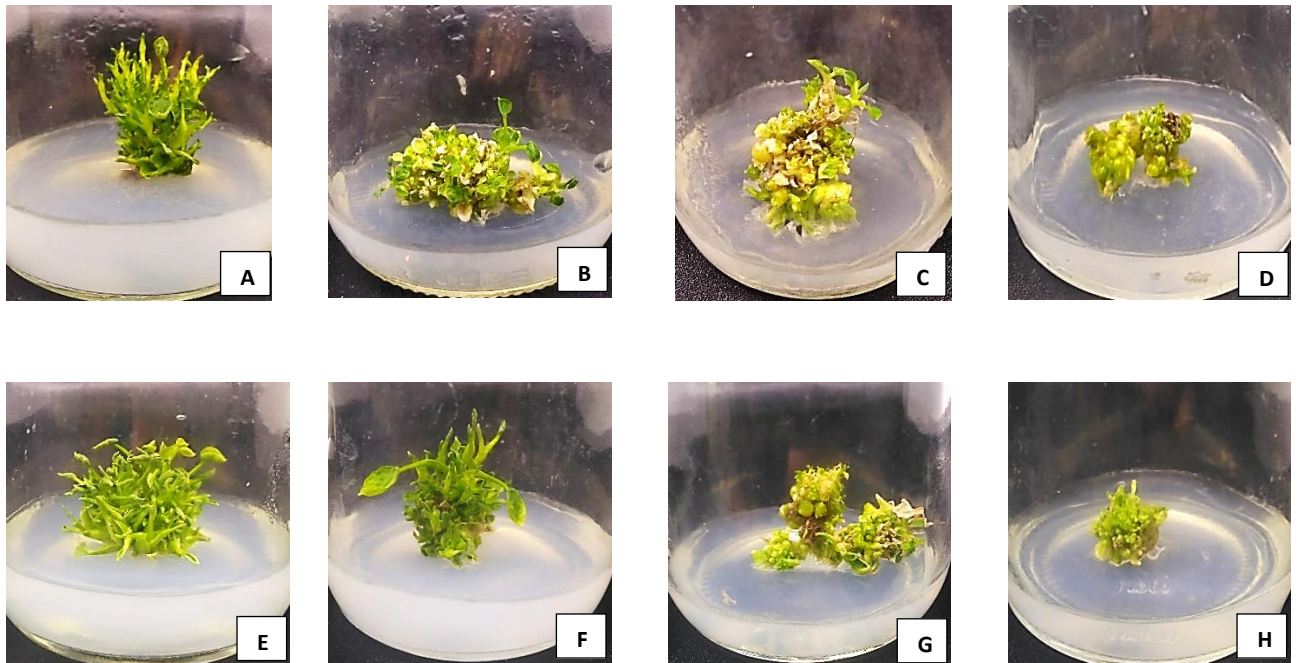
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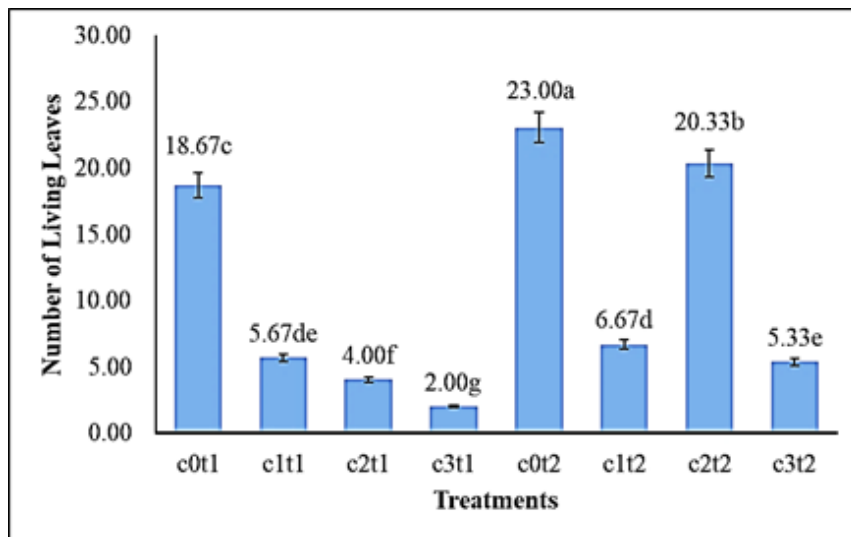
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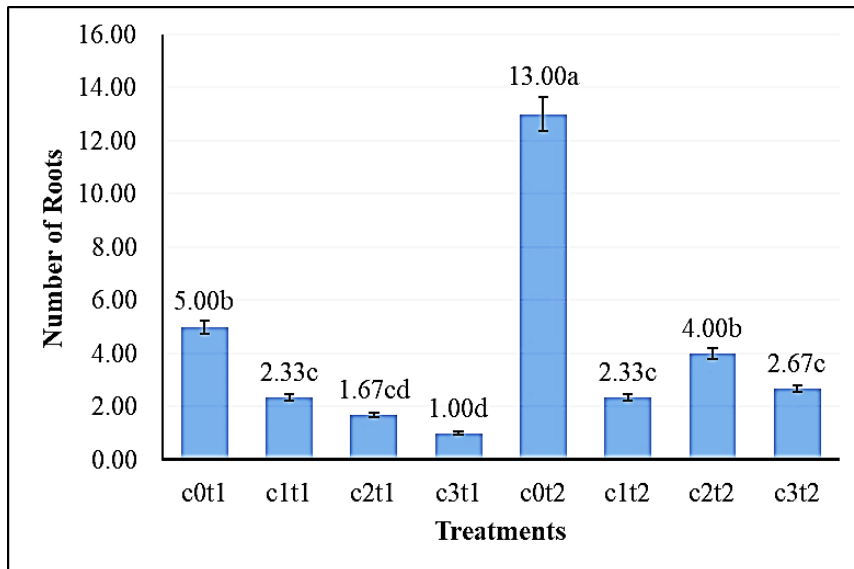
561 Figure 3. *Colocasia esculenta* var. *Antiquorum* Plants With Several Concentrations of Colchicine and In Vitro
 562 Immersion Time After 16 Weeks. (A) c0t1 (0% colchicine, 8 hours immersion); (B) c1t1 (Colchicine 0.05%,
 563 Immersion 8 hours); (C) c2t1 (Colchicine 0.075%, Immersion 8 hours); (D) c3t1(Colchicine 0.1%, Immersion 8
 564 hours); (E) c0t2 (0% Colchicine, Immersion 16 hours); (F) c1t2 (Colchicine 0.05%, Immersion 16 hours); (G)
 565 c2t2 (Colchicine 0.075%, Immersion 16 hours); (H) c3t2 (Colchicine 0.1%, Immersion 16 hours).

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 568 Figure 4. The Effect of Colchicine Concentration and Immersion Time on the Number of Living Leaves of
 569 *Colocasia esculenta* var *Antiquorum* Plants In Vitro. Numbers followed by the same letter (abcdefg) mean that
 570 they are not significantly different in the DMRT test with a level of = 0.05.

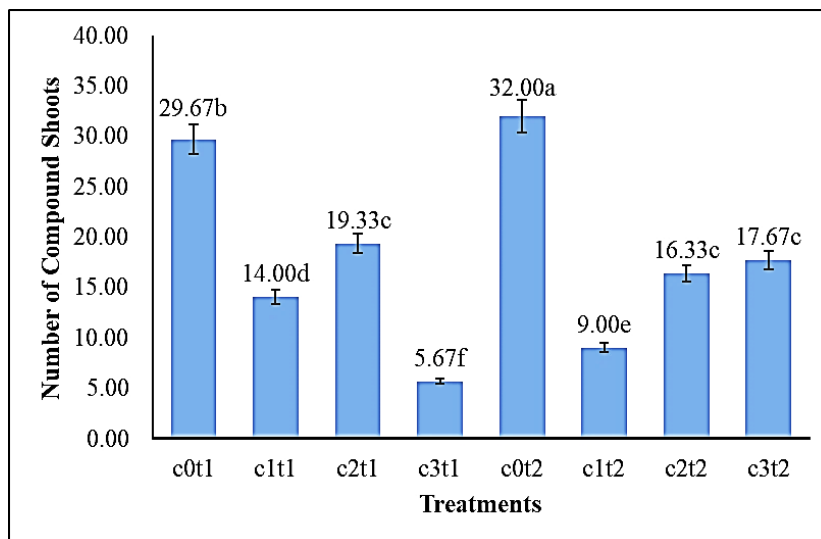
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573 Figure 5. Effect of Colchicine Concentration and Immersion Time on Root Number of *Colocasia esculenta* var.
 574 *Antiquorum* In Vitro. Numbers followed by the same letter (abcd) mean that they are not significantly different
 575 in the DMRT test with a level of = 0.05.

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578 Figure 6. The Effect of Colchicine Concentration and Immersion Time on the Number of Compound Shoots of
 579 *Colocasia esculenta* var. *Antiquorum* Plants In Vitro. Numbers followed by the same letter (abcdef) mean that
 580 they are not significantly different in the DMRT test with a level of = 0.05.

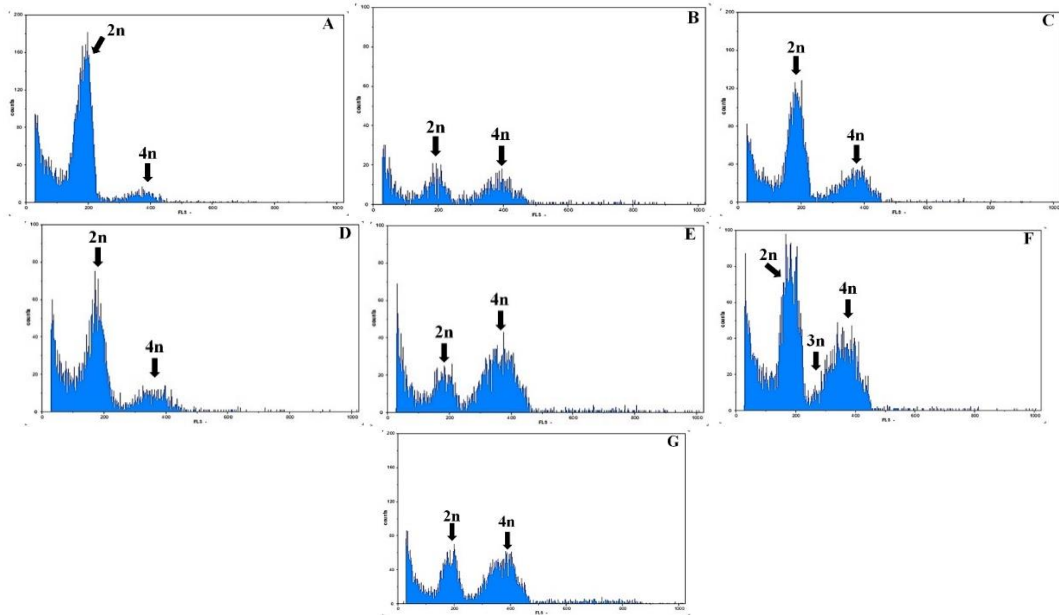
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588 Figure 7. Histogram of Ploidy Degree Results with Flow Cytometry of *Colocasia esculenta* var. *Antiquorum*
589 Plant Resulting in In Vitro Colchicine Induction. (A) c0t1 (0% Colchicine, Immersion 8 hours) and c0t2 (0%
590 Colchicine, Immersion 16 hours); (B) c1t1 (Colchicine 0.05%, Immersion 8 hours); (C) c2t1 (Colchicine
591 0.075%, Immersion 8 hours); (D) c3t1(Colchicine 0.1%, Immersion 8 hours); (E) c1t2 (Colchicine 0.05%,
592 Immersion 16 hours); (F) c2t2 (Colchicine 0.075%, Immersion 16 hours); (G) c3t2 (Colchicine 0.1%, Immersion
593 16 hours).

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Feedback Flow in review 1

609 Reviewer A

610 Please follow the correction according to feedback in the attached file and correct according
611 to journal format.

612 Author Responses

613 Thank you for your comment. We have revised the format according to your suggestion.

Reviewer Comment	Author response
I think this must be italic, please check with the journal format	We have revised according to your suggestion in line 6 and 28
25mm ² (Please correct)	We have revised according to your suggestion in line 105 (25 mm ²)
Please add references for correlation analysis	We have revised according to your suggestion in line 120 (Anshori <i>et al.</i> 2022)
Please use consistent format, it is different with the previous citation	We have revised according to your suggestion in line 273
Please check	We have revised according to your suggestion in line 277
Please follow journal format for references	We have revised according to your suggestion
Please correct Table 1	We have revised according to your suggestion and another reviewer
Please correct Table 2	We have revised according to your suggestion
C1t1 (please correct)	We have revised according to your suggestion in Table 3

614 Reviewer B

615 The manuscript entitled “The Morpho-Somatic and Chromosomal Changes in Colchicine
616 Polyploidy Induction *Colocasia esculenta* Var. *Antiquorum*” provided by Haring et al
617 describes that colchicine treatment resulted in the morphology such as number of shoot, root
618 and leaves, and polyploidy in *Colocasia esculenta* var. *Antiquorum*. Authors suggested that
619 the treatment of 0.05% colchicine concentration with a soaking time of 16 hours can be
620 recommended for the induction of polyploid mutations in *Colocasia esculenta* var.
621 *Antiquorum*.

622 Overall organization and description of experiments, references, and materials were properly
623 managed in the manuscript. However, this manuscript is needed overall editing by a
624 professional editor. Many scientific words are not properly written in the text. Some figure
625 citation is also not matching in the text.

