In vitro induction and identification of polyploid *Neolamarckia cadamba* plants by colchicine treatment

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Polyploidization has played a crucial role in plant breeding and crop improvement. However, studies on the polyploidization of tropical tree species are still very scarce in this region. This paper described the *in vitro* induction and identification of polyploid plants of *Neolamarckia cadamba* by colchicine treatment. *N. cadamba* belongs to the Rubiaceae family is a natural tetraploid plant with 44 chromosomes (2n = 4x = 44). Nodal segments were treated with colchicine (0.1%, 0.3% and 0.5%) for 24 h and 48 h before transferring to shoot regeneration medium. Flow cytometry (FCM) and chromosome count were employed to determine the ploidy level and chromosome number of the regenerants, respectively. Of 180 colchicine-treated nodal segments, 39, 14 and 22 were tetraploids, mixoploids and octoploids, respectively. The highest percentage of polyploidization (20% octoploids; 6.7% mixoploids) was observed after treated with 0.3% colchicine for 48 h. The DNA content of tetraploid (4C) and octoploid (8C) was 2.59 ± 0.09 pg and 5.35 ± 0.24 pg, respectively. Mixoploid plants are made up of mixed tetraploid and octoploid cells. Chromosome count confirmed that tetraploid cell has 44 chromosomes and colchicine-induced octoploid cell has 88 chromosomes. Both octoploids and mixoploids grew slower than tetraploids under *in vitro* conditions. Morphological characterizations showed that mixoploid and octoploid leaves had thicker leaf blades, thicker midrib, bigger stomata size, lower stomata density, higher SPAD value and smaller pith layer than tetraploids. This indicates that polyploidization has changed and resulted in traits that are predicted to increase photosynthetic capacity of *N. cadamba*. These novel polyploid plants could be valuable resources for advanced *N. cadamba* breeding programs to produce improved clones for planted forest development.

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Abstract

Polyploidization has played a crucial role in plant breeding and crop improvement. However, studies on the polyploidization of tropical tree species are still very scarce in this region. This paper described the *in vitro* induction and identification of polyploid plants of *Neolamarckia cadamba* by colchicine treatment. *N. cadamba* belongs to the Rubiaceae family is a natural tetraploid plant with 44 chromosomes (2n = 4x = 44). Nodal segments were treated with colchicine (0.1%, 0.3% and 0.5%) for 24 h and 48 h before transferring to shoot regeneration medium. Flow cytometry (FCM) and chromosome count were employed to determine the ploidy level and chromosome number of the regenerants, respectively. Of 180 colchicine-treated nodal segments, 39, 14 and 22 were tetraploids, mixoploids and octoploids, respectively. The highest percentage of polyploidization (20% octoploids; 6.7% mixoploids) was observed after treated with 0.3% colchicine for 48 h. The DNA content of tetraploid (4C) and octoploid (8C) was 2.59 ± 0.09 pg and 5.35 ± 0.24 pg, respectively. Mixoploid plants are made up of mixed tetraploid and octoploid cells. Chromosome count confirmed that tetraploid cell has 44 chromosomes and colchicine-induced octoploid cell has 88 chromosomes. Both octoploids and mixoploids grew slower than tetraploids under *in vitro* conditions. Morphological characterizations showed that mixoploid and octoploid leaves had thicker leaf blades, thicker midrib, bigger stomata size, lower stomata density, higher SPAD value and smaller pith layer than tetraploids. This indicates that polyploidization has changed and resulted in traits that are predicted to increase photosynthetic capacity of *N. cadamba*. These novel polyploid plants could be valuable resources for advanced *N. cadamba* breeding programs to produce improved clones for planted forest development.

48   **Keywords:** *Neolamarckia cadamba*; Planted Forest, *In Vitro* Culture, Polyploidization,

49   Colchicine, Polyploid

Introduction

51   *Neolamarckia cadamba* (Roxb.) Bosser or *Anthocephalus cadamba* Roxb synonym is a fast-

52   growing multipurpose tree, self-pruning and growing vigorously in exploited areas, such as

53   logged-over forests and plantations (Krisnawati *et al.*, 2011; Ho *et al*., 2014; Nordahlia *et al.*, 2014;

54   Ho *et al*., 2020). It belongs to the Rubiaceae family and is a natural tetraploid plant with 44

55   chromosomes (2n = 4x = 44) (Bedi et al., 1981; Kiehn & Lorence, 1996; Puangsomlee & Puff,

56   2001). It is a lightweight hardwood with a density of 290 - 560 kg/m^3 at 15% moisture content.

57   The wood has a moderately fine to medium texture that is easy to work with hand and machine

58   tools to give a perfect surface. This makes it an excellent raw material for plywood production,

59   light construction work and furniture manufacturing (Jøker, 2000; Lim & Chung, 2002; Orwa *et*

60   *al.*, 2009; Lal *et al.*, 2010). It is also suitable for reforestation and enrichment planting schemes

61   (Krishnapillay *et al.*, 2007). *N. cadamba* tree bark has also been used in traditional medicine to

62   treat various illnesses (Mondal *et al.*, 2009). Despite being a commercially important commodity,

63   attempts to improve *N. cadamba* have been limited to gene mutation study using ethyl

64   methanesulphonate (EMS) (Zayed *et al.*, 2014) and physical mutagens, such as gamma rays

65   (Zanzibar & Danu, 2015). Mutation at ploidy level or polyploidization has yet to be studied.

66   Colchicine is an inhibitor to polymerization of the tubulin in nucleus that can cause

67   polyploidization in cells. It binds with β-tubulin in the microtubule, resulting in disassociation and

68   destabilization of the microtubule (Lu *et al.*, 2012). Microtubule enables translocation of

69   chromosomes during cell division (Hammond *et al.*, 2008). The formation of polyploid is due to

70   the failure of chromosome pairs to separate during the anaphase of mitosis as mitosis ceases at

71   metaphase. This will result in two sets of chromosomes in a single cell or the formation of

72   polyploid cells (Inoue, 1952; Eigsti & Dustin, 1956). To date, colchicine is widely used in

73   polyploid induction with different explants, concentrations, treatment durations and methods

74   (Hassan *et al*., 2020; Julião *et al*., 2020; Li *et al*., 2020; Zhang & Gao, 2020). Polyploidization of

75   tropical woody tree species has been rare due to slow-growing habits, long life cycles and lack of

76   *in vitro* regeneration protocols.

77   The evolution and formation of new plant species are caused by natural and gradual

78   adaptation to changing environments over a long period of time. Polyploidization increases the

79   number of dominant alleles, diminishing the effects of recessive alleles that often result in

80   undesirable traits (Comai, 2005; Soltis *et al*., 2015). This hypothesis makes polyploidization an

81   attractive option for plant breeding to produce superior plants. Some induced polyploids were

82   proven to adapt and tolerate better than their progenitors under undesirable or hostile

83   environments. The induced tetraploid of *Plumbago auriculata* can tolerate cold environment at -

84   5ºC for 24 h (Jiang *et al*., 2020). In another study, the seed production of *Themeda triandra*

85   tetraploid was quadrupled and heavier than its diploid under a very dry and hot arid environment

86   (Godfree *et al.*, 2017). The acquired "giga" effects in polyploids increase the size of the cells,

87   followed by doubling in chromosome numbers (Sattler *et al.*, 2016). This "giga" effect may

88   contribute to