626 Figures and tables are redundantly used in the manuscript. Figure 1 and 2 is not different.
627 Table 1 is useless. I don’t understand what Table2 is meaning. Authors mentioned “tiller” and
628 “salinity stress”.

629 Significance of experiments, results and description is also poor. How many shoots (plant)
630 were regenerated in each condition? What ploidy of each plant were examined? At last, the
631 plant could be survival and fertile or not. These three questions should be addressed in the
632 manuscript. **Author**

633 **Responses**

634 Thank you for your responses. We glad to read this. We have revised our manuscript
635 according to your suggestion.

- 636 1. For the english editing, we can do it after we accepted. We think maybe there are
637 some revisions again for our manuscript. So maybe at final correction stage, we will
638 use english editing service.
- 639 2. Thank you for your comment about Figure 1 and 2; Table 1 and Table 2. We delete
640 Figure 1 because your suggestion. For Table 1, we change this table to be more
641 maningful than before.
- 642 3. For the last paragraph question, we have revised according to your suggestion. we
643 added some explanation for “How many shoots (plant) were regenerated in each
644 condition?” and “the plant could be survival and fertile or not?” in lines 125-131 and
645 Table 1. Meanwhile for the question “What ploidy of each plant were examined?”, we
646 have added in line 104-105. Samples from the best plantlet vigour on each treatment
647 that were taken from the treated plants' leaves of $\pm 25 \text{ mm}^2$.

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Revision 1

The Morpho-Somatic and Chromosomal Changes in Colchicine Polyploidy Induction *Colocasia esculenta* Var. Antiquorium

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Abstract

Increased productivity of *Colocasia esculenta* var. Antiquorium is very necessary to increase the mass of tubers. The polyploid mutation method using colchicine can increase tuber size by increasing the number of chromosomes. Therefore, the study aimed to determine the effect of colchicine on the morphology and polyploid changes of *Colocasia esculenta* var. Antiquorium at various concentrations and duration of immersion in vitro. The research was carried out as a completely randomized factorial design. The first factor is colchicine's concentration, consisting of 0.0%, 0.05%, 0.075%, and 0.1%. The second factor is the immersion time which consists of 8 and 16 hours. The results showed that the best combination of concentration and immersion time on somatic morphology was 0.075% colchicine with 8 hours and 16 hours of immersion. Meanwhile, the best these combinations of chromosomal changes were 0.05% with 16 hours of immersion. Therefore, these results indicate that the treatment of 0.05% colchicine concentration with a soaking time of 16 hours can be recommended for the induction of polyploid mutations in *Colocasia esculenta*.

Keywords: *Colocasia esculenta*, Polyploidy mutation, Colchicine, Diploid, Tetraploid, In vitro

Introduction

688 *Colocasia esculenta* var. Antiquorum is a type of taro with a small tuber size (small
689 corm taro), also known as Japanese taro (Maretta et al. 2016). *Colocasia esculenta* var.
690 Antiquorum tends to be more resistant to weather changes, where this plant can live again,
691 although with a different season character than before. Based on data from the Food Security
692 Service of South Sulawesi Province, the productivity of *Colocasia esculenta* var. Antiquorum
693 in 2020 was 7.214 tons. However, it resulted in exports of *Colocasia esculenta* L. tubers only
694 amounting to 300 tons/ha due to the low productivity of *Colocasia esculenta* L. (Nagano et al.
695 2016).

696 Increasing the productivity of *Colocasia esculenta* var. Antiquorum can be done through
697 plant breeding. One method of plant breeding is through the induction of polyploidy
698 mutations. Polyploidy induction aims to increase crop productivity because polyploid
699 (tetraploid) plants are known to have a more significant figure, fruit size, tuber, or flower than
700 diploid plants (Suryo 2007). Another advantage of tetraploid plants is their resistance to
701 specific environmental stresses (Deng et al. 2012).

702 Polyploidy can be done by giving chemical mutagens such as colchicine to plant
703 meristem tissue. Colchicine can change the number of plant chromosomes and cause gene
704 mutations on the scale of seeds and vegetatively propagated plants. In addition, it induces
705 polyploidy by inhibiting the formation of spindle fibers during cell division. In contrast, the
706 number of chromosomes increases, but cell division does not occur, producing polyploid cells
707 with a doubled number of chromosomes (Manzoor et al. 2018,2019 ; Ade et al. 2010).

708 Colchicine is an important mutagen that prevents microtubule formation and doubles the
709 number of chromosomes. It is commonly used to develop polyploid plants and functions as a
710 mitotic toxin, producing multiple plant mutagenic effects (El-Nashar et al., 2015). Colchicine
711 not only helps in doubling chromosomes but also causes plant mutations. Plants that have
712 mutated through colchicine are known as Colchis-mutants (Ari et al. 2015). The concentration

713 of colchicine for seed treatment is usually in the range of 0.1% - 0.8%, but high doses cause
714 malformations and reduce the production of tetraploid plants. So, it is recommended to use
715 colchicine with the lowest possible concentration (Pirkoohi et al. 2011).

716 The success of polyploidy induction depends on the explant, plant species, colchicine
717 concentration, and duration of exposure. Concentrations that are too high cause problems in
718 the form of abnormalities in developing seedlings (Manzoor et al. 2018.2019). So this needs
719 to be done very carefully by a plant breeder. Several studies on polyploid induction using
720 colchicine have been successful with different concentrations, such as *Papaver bracteatum* L.
721 (Esfahani et al. 2020), *Allium Sativum* L. (Ayu et al. 2019), *Colchicum chalcedonicum* Azn.
722 (Karlik et al. 2020), *Neolamarckia cadamba* (Eng et al. 2021), *Dionaea muscipula* Ellis (Jala,
723 2014), *Nigella sativa* (Gupta et al. 2021), and *Rhododendron fortunei* L. (Mo et al. 2020).
724 Therefore, polyploid induction can be used to create or select better offspring for further use.

725 Based on the description above, it is necessary to research the Induction of Polyploidy
726 Mutations in Plants *Colocasia esculenta* var *Antiquorum*. The aim is to determine the effect
727 of colchicine on morphology and chromosome polyploidy of *Colocasia esculenta* var
728 *Antiquorum* plantlets on the interaction of concentration and immersion time in vitro. This
729 study's novelty is the first study in the induction of polyploidy mutations in *Colocasia*
730 *esculenta* var. *Antiquorum* plant.

731

732 **Materials and Methods**

733 **Experimental design**

734 The study was performed at the Tissue Culture Laboratory, Department of
735 Agricultural Cultivation, Faculty of Agriculture, Hasanuddin University, Makassar, South
736 Sulawesi, 90245, Indonesia. This study was conducted as a 2-factor factorial (F2F)
737 experiment with a Completely Randomized Design (CRD) as the environmental design with

738 three replications in each of the eight treatments. The combination of treatment between
739 colchicine concentration c0 = 0%; c1 = 0.05%; c2 = 0.075%; c3 = 0.1%) and immersion time
740 (t1 = 8 hours; t2 = 16 hours). In one replication, there were three shoots. In this study, there
741 were 82 individual shoots, as the experimental unit observed.

742 **Experimental conditional**

743 The plantlets used as material for polyploidy induction were eight weeks old *Colocasia*
744 *esculenta* var Antiquorum shoots which were then used as explants. The in vitro shoots were
745 removed, and the petioles were cut to a size of 1-3 cm. The immersion is carried out in
746 laminar, and the equipment used is sterile. Immersion was done by immersion of the shoots in
747 colchicine solution at various concentrations of 0% (sterile distilled water) (c0), 0.05% (c1),
748 0.075% (c2), and 0.1% (c3). The shoots were immersed in a culture bottle containing 15 ml of
749 colchicine solution and shaken at 100 rpm on a shaker for 8 hours (c1) and 16 hours (c2).
750 After immersion, the explants were washed with sterile distilled water 3-4 times (sterile) and
751 then planted into shoot propagation media. The shoot propagation medium was MS media
752 with 2 mg/l BAP and 1 mg/l IBA. The medium contains sugar (30 g/l), and the pH of the
753 media is adjusted to 5.8 and compacted with 7 g/l agar. The shoots were kept in an incubation
754 room at a temperature of 25-26°C with 16 hours/day irradiation and an irradiation intensity of
755 ±1,000-2,000 lux (Modification of Dwiyani, 2015). Shoots were incubated for 16 week after
756 planting (WAP). Observations were made every week from outside the culture bottle for 16
757 WAP.

758 **Observation of parameters**

759 Several parameters observed from shoot explants were the percentage of living shoots
760 (%), the number of living leaves, the number of roots and the number of compound shoots.

$$761 \quad \text{Percentage of living explants} = \frac{\sum \text{explants planted} - \text{explants dead}}{\sum \text{explants planted}} \times 100\%$$

762 **Chromosome analysis with flow cytometry**

763 Polyploidy level analysis was performed using flow cytometry. Samples from the best
764 plantlet vigour on each treatment that were taken from the treated plants' leaves of $\pm 25 \text{ mm}^2$.
765 The sample was then ground using a razor blade in a petri dish with 0.2 ml of extraction
766 buffer (solution A from Partec Kit), then incubated for 10 minutes at room temperature. After
767 that, the supernatants from the sample were filtered using nylon kesh measuring 30 m and
768 placed in a cuvette which was then added to 1 ml of DAPI solution. The sample was
769 incubated again for 1 minute. The sample is then inserted into the Flow cytometry tool. The
770 tool will read the light intensity of the measured sample to show the number of ploidy
771 changes that occur, which will be shown through the graph displayed in the tool (Escobedo-
772 Gracia-Medrano et al. 2018).

773 **Data analysis**

774 The F test analyzed data to determine the interaction between colchicine concentration
775 and immersion time. If the variance obtained has a significant effect, perform the Duncan's
776 Multiple Range Test (DMRT) tests at a 5% significance level to determine the effect of
777 differences between treatments. Data processing used the STAR (Statistical Tool for
778 Agricultural Research) program and Microsoft Office Excel 2019. Data were analyzed by
779 correlation analysis to see the relationship between the parameters (Anshori *et al.* 2022).

780

781 **Results**

782

783 **Percentage of Living Shoots of *Colocasia esculenta* var *Antiuorum* In Vitro**

784 The results of the living shoots are shown in Table 1. Based on this table, control shoots
785 had a very high percentage of survival compared to the presentation of the combination of
786 concentration and immersion time. In this study, there were differences in the number of
787 shoots planted due to contamination of the media before treatment, so from the 18 shoots that

788 were propagated, there was a variation in the number of shoots that could be grown before
789 colchicine immersion treatment. However, this can be corrected through the percentage of
790 living shoots from these shoots, which are analyzed via graphs.

791 Based on the graph of living shoot percentage, this study showed a negative graph with
792 the increase of colchicine concentration and time of immersion. The graph has formed a linear
793 regression with the formula $Y = 7.2751x + 82.378$, and the coefficient of determination (R²)
794 was 0.3131 (Fig 1). Based on this formula, the lethal 50 is in 0.1% colchicine concentration
795 with 8 hours of immersion. The relationship between the percentage of surviving shoot
796 explants was inversely proportional to the concentration and duration of colchicine
797 immersion. The higher the concentration and the longer the immersion of colchicine, the
798 lower the number of explants that survived. However, the survival plantlet in the chemical
799 mutants has unique traits change. So, this mutation process needs a meeting point between the
800 plantlet regeneration and optimum diversity in chemical mutation induction. Therefore, the
801 lethal 50 is a practical point to meet both.

802 The analysis results of the mean square of variance in the polyploid mutation induction
803 were shown in Table 2. The analysis showed that the treatment duration of colchicine
804 immersion in *Colocasia esculenta* var *Antiquorum* significantly affected all observation
805 parameters. The colchicine concentration level also showed a significant effect for all
806 observation parameters. The interaction effect between concentration level (c) and immersion
807 time (t) also significantly affected the number of leaves, roots, and shoots. In addition to these
808 parameters, it is also seen in the coefficient of diversity in the medium category.

809 **Number of living leaves**

810 The results of the DMRT test ($\alpha = 0.05$) Fig. 2 showed the number of living leaves of
811 *Colocasia esculenta* var. *Antiquorum* in the best treatment, c0t2 (23.00 leaves), was
812 significantly different from c2t2 (20.33 leaves), c1t2 (6.67 leaves), and c3t2 (5.33 leaves).

813 The lowest mean number of living leaves of *Colocasia esculenta* var *Antiquorum* in the
814 treatment c3t1 (2.00 leaves) was significantly different from c2t1 (4.00 leaves), c1t1 (5.67
815 leaves), c0t1 (18.67 leaves).

816 **Number of roots**

817 The results of the DMRT test ($\alpha = 0.05$) Fig. 3 showed the number of roots of the best
818 *Colocasia esculenta* var *Antiquorum* in the treatment c0t2 (13.00 roots) was significantly
819 different from c2t2 (4.00 roots), c3t2 (2.67 roots), c1t2 (2.33 leaves). The lowest mean
820 number of roots of *Colocasia esculenta* var *Antiquorum* in the treatment c3t1 (1.00 roots) was
821 significantly different from c0t1 (5.00 roots) but not significantly different from c1t1 (2.33
822 roots), c2t1 (1.67 roots).

823 **Number of compound shoots**

824 The results of the DMRT test ($\alpha = 0.05$) Fig. 4 showed the number of the best
825 *Colocasia esculenta* var. *Antiquorum* compound shoots in the treatment c0t2 (32.00 shoots)
826 were significantly different from c3t2 (17.67 shoots), c2t2 (16.33 shoots), c1t2 (9.00 shoots),
827 but c3t2 (17.67 shoots) was not significantly different from c2t2 (16.33 shoots). The lowest
828 average number of shoots of *Colocasia esculenta* var. *Antiquorum* in the treatment c3t1 (5.67
829 shoots) was significantly different from c1t1 (14.00 shoots), c2t1 (19.33 shoots), c0t1 (29.67
830 shoots).

831 **Ploidy analysis of plants *Colocasia esculenta* var. *Antiquorum***

832 The ploidy analysis using flow cytometry on *Colocasia esculenta* var plant samples.
833 *Antiquorum* in Table 3 shows that the difference in concentration and duration of colchicine
834 immersion can affect plant chromosomes. Chromosomes increased in the treatment c0t1, c1t1,
835 c2t1, c3t1, c0t2, c1t2, and c3t1b showed that the chromosomes detected were $2n-4n= 24$
836 (diploid) to 48 (tetraploid). In the c2t2 treatment (0.075% colchicine, immersion 16 hours)
837 showed, chromosomes detected $2n-3n-4n= 24$ (diploid), 36 (triploid) to 48 (tetraploid).

838 Based on the percentage of gated chromosomes, it was found that in the treatment c0t1 and
839 c0t2, the dominant chromosome was 2n (diploid) with a percentage of 67.70% and very much
840 different from the 4n chromosome (tetraploid).) with a percentage of 4.17%, so it is diploid.
841 In the c1t1 treatment (0.05% colchicine, immersion 8 hours), the dominant chromosome was
842 4n (tetraploid) with a percentage of 36.97% but not much different from chromosome 2n
843 (diploid) with a percentage of 28.35% so that it was putative-tetraploid because it allowed
844 ploidy selection to occur. In the treatment c2t1 and c3t1, the dominant chromosomes were 2n
845 (diploid) with a percentage of 52.94% and 55.26%, different from chromosome 4n
846 (tetraploid) (with a percentage of 24.99% and 16.25%, so it is mixoploid. In the treatment
847 c1t2 and c3t2, the dominant chromosome was 4n (tetraploid) with a percentage of 54.10% and
848 44.36%, very much different from chromosome 2n (diploid) with a percentage of 20.27% and
849 28.24% which do not allow ploidy selection to occur so that they are tetraploid. In treatment
850 c2t2 (0.075% colchicine, immersion 16 hours) showed the dominant chromosome is 4n
851 (tetraploid) with a percentage of 35.50% but not much different from chromosome 2n
852 (diploid) with a percentage of 29.94% and 3n (triploid) with a percentage of 15.39% so that it
853 is putative -tetraploid because it allows ploidy selection.

854 The results of the flow cytometry analysis in Fig. 5 show the chromosome peaks in *Colocasia*
855 *esculenta* var. *Antiquorum* plants were seen in all treatments interpreted as standards for
856 diploid (2n) and tetraploid (4n) cells. Apart from diploid (2n) and tetraploid (4n) cells, triploid
857 cells (3n) were also seen. Although the seven treatments found diploid (2n), triploid (3n), and
858 tetraploid (4n) cells, the peak height of each cell was different in each treatment.

859 **Correlation analysis**

860 The results of the correlation analysis in Table 4 show the relationship between the
861 number of leaf parameters in the in vitro polyploidy of *Colocasia esculenta* var. *Antiquorum*.
862 The number of leaves parameter is a parameter that can be evaluated in the polyploidy of

863 *Colocasia esculenta* var. *Antiquorum* in vitro. The number of leaves was positively correlated
864 with the number of roots (0.80) and positively correlated with the number of compound
865 shoots (0.75).

866

867 **Discussion**

868

869 Polyploidy is a plant breeding technique carried out to get plants with a chromosome
870 number of more than $2n$ (diploid). Polyploidy can be done using mutagens, one of which is
871 colchicine. Colchicine is one of the chemicals known to induce somaclonal variation in
872 polyploidization in cultured plant cells. In plants, colchicine binds to tubulin, one of the main
873 constituents of microtubules, thereby inhibiting the development of spindle fibers and
874 inhibiting further mitotic division leading to the induction of polyploidy (Kharde et al. 2017).
875 Colchicine is used to induce polyploidy, which increases the potential for metabolite
876 production and has been used for many years to produce valuable compounds in plants
877 (Bernard et al. 2012).

878 Flow cytometry analysis is a modern method for determining the ploidy level in plants.
879 Flow cytometry has proven to be a fast and efficient method for estimating ploidy levels in
880 almost all plant tissue regeneration and is particularly suitable for studies involving large
881 sample sizes (De et al. 2010; Eeckhaut et al. 2005). For example, Jones et al. (2008) and De et
882 al. (2004) used flow cytometry to determine the ploidy level and genome size of 200 diverse
883 species and *Rhododendron* cultivars, now a valuable database for breeders. Flow cytometry
884 also determined the ploidy level of induced polyploid adventitious shoots from three
885 *Rhododendron* hybrids.

886 Based on the results of flow cytometry analysis in this study (Table 3 and Fig. 6), the
887 number of chromosomes in the plant cell *Colocasia esculenta* var. *Antiquorum* doubled to

888 tetraploid (4n). It indicates that *Colocasia esculenta* var. *Antiquorum* can be induced by
889 polyploidy by treating with colchicine concentration and immersion time. In addition, the
890 multiplication of chromosomes varied in each colchicine concentration and immersion time
891 treatment. Visually, the plantlets (Fig. 2) can confirm the doubling of chromosomes in the
892 *Colocasia esculenta* var. *Antiquorum* plant. The increase in leaf size and petiole in *Colocasia*
893 *esculenta* var. *Antiquorum* was seen in the c1t2 treatment (0.05% colchicine, immersion 16
894 hours). In addition, an increase also occurred in the size of shoots formed in the colchicine
895 treatment. So it is by the statement of Mahyuni et al. (2015) stated that diploid plants have a
896 smaller morphological size than polyploid plants.

897 This study showed that the doubling of the chromosomes formed occurred randomly,
898 giving a non-uniform effect on each cell in an individual. The best chromosomal doubling
899 occurred at low concentrations, namely 0.05% colchicine concentration with 16 hours
900 immersion time of 54.10% higher than 44.36% colchicine 0.1% concentration and 0.075%
901 colchicine concentration of 35.50%. The same thing happened at 8 hours of immersion; more
902 tetraploid chromosomes were formed at a low concentration of 0.05% by 36.97%. This proves
903 that mutations in plants are different even in the same plant. This follows the results of the
904 polyploidization study of Heo et al. (2016), who concluded that the most prolonged
905 immersion with low colchicine concentrations induced the highest percentage of polyploidy
906 production efficiency among other treatments on *L. leichtlinii* plants. In orchid plants, higher
907 concentrations of colchicine resulted in shorter pods and some dry and dead pods due to
908 colchicine toxicity at high concentrations (1000 and 2000 mg/L) (Sudirman et al. 2021).

909 Table 3 and Fig. 6 show that the doubling of the chromosomes formed is diploid,
910 mixoploid, and tetraploid. Mixoploid plants have diploid and tetraploid nuclei, assessed based
911 on the relative number of nuclei (Cimen 2020; Koutoulis et al. 2005). In polyploidy induction,
912 a high percentage of mixoploid yield is generally known as a procedure drawback because the

913 unstable polyploidy state often returns wholly or partially to the diploid state after successive
914 cell division cycles (Esfahani et al., 2020). In addition, developing two different cells in a
915 mixoploid bud causes competition during its growth. According to Kainth and Grosser
916 (2010), diploid cells have a faster division rate than autotetraploid cells, allowing the resulting
917 mixoploid shoots to become diploid again.

918 A tetraploid is a polyploid containing four sets of chromosomes. Compared to diploids,
919 tetraploids produce more secondary metabolites and biomass, and tetraploids are drought-
920 tolerant and disease-resistant (Sattler et al. 2016; Xu et al. 2014). Tetraploids can be
921 artificially induced using mitotic spindle inhibitors such as colchicine (Wang et al. 2016).
922 Tetraploid plants have giant leaf and shoot sizes than diploid plants (Kurtz et al. 2020). The
923 study of Wulandari et al. (2015) concluded that 0.1% colchicine treatment for 1 hour was the
924 most efficient in producing tetraploid Pumelo Nambangan citrus plants.

925 Asif (2001) and Tesfaye (2005) later reported that chromosomes were doubled in *Musa*
926 *balbisiana*, various *Musa acuminata* subspecies, and *Ensete ventricosum* species using seeds,
927 respectively. In polyploid induction, colchicine is very efficient inducing (Ganga 2002).
928 Chromosomal induction was doubled depending on the variable amount of media, antimetabolic
929 agent, type of explant, exposure time, and concentration. Flow cytometry is the pre-eminent
930 method for induced evaluation polyploidization. Alternative confirmation methods were also
931 used, such as chromosome number and morphological observations.

932 Urwin (2014) in his study reported that a concentration of 0.1% and 6 hours of
933 immersion with the immersion method could duplicate chromosomes in Lavandins plants
934 (*Lavandula × intermedia* cvs. Grosso and Seal). Sadhukhan et al. (2014) reported that
935 colchicine at a concentration of 0.0005% with 6 hours of immersion could duplicate
936 chromosomes in African marigold (*Tagetes erecta*) plants. The method is carried out by

937 placing the entire plant with roots in a colchicine solution and applying it to the apical shoots
938 on cotton soaked in colchicine.

939 In this study, different concentrations of colchicine treatment and immersion time were
940 found to affect the morphological characteristics of regenerated plants under in vitro
941 conditions. This follows a study conducted by Jala (2014), concluding that the effect of
942 colchicine concentration and duration of time for immersion in *D. muscipula* can be observed
943 in changes in morphology and growth rate, survival rate, and variations that occur at high
944 concentrations of colchicine and immersion time. Longer than the control.

945 Based on the results of the DMRT analysis, there were differences in the number of
946 living leaves (Fig. 3), the number of roots (Fig. 4), and the number of compound shoots (Fig.
947 5). Based on the graph, it shows that the highest data was obtained in the c0t2 treatment (0.0%
948 colchicine, immersion 16 hours) and the lowest data was obtained in the c3t1 treatment (0.1%
949 colchicine, immersion 8 hours). This indicates that adding a small concentration of colchicine
950 and increasing immersion time will significantly affect the morphology of *Colocasia*
951 *esculenta* var. *Antiquorum*. In addition, the increase in concentration and duration of
952 immersion will also cause more exposed cells to be damaged or fail in the growth process.
953 This is the opinion of Allum et al. (2007), who states that if the concentration is correct,
954 chromosomes will be doubled in the cell.

955 In this study, a concentration of 0.0% showed the best morphology. It happened
956 because, in the colchicine treatment, there was stress on the plant, affecting its morphology.
957 However, in subsequent subcultures, other differences can be seen between the concentration
958 of 0.0% and the concentration of colchicine (0.05%, 0.075%, and 0.1%) because the plants
959 were no longer under stress due to colchicine administration. The nature of the colchicine
960 mutagen influenced the morphological differences in each treatment. Colchicine is highly
961 toxic to plants. Therefore low doses with a long exposure period are considered reliable to

962 reduce its toxic effect and increase the rate of polyploid production (Sajjad et al. 2013). The
963 optimal amount of colchicine used of polyploid output varies widely, with concentrations
964 ranging from 0.01% (Thao et al. 2003) to 1.0% (Demtsu et al. 2013).

965 In the correlation analysis in Table 4, the researcher measures the closeness between the
966 variables without paying attention to the affected variables. These variables influence or the
967 magnitude of the influence of one variable on other variables. In this study, the main character
968 is the number of leaves because it can be used as a character to identify tetraploid plants. The
969 results of the correlation analysis showed that four characters were positively correlated,
970 including a very significant positive correlation on the number of roots (0.80) and a
971 significantly positive correlation on the parameters of the number of shoots (0.75), speed of
972 shoots (0.69) and speed of rooting (0.66). Correlation proves the extent to which a
973 relationship or close relationship exists between one or more variables (Astuti, 2017).
974 Correlation with strong character conditions can be used as an indirect selection tool for the
975 main character (Rohaeni 2012).

976 In conclusion, the colchicine had the best effect on the formation of polyploids of Safira
977 taro was the interaction between 0.05% colchicine concentration and 16 hours immersion
978 time (c1t2) which resulted in tetraploid (4n) of 54.10% and colchicine concentration of 0.1%
979 with 16 hours of immersion (c3t2) which yielding a tetraploid (4n) of 44.36%. Colchicine
980 which gives the best effect on morphological parameters, is the interaction between colchicine
981 concentration of 0.075% with 16 hours of immersion (c2t2) which produces several leaves as
982 much as 20.33 leaves, colchicine concentration of 0.075% with 16 hours of immersion (c2t2)
983 which produces a total number of 4.00 roots and colchicine concentration of 0.075% with 8
984 hours of immersion (c2t1) which resulted in the number of shoots as many as 19.33 shoots.

985

986

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1143 Table 1. *Percentage of living shoots on each treatment of Colchicine concentration and time*
1144 *of Immersion.*

Treatments	Shoot planted	Living shoots	Dead shoots	living Percentage (%)
c0t1	13	13	0	100.00
c1t1	9	4	5	44.44
c2t1	9	4	5	44.44
c3t1	12	4	8	33.33
c0t2	13	13	0	100.00
c1t2	18	5	13	27.78
c2t2	18	5	13	27.78
c3t2	18	4	12	22.22
Total	110	52	56	47.27

1145 Notes: c0t1 (0% Colchicine, Immersion 8 hours); c1t1 (Colchicine 0.05%, Immersion 8 hours); c2t1 (Colchicine
1146 0.075%, Immersion 8 hours); c3t1(Colchicine 0.1%, Immersion 8 hours); c0t2 (0% Colchicine, Immersion 16
1147 hours); c1t2 (Colchicine 0.05%, Immersion 16 hours); c2t2 (Colchicine 0.075%, Immersion 16 hours); c3t2
1148 (Colchicine 0.1%, Immersion 16 hours).

1149
1150 Table 2. *Mean square analysis of variance on several observation parameters in in vitro*
1151 *polyploid induction experiments of Colocasia esculenta var. Antiquorum plants.*

Source	DF	Mean Square in <i>Annalysis of Variance</i> (ANNOVA)		
		Number of Living Leaves	Number of Roots	Number of Compound

				Shoots
Time Immersion (T)	1	234.375**	54.000**	15.042 ^{ns}
Colchicine Concentration (C)	3	349.708**	67.667**	494.153**
Interaction of T x C	3	70.708**	18.111**	86.708**
Error	16	4.333	0.542	12.458
CV (%)		16.82%	19.44%	18.40%

1152 Note: **: Very Significant ($P \leq 0.01$), *: Significant ($P \leq 0.05$), ns : No Significant, DF: Degree of Free, CV:

1153 Coefficient of Variance

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1159 Table 3. Results of polyploidy analysis with flow cytometry in *Colocasia esculenta* var.

1160 *Antiquorum* plants in vitro induction of colchicine.

Treatment	Chromosomes Detected	% Gated	Dominant Chromosomes	Explanation ^(*)
Control (c0t1, c0t2)	2n-4n	67.70 + 4.17	2n	Diploid
c1t1	2n-4n	28.35 + 36.97	4n	Putatif-Tetraploid
c2t1	2n-4n	52.94 + 24.99	2n	Mixoploid
c3t1	2n-4n	55.26 + 16.25	2n	Mixoploid
c1t2	2n-4n	20.27 + 54.10	4n	Tetraploid
c2t2	2n-3n-4n	29.94 + 15.39 + 35.50	4n	Putatif-Tetraploid
c3t2	2n-4n	28.24 + 44.36	4n	Tetraploid

1161 Note: 2n (Diploid); 3n (Triploid); 4n (Tetraploid); c0t1 (0% Colchicine, Immersion 8 hours); c1t1 (Colchicine

1162 0.05%, Immersion 8 hours); c2t1 (Colchicine 0.075%, Immersion 8 hours); c3t1(Colchicine 0.1%, Immersion 8

1163 hours); c0t2 (0% Colchicine, Immersion 16 hours); c1t2 (Colchicine 0.05%, Immersion 16 hours); c2t2

1164 (Colchicine 0.075%, Immersion 16 hours); c3t2 (Colchicine 0.1%, Immersion 16 hours). (*) refers to Shahriari-

1165 Ahmadi *et al.* 2008 and based on the difference between diploid and tetraploid ≤ 1.50)

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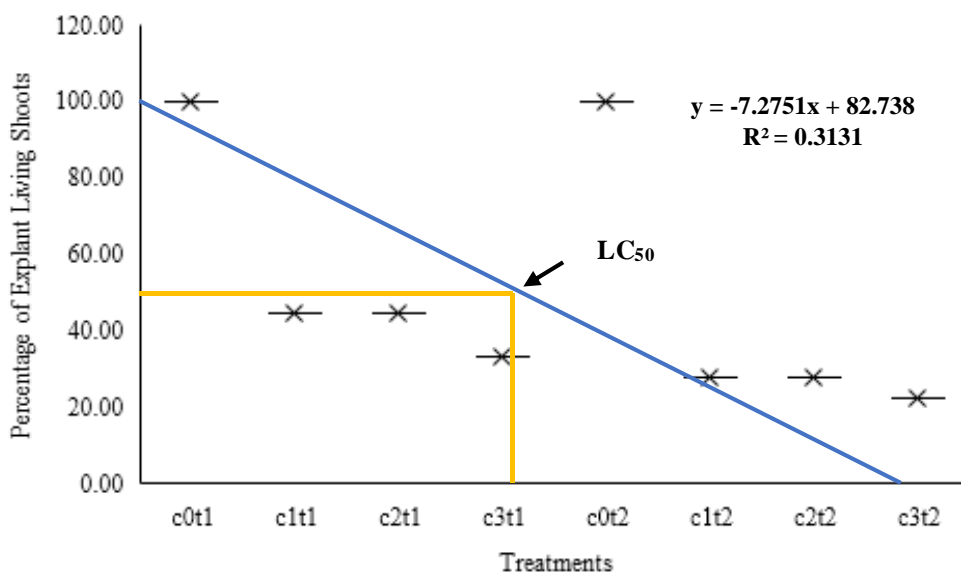
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1168 Table 4. Results of correlation analysis on all observation parameters of *Colocasia esculenta*
 1169 var. *Antiquorum* plants at various concentrations and duration of in vitro immersion of
 1170 colchicine.

	Number of Compound Shoots	Number of Roots	Number of Living Leaves
Number of Compound Shoots	1.00	0.79**	0.75*
Number of Roots		1.00	0.80**
Number of Leaves			1.00

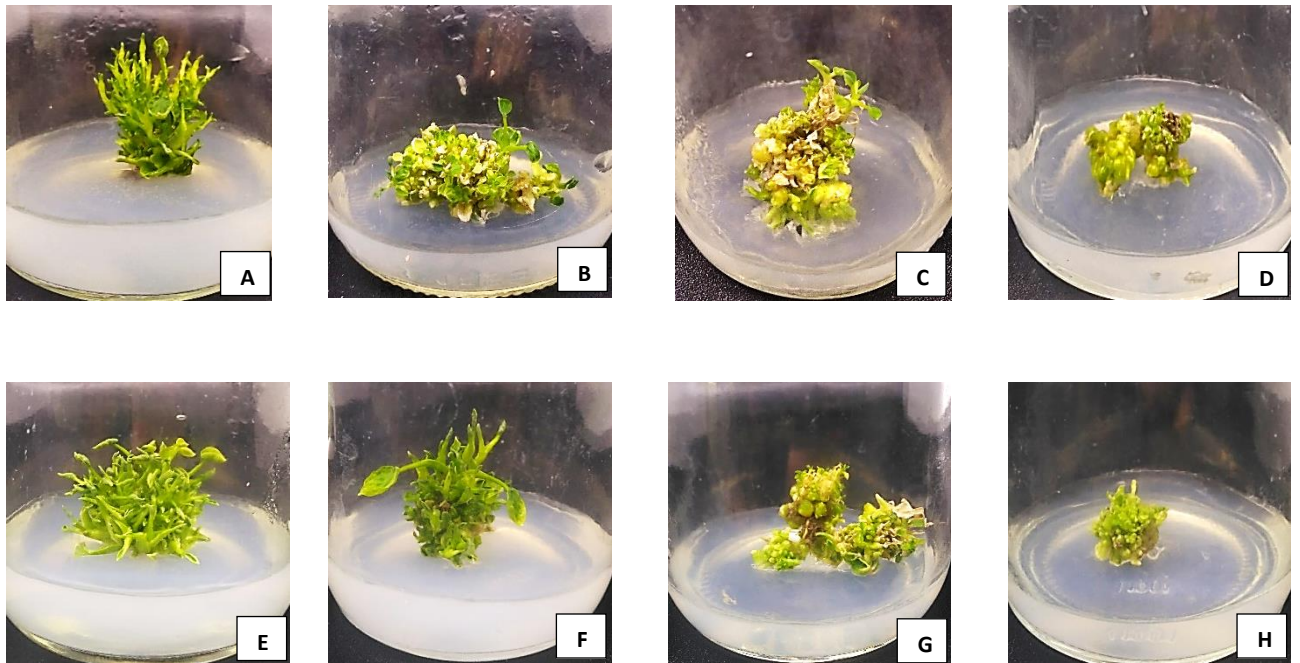
1171 Note: Numbers followed by a sign mean that they are significantly different from the table $r_{0.05} = 0.62$ (*); $r_{0.01} = 0.79$ (**).

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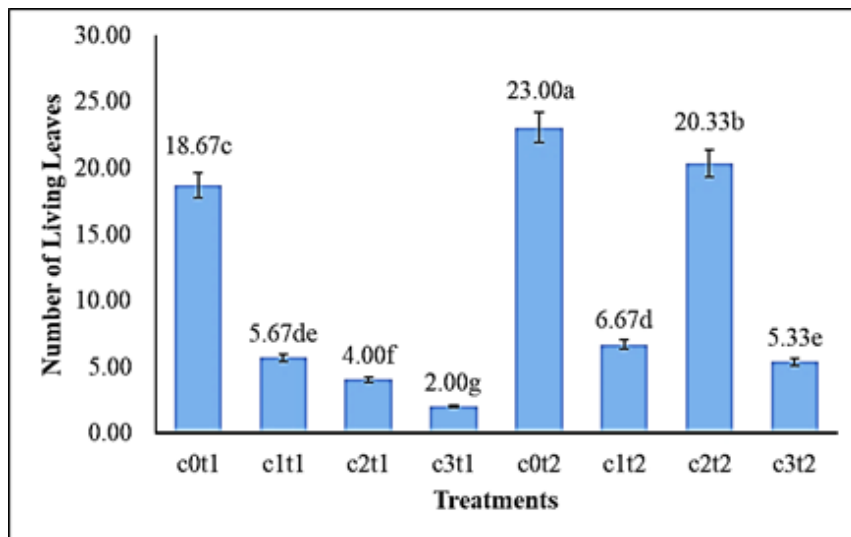
1176
 1177 Figure. 1. Graph Letal Concentrasion of Living Shooting Percentage of *Colocasia esculenta* var. *Antiquorum*
 1178 from First Week to Sixteenth Week After In Vitro Induction. c0t1 (0% Colchicine, Immersion 8 hours); c1t1
 1179 (Colchicine 0.05%, Immersion 8 hours); c2t1 (Colchicine 0.075%, Immersion 8 hours); c3t1(Colchicine 0.1%,
 1180 Immersion 8 hours); c0t2 (0% Colchicine, Immersion 16 hours); c1t2 (Colchicine 0.05%, Immersion 16 hours);
 1181 c2t2 (Colchicine 0.075%, Immersion 16 hours); c3t2 (Colchicine 0.1%, Immersion 16 hours).

1182



1183 Figure 2. *Colocasia esculenta* var. Antiquorum Plants With Several Concentrations of Colchicine and In Vitro
 1184 Immersion Time After 16 Weeks. (A) c0t1 (0% colchicine, 8 hours immersion); (B) c1t1 (Colchicine 0.05%,
 1185 Immersion 8 hours); (C) c2t1 (Colchicine 0.075%, Immersion 8 hours); (D) c3t1(Colchicine 0.1%, Immersion 8
 1186 hours); (E) c0t2 (0% Colchicine, Immersion 16 hours); (F) c1t2 (Colchicine 0.05%, Immersion 16 hours); (G)
 1187 c2t2 (Colchicine 0.075%, Immersion 16 hours); (H) c3t2 (Colchicine 0.1%, Immersion 16 hours).

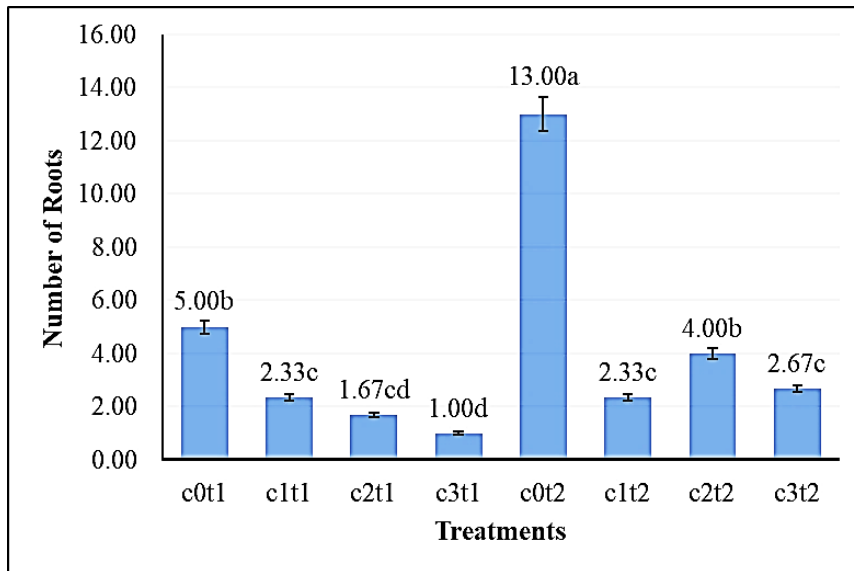
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1190 Figure 3. The Effect of Colchicine Concentration and Immersion Time on the Number of Living Leaves of
 1191 *Colocasia esculenta* var Antiquorum Plants In Vitro. Numbers followed by the same letter (abcdefg) mean that
 1192 they are not significantly different in the DMRT test with a level of = 0.05.

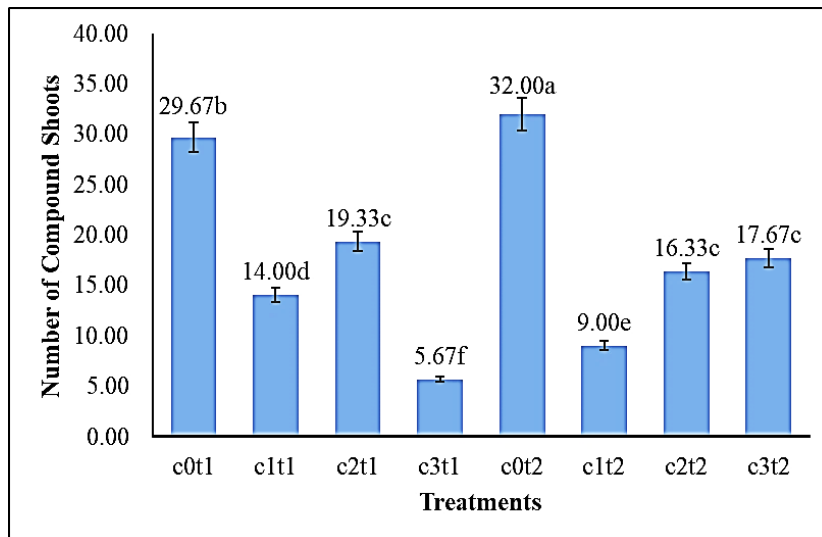
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1195 Figure 4. Effect of Colchicine Concentration and Immersion Time on Root Number of *Colocasia esculenta* var.
 1196 Antiquorum In Vitro. Numbers followed by the same letter (abcd) mean that they are not significantly different
 1197 in the DMRT test with a level of = 0.05.

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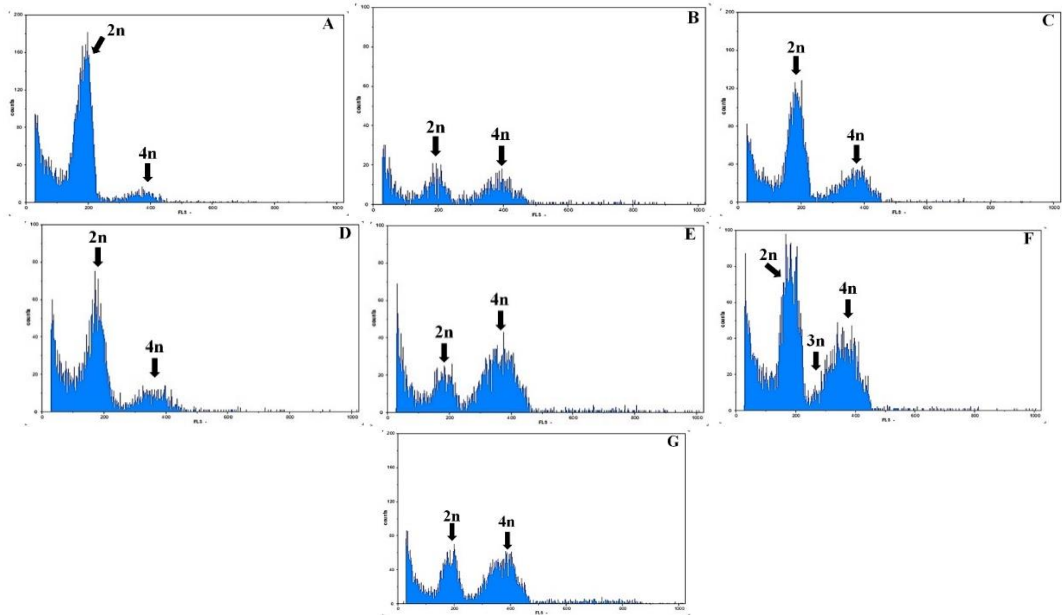


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1200 Figure 5. The Effect of Colchicine Concentration and Immersion Time on the Number of Compound Shoots of
 1201 *Colocasia esculenta* var. Antiquorum Plants In Vitro. Numbers followed by the same letter (abcdef) mean that
 1202 they are not significantly different in the DMRT test with a level of = 0.05.

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1207 Figure 6. Histogram of Ploidy Degree Results with Flow Cytometry of *Colocasia esculenta* var. Antiquorum
1208 Plant Resulting in In Vitro Colchicine Induction. (A) c0t1 (0% Colchicine, Immersion 8 hours) and c0t2 (0%
1209 Colchicine, Immersion 16 hours); (B) c1t1 (Colchicine 0.05%, Immersion 8 hours); (C) c2t1 (Colchicine
1210 0.075%, Immersion 8 hours); (D) c3t1(Colchicine 0.1%, Immersion 8 hours); (E) c1t2 (Colchicine 0.05%,
1211 Immersion 16 hours); (F) c2t2 (Colchicine 0.075%, Immersion 16 hours); (G) c3t2 (Colchicine 0.1%, Immersion
1212 16 hours).

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Feedback flow in Review 2

1228 Reviewer C

1229 The manuscript revision improved the figure arrangement and description to send the main
1230 message that the treatment of 0.05% colchicine concentration with a soaking time of 16 hours
1231 can be recommended for the induction of polyploid mutations in *Colocasia esculenta*.

1232 This revised manuscript is worthwhile to publish in PBB when improving the following
1233 minor comments.

1234 Author Responses

1235 Thank you for your comments and suggestions. We have revised the according to your
1236 suggestion and edited the English manuscript with a professional English editor. A certificate
1237 of proofreading attaches to the review process.

Reviewer Comment	Author response
This revision still includes some points to be edited. For example, “in vitro” is italic font. Initials and abbreviations appear in parentheses once in the description, followed by the abbreviation.	We have revised it according to your suggestion in the manuscript. Example: line 14 <i>in vitro</i> and line 124-125 Statistical Tool for Agricultural Research (STAR).
Line 172-198: Double-check your sentences for spacing and redundancy.	We have revised it according to your suggestion.
To make it easier to read, Enlarge the font inside that refers to the section in Figure 6, or put it outside of each	We have resized of all figure 6 according to your suggestion.

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Revision 2

The Morpho-Somatic and Chromosomal Changes in Colchicine Polyploidy Induction *Colocasia esculenta* Var. Antiquorium

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Abstract

Increasing the productivity of *Colocasia esculenta* var. Antiquorium is very necessary to enhance the mass of tubers. This can be achieved through polyploid mutation method using colchicine to improve tuber size by increasing the number of chromosomes. Therefore, this study aimed to determine the effect of colchicine on morphology and polyploid changes of *Colocasia esculenta* var. Antiquorium at various concentrations and duration of immersion *In vitro*. The analysis was carried out as a completely randomized factorial design, with the first factor being colchicine concentration, consisting of 0.0%, 0.05%, 0.075%, and 0.1%. The second factor was the immersion time which consisted of 8 and 16 hours. The results showed that the best combination of concentration and immersion time on somatic morphology was 0.075% colchicine with 8 hours and 16 hours of immersion. Meanwhile, the best combination of chromosomal changes was 0.05% colchicine with 16 hours of immersion. These results indicated that the treatment of 0.05% colchicine concentration with a soaking time of 16 hours can be recommended for induction of polyploid mutations in *Colocasia esculenta*.

Keywords: *Colocasia esculenta*, Polyploid Mutation, Colchicine, Diploid, Tetraploid, *In vitro*

Introduction

1275 *Colocasia esculenta* var. Antiquorum is a type of taro with a small tuber size (small
1276 corm taro), also known as Japanese taro (Maretta et al. 2016). The plant exhibits a high level
1277 of resistance to weather changes, which allows it to adapt to different seasons. Based on the
1278 data from the Food Security Service of South Sulawesi Province, the productivity of
1279 *Colocasia esculenta* var. Antiquorum in 2020 was 7.214 tons. However, the exports of
1280 *Colocasia esculenta* L. tubers only amount to 300 tons/ha due to the low productivity of the
1281 plant (Nagano et al. 2016).

1282 Increasing the productivity of *Colocasia esculenta* var. Antiquorum can be done through
1283 plant breeding by induction of polyploid mutations. Polyploid induction aims to increase crop
1284 productivity because polyploid (tetraploid) plants have a more significant figure, fruit size,
1285 tuber, and flower than diploid plants (Suryo 2007) and are also resistant to specific
1286 environmental stresses (Deng et al. 2012).

1287 Polyploid can be carried out by giving chemical mutagens such as colchicine to plant
1288 meristem tissue. Colchicine changes the number of plant chromosomes and causes gene
1289 mutations on the scale of seeds and vegetatively propagated plants. It also induces polyploid
1290 by inhibiting the formation of spindle fibers during cell division. However, the number of
1291 chromosomes increases, but cell division does not occur, thereby producing polyploid cells
1292 with a doubled number of chromosomes (Manzoor et al. 2018,2019; Ade et al. 2010).

1293 Colchicine is an important mutagen that prevents microtubule formation and doubles the
1294 number of chromosomes. It is commonly used to develop polyploid plants and functions as a
1295 mitotic toxin, which produces multiple plant mutagenic effects (El-Nashar et al., 2015).
1296 Colchicine not only helps in doubling chromosomes but also causes plant mutations, resulting
1297 in the formation of Colchis-mutants (Ari et al. 2015). The concentration of colchicine for seed
1298 treatment usually ranges from 0.1% to 0.8%, but high doses cause malformations and reduce

1299 the production of tetraploid plants. Therefore, it is recommended to use colchicine with the
1300 lowest possible concentration (Pirkoohi et al. 2011).

1301 The success of polyploid induction depends on the explant, plant species, colchicine
1302 concentration, and duration of exposure. However, induction must be carried out carefully by
1303 plant breeders as excessively high concentrations cause problems in the form of abnormalities
1304 in developing seedlings (Manzoor et al. 2018,2019). Several studies on polyploid induction
1305 using colchicine have been successful with different concentrations, such as *Papaver*
1306 *bracteatum* L. (Esfahani et al. 2020), *Allium Sativum* L. (Ayu et al. 2019), *Colchicum*
1307 *chalcedonicum* Azn. (Karlik et al. 2020), *Neolamarckia cadamba* (Eng et al. 2021), *Dionaea*
1308 *muscipula* Ellis (Jala, 2014), *Nigella sativa* (Gupta et al. 2021), and *Rhododendron fortunei* L.
1309 (Mo et al. 2020). This shows that polyploid induction can be used to create or select better
1310 offspring for further use.

1311 Based on the description above, it is necessary to investigate induction of polyploid
1312 mutations in *Colocasia esculenta* var. *Antiquorum*. Therefore, this study aims to determine
1313 the effect of colchicine on morphology and chromosome polyploid of *Colocasia esculenta*
1314 var. *Antiquorum* plantlets on the interaction of concentration and immersion time *In vitro*.
1315 The novelty of this study is that it is the first to explore induction of polyploid mutations in
1316 *Colocasia esculenta* var. *Antiquorum*.

1317

1318 **Materials and Methods**

1319 **Experimental design**

1320 This study was performed at the Tissue Culture Laboratory, Department of Agricultural
1321 Cultivation, Faculty of Agriculture, Hasanuddin University, Makassar, South Sulawesi,
1322 90245, Indonesia. The analysis employed a 2-factor factorial (F2F) experiment with a
1323 Completely Randomized Design (CRD) as the environmental design, with three replications

1324 in each of the eight treatments. The combination of treatment between colchicine
1325 concentration (c0 = 0%, c1 = 0.05%, c2 = 0.075%, c3 = 0.1%) and immersion time (t1 = 8
1326 hours; t2 = 16 hours). Each replication consisted of three shoots, resulting in a total of 82
1327 individual shoots, as the experimental unit observed.

1328 **Experimental conditional**

1329 The plantlets used as material for polyploid induction were eight weeks old *Colocasia*
1330 *esculenta* var. Antiquorum shoots, which were selected as explants. The in vitro shoots were
1331 removed and the petioles were cut to a size of 1-3 cm. The immersion was carried out in
1332 laminar and the equipment used was sterile. The shoots were immersed in different
1333 concentrations of colchicine solution, namely 0% (sterile distilled water) (c0), 0.05% (c1),
1334 0.075% (c2), and 0.1% (c3). Each shoot was immersed in a culture bottle containing 15 ml of
1335 colchicine solution and shaken at 100 rpm on a shaker for 8 hours (c1) and 16 hours (c2).
1336 After immersion, the explants were washed with sterile distilled water 3-4 times and planted
1337 into shoot propagation medium, namely MS media with 2 mg L⁻¹ BAP and 1 mg L⁻¹ IBA. The
1338 medium contained sugar (30 g L⁻¹), with the pH being adjusted to 5.8, and compacted with 7 g
1339 L⁻¹ agar. The shoots were kept in an incubation room at a temperature of 25-26°C with 16
1340 hours/day irradiation and an irradiation intensity of ± 1,000-2,000 lux (Modification of
1341 Dwiyani, 2015). Subsequently, the shoots were incubated for 16 Weeks After Planting (WAP)
1342 and observations were made every week from outside the culture bottle for 16 WAP

1343 **Observation of parameters**

1344 Several parameters observed from shoot explants were the percentage of living shoots
1345 (%), the number of living leaves, the number of roots and the number of compound shoots.

1346 Percentage of living explants =
$$\frac{\sum \text{explants planted} - \text{explants dead}}{\sum \text{explants planted}} \times 100\%$$

1347 **Chromosome analysis with flow cytometry**

1348 Polyploid level analysis was performed using flow cytometry. For each treatment,
1349 samples from the best plantlet vigor were collected from the leaves of the treated plants,
1350 measuring $\pm 25 \text{ mm}^2$. The samples were ground using a razor blade in a petri dish with 0.2 ml
1351 of extraction buffer (solution A from Partec Kit) and incubated for 10 minutes at room
1352 temperature. Subsequently, the supernatants from the samples were filtered using nylon kesh
1353 measuring 30 m and placed in a cuvette, which was added to 1 ml of DAPI solution. The
1354 samples were incubated again for 1 minute and inserted into the Flow cytometry tool. The
1355 tool read the light intensity of the measured sample through the graph displayed to show the
1356 number of ploidy changes that occurred (Escobedo-Gracia-Medrano et al. 2018).

1357 **Data analysis**

1358 The data analysis was carried out using the F test to determine the interaction between
1359 colchicine concentration and immersion time. When the variance obtained had a significant
1360 effect, further analysis was conducted using the Duncan's Multiple Range Test (DMRT) at a
1361 5% significance level to determine the effect of differences between treatments. Data
1362 processing was carried out using the Statistical Tool for Agricultural Research (STAR)
1363 program and Microsoft Office Excel 2019. The data were analyzed by correlation analysis to
1364 determine the relationship between the parameters (Anshori *et al.* 2022).

1365

1366 **Results**

1367 **Percentage of Living Shoots of *Colocasia esculenta* var *Antiuorum* In Vitro**

1368 The results of the living shoots in Table 1 showed that the control shoots had a very high
1369 percentage of survival compared to the combination of concentration and immersion time. In
1370 this study, there were differences in the number of shoots planted due to contamination of the
1371 media before treatment. Therefore, out of the 18 propagated shoots, there was a variation in

1372 the number of shoots that grew before colchicine immersion treatment. However, this can be
1373 corrected through the percentage of living shoots, which were analyzed via graphs.

1374 Based on the graph of living shoot percentage, this study showed a negative graph with
1375 an increase in colchicine concentration and time of immersion. The graph formed a linear
1376 regression with the formula $Y = 7.2751x + 82.378$, and the coefficient of determination (R²)
1377 was 0.3131, as presented in Figure 1. Based on this formula, the lethal 50 was in 0.1%
1378 colchicine concentration with 8 hours of immersion. The relationship between the percentage
1379 of surviving shoot explants was inversely proportional to the concentration and duration of
1380 colchicine immersion. As the concentration and duration of immersion of colchicine
1381 increased, the number of explants that survived decreased. However, the survival plantlet in
1382 the chemical mutants had unique trait changes, making it necessary to achieve a balance
1383 between the plantlet regeneration and optimum diversity in chemical mutation induction.
1384 Therefore, the lethal 50 served as a practical point to meet both of these objectives.

1385 The analysis of the mean square of variance in polyploid mutation induction were
1386 presented in Table 2. The results showed that the treatment duration of colchicine immersion
1387 in *Colocasia esculenta* var. Antiquorum and concentration levels significantly affected all
1388 observation parameters. The interaction effect between concentration level (c) and immersion
1389 time (t) also significantly affected the number of leaves, roots, and shoots, as well as the
1390 coefficient of diversity in the medium category.

1391 **Number of living leaves**

1392 The results of the DMRT test ($\alpha = 0.05$) in Figure 2 showed the number of living leaves
1393 of *Colocasia esculenta* var. Antiquorum in different treatments. The best treatment was c0t2
1394 with 23.00 leaves, compared to c2t2, c1t2, and c3t2 with 20.33, 6.67, and 5.33 leaves,
1395 respectively. The lowest mean number of living leaves of *Colocasia esculenta* var

1396 Antiquorum in the treatment c3t1 (2.00 leaves) was significantly different from c2t1 (4.00
1397 leaves), c1t1 (5.67 leaves), c0t1 (18.67 leaves).

1398 **Number of roots**

1399 The results of the DMRT test ($\alpha = 0.05$) in Figure 3 showed the number of roots of the
1400 best *Colocasia esculenta* var Antiquorum in the treatment c0t2 with 13.00 roots were
1401 significantly different from c2t2, c3t2, and c1t2 with 4.00, 2.67, and 2.33 leaves, respectively.
1402 The lowest mean number of roots of *Colocasia esculenta* var Antiquorum in the treatment
1403 c3t1 with 1.00 roots was significantly different from the 5.00 roots of c0t1 but not from c1t1
1404 and c2t1 at 2.33 and 1.67 roots, respectively.

1405 **Number of compound shoots**

1406 The results of the DMRT test ($\alpha = 0.05$) in Figure 4 showed the number of the best
1407 *Colocasia esculenta* var. Antiquorum compound shoots in the treatment c0t2 with 32.00
1408 shoots were significantly different from c3t2 (17.67 shoots), c2t2 (16.33 shoots), c1t2 (9.00
1409 shoots), but c3t2 (17.67 shoots) was not different from c2t2 (16.33 shoots). The lowest
1410 average number of shoots of *Colocasia esculenta* var. Antiquorum in the treatment c3t1 with
1411 5.67 shoots was significantly different from c1t1, c2t1, and c0t1 with 14.00, 19.33, and 29.67
1412 shoots, respectively.

1413 **Ployploid analysis of plants *Colocasia esculenta* var. Antiquorum**

1414 Polyploid analysis using flow cytometry on *Colocasia esculenta* var. Antiquorum in
1415 Table 3 showed that the difference in concentration and duration of colchicine immersion
1416 affected plant chromosomes. The treatments, namely c0t1, c1t1, c2t1, c3t1, c0t2, c1t2, and
1417 c3t1b showed an increase in the chromosomes from $2n-4n= 24$ (diploid) to 48 (tetraploid). In
1418 the c2t2 treatment (0.075% colchicine, immersion 16 hours) the detected chromosomes
1419 ranged from $2n-3n-4n= 24$ (diploid), 36 (triploid) to 48 (tetraploid).

1420 Based on the percentage of gated chromosomes in the treatment c0t1 and c0t2, the
1421 dominant chromosome was 2n (diploid) with a percentage of 67.70%, which was significantly
1422 different from the 4n chromosome (tetraploid) at 4.17%. Therefore, it can be concluded that
1423 the chromosomes in the treatments were diploid. In the c1t1 treatment (0.05% colchicine,
1424 immersion 8 hours), the dominant chromosome was 4n (tetraploid) with a percentage of
1425 36.97% but not significantly different from 2n (diploid) at 28.35%. This showed that the
1426 chromosome was putative tetraploid because it allowed ploidy selection to occur. In c2t1 and
1427 c3t1, the dominant chromosomes were 2n (diploid) with a percentage of 52.94% and 55.26%,
1428 different from 4n (tetraploid) at 24.99% and 16.25%, which was mixoploid. In c1t2 and c3t2,
1429 the dominant chromosome was 4n (tetraploid) with a percentage of 54.10% and 44.36%,
1430 significantly different from 2n (diploid) at 20.27% and 28.24%, which did not allow ploidy
1431 selection to occur, namely tetraploid. In c2t2 treatment (0.075% colchicine, immersion 16
1432 hours), the dominant chromosome was 4n (tetraploid) at 35.50% but not significantly
1433 different from chromosome 2n (diploid) at 29.94% and 3n (triploid) at 15.39%, making it
1434 putative-tetraploid because it allowed ploidy selection.

1435 The results of the flow cytometry analysis in Figure 5 showed the chromosome peaks in
1436 *Colocasia esculenta* var. Antiquorum plants in all treatments, which were interpreted as
1437 standards for diploid (2n), tetraploid (4n), and triploid cells (3n) were also seen. Although all
1438 seven treatments exhibited diploid (2n), triploid (3n), and tetraploid (4n) cells, the peak height
1439 of each cell was different among treatments.

1440 **Correlation analysis**

1441 The results of the correlation analysis in Table 4 showed the relationship between the
1442 number of leaf parameters, which can be evaluated *In vitro*, in polyploid of *Colocasia*
1443 *esculenta* var. Antiquorum. The number of leaves was positively correlated with the number
1444 of roots and compound shoots at 0.80 and 0.75, respectively.

1445

1446 **Discussion**

1447 Polyploid is a plant breeding technique carried out using mutagens such as colchicine to
1448 obtain plants with a chromosome number of more than $2n$ (diploid). Colchicine is one of the
1449 chemicals known to induce somaclonal variation in polyploidization in cultured plant cells. In
1450 plants, colchicine binds to tubulin, one of the main constituents of microtubules, which
1451 inhibits the development of spindle fibers and further mitotic division, leading to induction of
1452 polyploid (Kharde et al. 2017). It has also been used for several years to induce polyploid,
1453 which increases the potential for metabolite production and facilitates the generation of
1454 valuable compounds in plants (Bernard et al. 2012).

1455 Flow cytometry analysis is a fast and efficient modern method for determining the
1456 ploidy level in almost all plant tissue regeneration and is particularly suitable for studies
1457 involving large sample sizes (De et al. 2010; Eeckhaut et al. 2005). For example, Jones et al.
1458 (2008) and De et al. (2004) used flow cytometry to determine the ploidy level and genome
1459 size of 200 diverse species and *Rhododendron* cultivars, creating a valuable database for
1460 breeders. This method has also been used to determine the ploidy level of induced polyploid
1461 adventitious shoots from three *Rhododendron* hybrids.

1462 The results of flow cytometry analysis in Table 3 and Figure 6 showed that the number
1463 of chromosomes in the plant cell of *Colocasia esculenta* var. *Antiquorum* doubled to
1464 tetraploid ($4n$). This indicated that the plant can be induced using polyploid by treating with
1465 colchicine concentration and immersion time, with the multiplication of chromosomes varied
1466 in each treatment. Visual observations of plantlets in Figure 2 confirmed the doubling of
1467 chromosomes in *Colocasia esculenta* var. *Antiquorum* plant. The increase in leaf size and
1468 petiole was observed in the c1t2 treatment (0.05% colchicine, immersion 16 hours), with an

1469 improvement in the size of shoots formed. This result was supported by Mahyuni et al.
1470 (2015), who stated that diploid plants had a smaller morphology size than polyploid plants.

1471 This study showed that the doubling of the chromosomes occurred randomly, leading to
1472 a non-uniform effect on an individual cell. The best chromosomal doubling occurred at low
1473 concentrations, namely 0.05% colchicine with 16 hours immersion time. This led to a
1474 significantly higher percentage of 54.10% compared to 44.36% and 35.50% at 0.1% and
1475 0.075% colchicine concentration, respectively. Similarly, at 8 hours of immersion, more
1476 tetraploid chromosomes were formed at a low concentration of 0.05% by 36.97%, indicating
1477 different mutations in the same plant. Heo et al. (2016) also concluded that the most
1478 prolonged immersion with low colchicine concentrations induced the highest percentage of
1479 polyploid production efficiency among other treatments on *L. leichtlinii* plants. In orchid
1480 plants, higher concentrations of colchicine produced shorter pods, with some dry and dead
1481 pods due to colchicine toxicity at high concentrations (1000 and 2000 mg/L) (Sudirman et al.
1482 2021).

1483 Table 3 and Figure 6 showed that the doubling of the chromosomes formed were
1484 diploid, mixoploid, and tetraploid. Mixoploid plants had diploid and tetraploid nuclei,
1485 assessed based on the relative number of nuclei (Cimen 2020; Koutoulis et al. 2005). In
1486 polyploid induction, a high percentage of mixoploid yield was a procedure drawback because
1487 the unstable polyploid state often returned wholly or partially to diploid state after successive
1488 cell division cycles (Esfahani et al., 2020). Furthermore, developing two different cells in a
1489 mixoploid bud caused competition during its growth. According to Kainth and Grosser
1490 (2010), diploid cells had a faster division rate than autotetraploid cells, allowing the resulting
1491 mixoploid shoots to become diploid again.

1492 Tetraploid are polyploid containing four sets of chromosomes that can produce more
1493 secondary metabolites and biomass compared to diploid and are also drought-tolerant and

1494 disease-resistant (Sattler et al. 2016; Xu et al. 2014). Tetraploid can be artificially induced
1495 using mitotic spindle inhibitors such as colchicine (Wang et al. 2016), with their plants having
1496 giant leaf and shoot sizes than diploid (Kurtz et al. 2020). Wulandari et al. (2015) conclude
1497 that 0.1% colchicine treatment for 1-hour is the most efficient in producing tetraploid Pumelo
1498 Nambangan citrus plants.

1499 Asif (2001) and Tesfaye (2005) reported that chromosomes were doubled in *Musa*
1500 *balbisiana*, various *Musa acuminata* subspecies, and *Ensete ventricosum* species using seeds,
1501 respectively. In polyploid induction, colchicine was found to be very efficient (Ganga 2002)
1502 depending on the variable amount of media, antimitotic agent, type of explant, exposure time,
1503 and concentration. Flow cytometry was the pre-eminent method used to induce evaluation
1504 polyploidization, while other methods such as chromosome number and morphology
1505 observations had also been applied.

1506 Urwin (2014) reported that a concentration of 0.1% and 6 hours of immersion can
1507 duplicate chromosomes in Lavandins plants (*Lavandula × intermedia* cvs. Grosso and Seal).
1508 According to Sadhukhan et al. (2014), colchicine at a concentration of 0.0005% with 6 hours
1509 of immersion duplicated chromosomes in African marigold (*Tagetes erecta*) plants. The
1510 method was carried out by placing the entire plant with roots in colchicine solution and
1511 applying it to the apical shoots on cotton soaked in colchicine.

1512 In this study, different concentrations of colchicine treatment and immersion time were
1513 found to affect morphology characteristics of regenerated plants under *In vitro* conditions.
1514 According to Jala (2014), the effect of colchicine concentration and duration of time for
1515 immersion in *D. muscipula* can be observed in changes in morphology and growth rate,
1516 survival rate, and variations that occurred at high concentrations of colchicine and immersion
1517 time.

1518 The results of the DMRT analysis in Figures 3, 4, and 5 showed differences in the
1519 number of living leaves, roots, and compound shoots. The graph indicated that the highest
1520 data were obtained in the c0t2 treatment (0.0% colchicine, immersion 16 hours) and the
1521 lowest was in the c3t1 treatment (0.1% colchicine, immersion 8 hours). This indicated that
1522 adding a small concentration of colchicine and increasing immersion time significantly
1523 affected morphology of *Colocasia esculenta* var. *Antiquorum*, caused more exposed cells to
1524 be damaged or fail during growth. This was supported by Allum et al. (2007), who stated that
1525 at the concentration, the chromosomes will be doubled in the cell.

1526 The best morphology in this study was obtained at a concentration of 0.0% due to the
1527 stress on the plant in colchicine treatment. However, in subsequent subcultures, other
1528 differences were observed between the concentration of 0.0% and colchicine at 0.05%,
1529 0.075%, and 0.1% because the plants were no longer under stress due to colchicine
1530 administration. The nature of colchicine mutagen influenced morphology differences in each
1531 treatment due to its high toxicity to plants. Therefore, low doses with a long exposure period
1532 were considered reliable to reduce its toxic effect and increase the rate of polyploid
1533 production (Sajjad et al. 2013). The optimal amount of colchicine used for polyploid output
1534 varied widely, with concentrations ranging from 0.01% (Thao et al. 2003) to 1.0% (Demtsu et
1535 al. 2013).

1536 In the correlation analysis in Table 4, the closeness between the variables was measured
1537 without considering the influence or the magnitude of one variable on others. In this study,
1538 the main character was the number of leaves because it can be used as a character to identify
1539 tetraploid plants. The results of the correlation analysis showed that four characters were
1540 positively correlated, including a very significant positive correlation on the number of roots
1541 (0.80) and shoots (0.75), speed of shoots (0.69), and speed of rooting (0.66). This correlation
1542 proved the extent to which a relationship exists between one or more variables (Astuti, 2017).

1543 According to (Rohaeni 2012), correlation with strong character conditions can be used as an
1544 indirect selection tool for the main character.

1545 Based on the results, colchicine with the best effect on the formation of polyploid of
1546 Safira taro was the interaction between 0.05% colchicine concentration and 16 hours
1547 immersion time (c1t2), which resulted in tetraploid (4n) of 54.10%. This was followed by the
1548 combination of 0.1% colchicine concentration with 16 hours of immersion (c3t2), yielding
1549 tetraploid (4n) of 44.36%. Furthermore, colchicine with the best effect on morphology
1550 parameters was obtained from the interaction between colchicine concentration of 0.075%
1551 with 16 hours of immersion (c2t2), which produced 20.33 leaves and 4.00 roots, while a
1552 concentration of 0.075% with 8 hours of immersion (c2t1) yielded 19.33 shoots.

1553

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1702 *Table 1. Percentage of living shoots on each treatment of Colchicine concentration and time*
 1703 *of Immersion.*

Treatments	Shoot planted	Living shoots	Dead shoots	living Percentage (%)
c0t1	13	13	0	100.00
c1t1	9	4	5	44.44
c2t1	9	4	5	44.44
c3t1	12	4	8	33.33
c0t2	13	13	0	100.00
c1t2	18	5	13	27.78
c2t2	18	5	13	27.78
c3t2	18	4	12	22.22
Total	110	52	56	47.27

1704 Notes: c0t1 (0% Colchicine, Immersion 8 hours); c1t1 (Colchicine 0.05%, Immersion 8 hours); c2t1 (Colchicine
 1705 0.075%, Immersion 8 hours); c3t1(Colchicine 0.1%, Immersion 8 hours); c0t2 (0% Colchicine, Immersion 16
 1706 hours); c1t2 (Colchicine 0.05%, Immersion 16 hours); c2t2 (Colchicine 0.075%, Immersion 16 hours); c3t2
 1707 (Colchicine 0.1%, Immersion 16 hours).

1708

1709 *Table 2. Mean square analysis of variance on several observation parameters in In vitro*
 1710 *polyploid induction experiments of Colocasia esculenta var. Antiquorum plants.*

Source	DF	Mean Square in <i>Annalysis of Variance</i> (ANNOVA)		
		Number of Living Leaves	Number of Roots	Number of Compound Shoots
Time Immersion (T)	1	234.375**	54.000**	15.042 ^{ns}
Colchicine Concentration (C)	3	349.708**	67.667**	494.153**
Interaction of T x C	3	70.708**	18.111**	86.708**
Error	16	4.333	0.542	12.458
CV (%)		16.82%	19.44%	18.40%

1711 Note: **: Very Significant ($P \leq 0.01$), *: Significant ($P \leq 0.05$), ns : No Significant, DF: Degree of Free, CV:

1712 Coefficient of Variance

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1718 Table 3. *Results of polyploidy analysis with flow cytometry in Colocasia esculenta var.*

1719 *Antiquorum plants In vitro induction of colchicine.*

Treatment	Chromosomes Detected	% Gated	Dominant Chromosomes	Explanation ^(*)
Control (c0t1, c0t2)	2n-4n	67.70 + 4.17	2n	Diploid
c1t1	2n-4n	28.35 + 36.97	4n	Putatif-Tetraploid
c2t1	2n-4n	52.94 + 24.99	2n	Mixoploid
c3t1	2n-4n	55.26 + 16.25	2n	Mixoploid
c1t2	2n-4n	20.27 + 54.10	4n	Tetraploid
c2t2	2n-3n-4n	29.94 + 15.39 + 35.50	4n	Putatif-Tetraploid
c3t2	2n-4n	28.24 + 44.36	4n	Tetraploid

1720 Note: 2n (Diploid); 3n (Triploid); 4n (Tetraploid); c0t1 (0% Colchicine, Immersion 8 hours); c1t1 (Colchicine

1721 0.05%, Immersion 8 hours); c2t1 (Colchicine 0.075%, Immersion 8 hours); c3t1(Colchicine 0.1%, Immersion 8

1722 hours); c0t2 (0% Colchicine, Immersion 16 hours); c1t2 (Colchicine 0.05%, Immersion 16 hours); c2t2

1723 (Colchicine 0.075%, Immersion 16 hours); c3t2 (Colchicine 0.1%, Immersion 16 hours). (*) refers to Shahriari-

1724 Ahmadi *et al.* 2008 and based on the difference between diploid and tetraploid ≤ 1.50)

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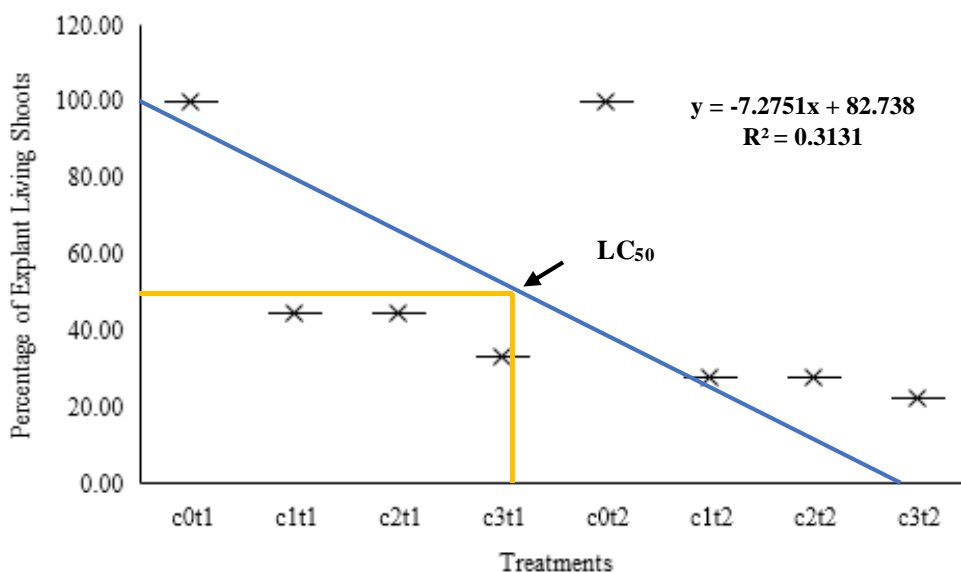
1726

1727 Table 4. Results of correlation analysis on all observation parameters of *Colocasia esculenta*
 1728 var. *Antiquorum* plants at various concentrations and duration of *In vitro* immersion of
 1729 colchicine.

	Number of Compound Shoots	Number of Roots	Number of Living Leaves
Number of Compound Shoots	1.00	0.79**	0.75*
Number of Roots		1.00	0.80**
Number of Leaves			1.00

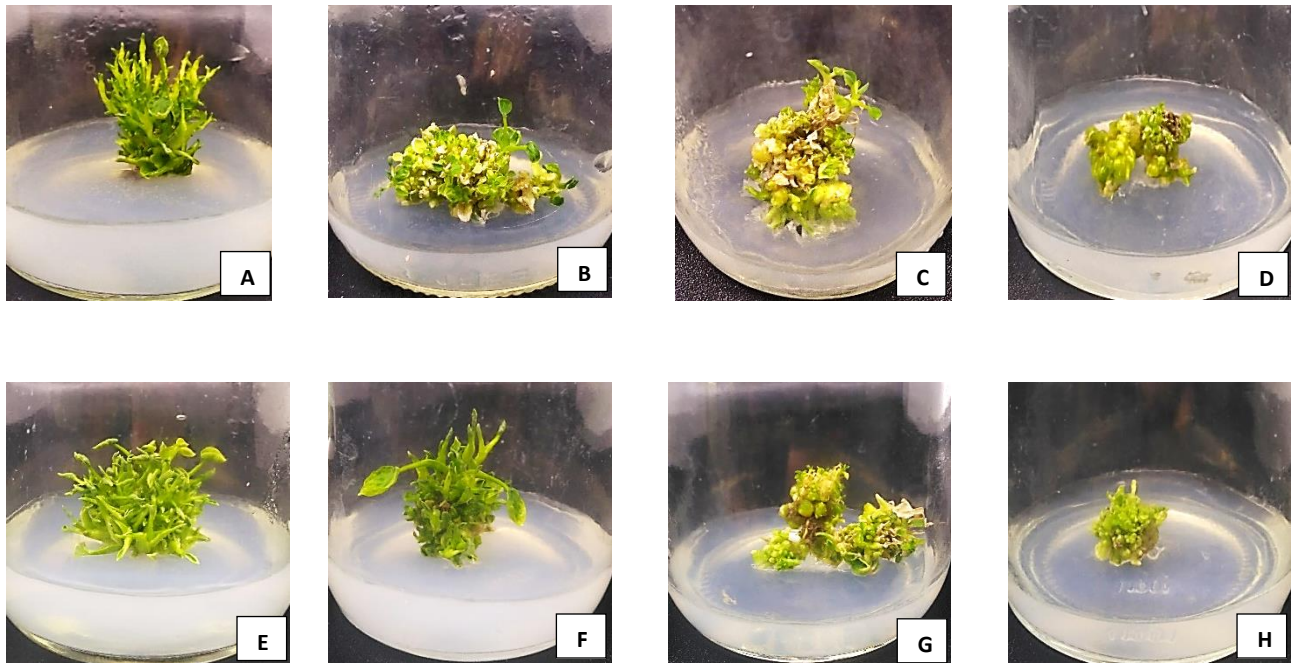
1730 Note: Numbers followed by a sign mean that they are significantly different from the table $r_{0.05} = 0.62$ (*); $r_{0.01} = 0.79$ (**).

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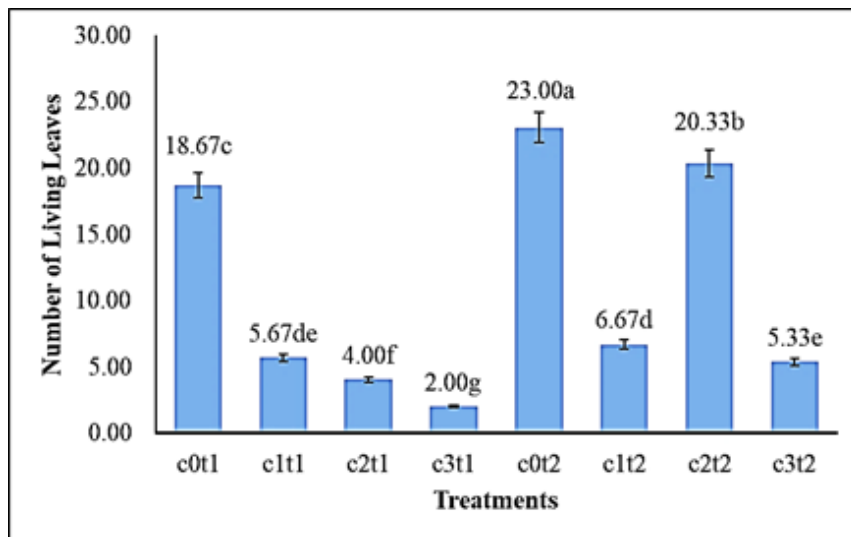


1735
 1736 Figure. 1. Graph Letal Concentrasion of Living Shooting Percentage of *Colocasia esculenta* var. *Antiquorum*
 1737 from First Week to Sixteenth Week After *In vitro* Induction. c0t1 (0% Colchicine, Immersion 8 hours); c1t1
 1738 (Colchicine 0.05%, Immersion 8 hours); c2t1 (Colchicine 0.075%, Immersion 8 hours); c3t1(Colchicine 0.1%,
 1739 Immersion 8 hours); c0t2 (0% Colchicine, Immersion 16 hours); c1t2 (Colchicine 0.05%, Immersion 16 hours);
 1740 c2t2 (Colchicine 0.075%, Immersion 16 hours); c3t2 (Colchicine 0.1%, Immersion 16 hours).

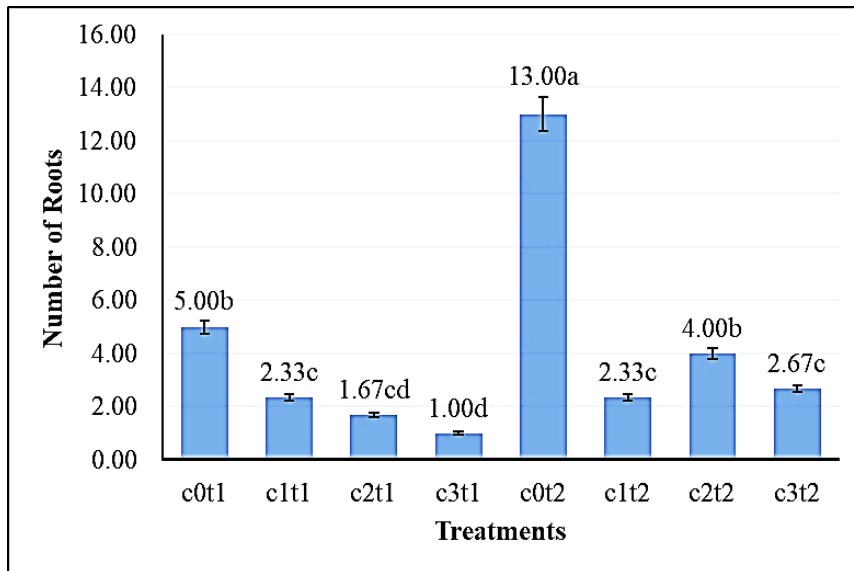
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1742 Figure 2. *Colocasia esculenta* var. Antiquorum Plants With Several Concentrations of Colchicine and *In vitro*
 1743 Immersion Time After 16 Weeks. (A) c0t1 (0% colchicine, 8 hours immersion); (B) c1t1 (Colchicine 0.05%,
 1744 Immersion 8 hours); (C) c2t1 (Colchicine 0.075%, Immersion 8 hours); (D) c3t1(Colchicine 0.1%, Immersion 8
 1745 hours); (E) c0t2 (0% Colchicine, Immersion 16 hours); (F) c1t2 (Colchicine 0.05%, Immersion 16 hours); (G)
 1746 c2t2 (Colchicine 0.075%, Immersion 16 hours); (H) c3t2 (Colchicine 0.1%, Immersion 16 hours).
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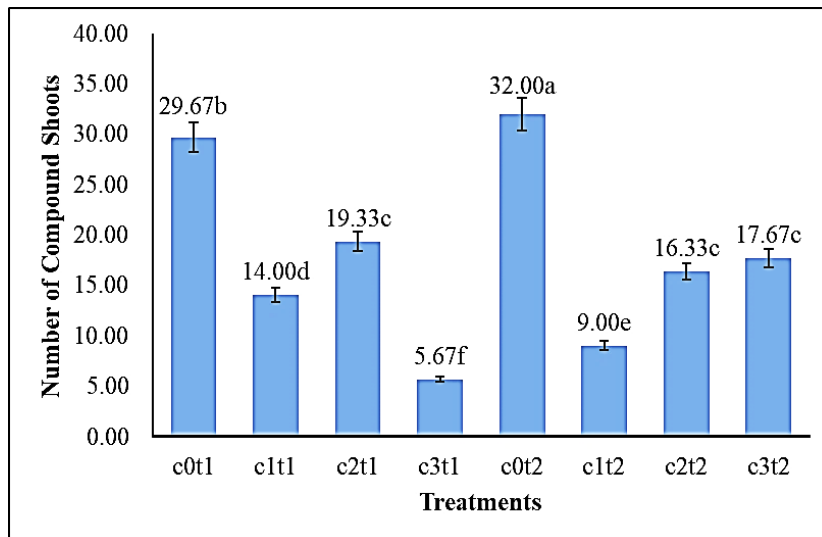
1748
 1749 Figure 3. The Effect of Colchicine Concentration and Immersion Time on the Number of Living Leaves of
 1750 *Colocasia esculenta* var Antiquorum Plants *In vitro*. Numbers followed by the same letter (abcdefg) mean that
 1751 they are not significantly different in the DMRT test with a level of = 0.05.
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1754 Figure 4. Effect of Colchicine Concentration and Immersion Time on Root Number of *Colocasia esculenta* var.
 1755 *Antiquorum In vitro*. Numbers followed by the same letter (abcd) mean that they are not significantly different in
 1756 the DMRT test with a level of = 0.05.

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1758

1759 Figure 5. The Effect of Colchicine Concentration and Immersion Time on the Number of Compound Shoots of
 1760 *Colocasia esculenta* var. *Antiquorum* Plants *In vitro*. Numbers followed by the same letter (abcdef) mean that
 1761 they are not significantly different in the DMRT test with a level of = 0.05.

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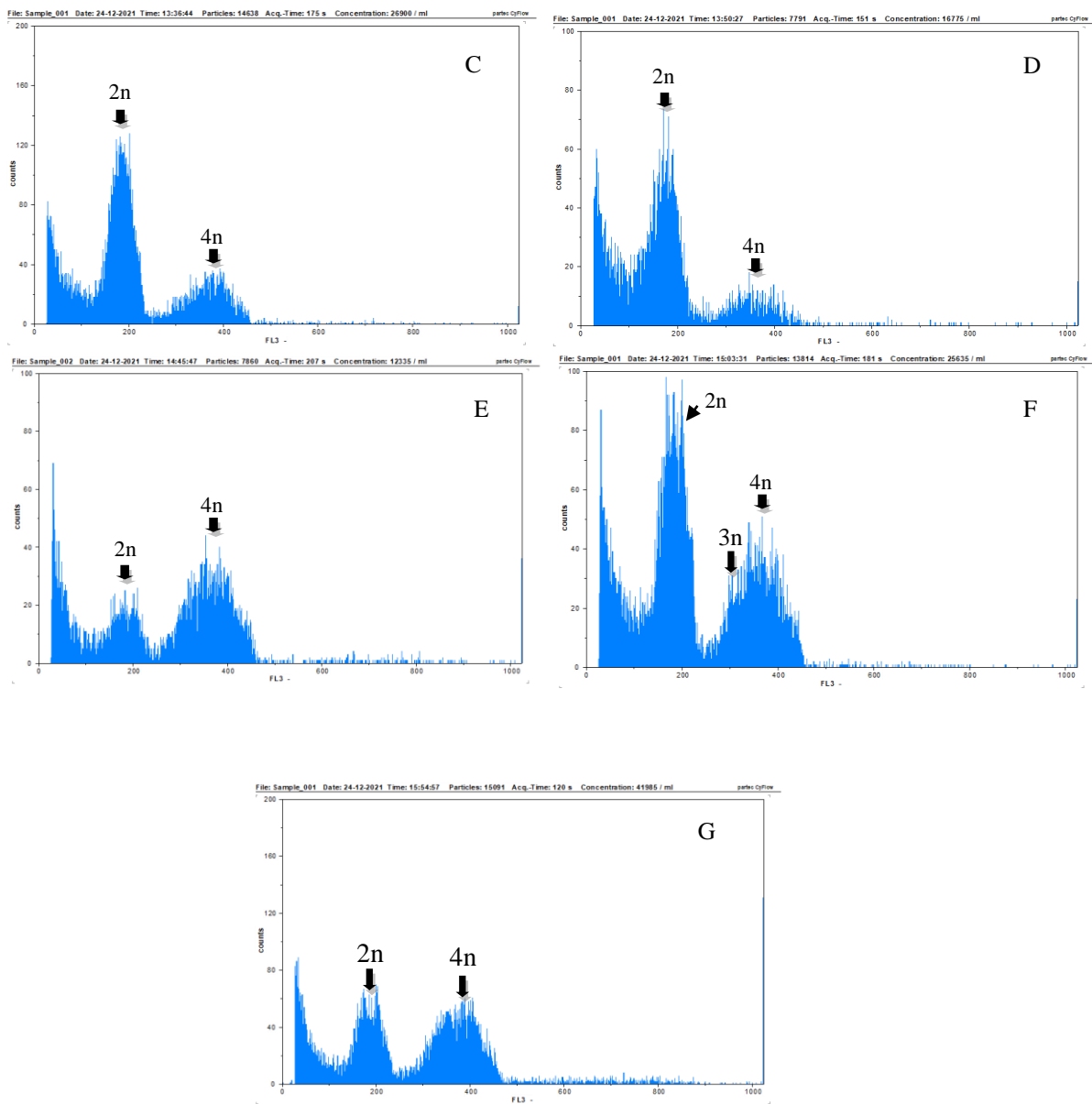


Figure 6. Histogram of Ploidy Degree Results with Flow Cytometry of *Colocasia esculenta* var. Antiquorum Plant Resulting in *In vitro* Colchicine Induction. (A) c0t1 (0% Colchicine, Immersion 8 hours) and c0t2 (0% Colchicine, Immersion 16 hours); (B) c1t1 (Colchicine 0.05%, Immersion 8 hours); (C) c2t1 (Colchicine 0.075%, Immersion 8 hours); (D) c3t1(Colchicine 0.1%, Immersion 8 hours); (E) c1t2 (Colchicine 0.05%, Immersion 16 hours); (F) c2t2 (Colchicine 0.075%, Immersion 16 hours); (G) c3t2 (Colchicine 0.1%, Immersion 16 hours).

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Accepted Status

The screenshot shows an email from 'Plant Breeding and Biotechnology' with the subject 'Plant Breeding and Biotechnology'. The email content includes:

Manuscript ID : PBB-22-1043
Manuscript Type : Research Article
Manuscript Title : The Morpho-Somatic and Chromosomal Changes in Colchicine Polyploidy Induction Colocasia esculenta Var. Antiquorum

Dear Dr. Muh Farid:

We are very pleased to inform you that the article, "The Morpho-Somatic and Chromosomal Changes in Colchicine Polyploidy Induction Colocasia esculenta Var. Antiquorum", has been accepted for publication to Plant Breeding and Biotechnology. To finalize the publication procedure of the accepted manuscript, please take the following steps to prepare a ZIP file to upload :

Step 1: Please make final corrections through the manuscript according to the reviewers' comments.
 Step 2: Make sure that all tables with its legend, and figure legends are placed after "References" in the final manuscript.
 Step 3: Each "Figure" should be saved as a single image file in jpg, gif, or tiff format over 300 dpi or 3 million pixels. File name should also be as same as figure name, e.g. image file for "Figure 1" should be "Figure1.jpg"
 Step 4: Combine the final revision and all figure image files from above steps into a single ZIP file and name it as your manuscript submission number (PBB-xx-xxxx.zip).

Once the file is ready, please log on to the Author Center and upload the ZIP file at "submissions with a Decision" under "Decision" menu.

Plant Breeding and Biotechnology sincerely appreciates your contribution to the journal. We also look forward to your continued support and contribution.
 Thank you.

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The screenshot shows the 'Step 1: Type, Title & Abstract' section of the author center. It contains the following information:

MS ID	PBB-22-1043	Date Submitted	Dec 28, 2022	Date Received	Dec 29, 2022
** Manuscript Type	Research Article	Date Revised	[1] Feb 28, 2023 [2] May 20, 2023	Date Accepted	May 22, 2023
Research Field	biotechnology				
Status	Accepted				
** Title	The Morpho-Somatic and Chromosomal Changes in Colchicine Polyploidy Induction Colocasia esculenta Var. Antiquorum				
Running Title	Colchicine Polyploidy Induction Colocasia esculenta				
** Abstract	Increased productivity of Colocasia esculenta var. Antiquorum is very necessary to increase the mass of tubers. The polyploid mutation method using colchicine can increase tuber size by increasing the number of chromosomes. Therefore, the study aimed to determine the effect of colchicine on the morphology and polyploid changes of Colocasia esculenta var. Antiquorum at various concentrations and duration of immersion in vitro. The research was carried out as a completely randomized factorial design. The first factor is colchicine's concentration, consisting of a concentration of 0.0%, 0.05%, 0.075%, and 0.1%. The second factor is the immersion time which consists of 8 hours of immersion and 16 hours of immersion. The results showed that the best combination of concentration and immersion time on somatic morphology was 0.075% colchicine with 8 hours and 16 hours of immersion. Meanwhile, the best these combination to chromosomal changes was 0.05% with 16 hours of immersion. Therefore, these results indicate that the treatment of 0.05% colchicine concentration with a soaking time of 16 hours can be recommended for the induction of polyploid mutations in Colocasia esculenta.				
** Keywords	Colocasia esculenta, Polyploidy mutation, Colchicin, Diploid, Tetraploid, In vitro				
** English Proofreading	Yes (PBB-22-1043_EngProof.pdf)				
Conflict of Interest	No potential conflict of interest relevant to this article was reported.				

1797

The screenshot shows the 'Step 2: Authors & Institutions', 'Step 3: File Upload', and 'Step 4: PDF File' sections of the author center.

Step 2: Authors & Institutions

Institution: Hasanuddin University
Authors: Feranita Haring¹, Muh Farid^{2*}, Sudirman Sudirman³ and Muhammad Fuad Anshori
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Step 3: File Upload

Order	File Name	File Item	Date
Original File	PBB-20-1058_final revision (Feranita et al).doc (4,817kb)	Manuscript File	Dec 28, 2022
1st Revision	PBB-22-1043_revised.doc (4,802kb)	Manuscript File	Feb 28, 2023
2nd Revision	PBB-22-1043_revised 2.doc (4,977kb)	Manuscript File	May 20, 2023
Sup. 1	Cover letter_fera.docx (38kb)	Supplemental	Dec 28, 2022

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Order	File Name
Original File	PBB-22-1043.pdf
1st Revision	PBB-22-1043_R1.pdf
2nd Revision	PBB-22-1043_R2.pdf

Author's Final File

Final File	Date
PBB-22-1043.zip (5,512kb)	May 24, 2023

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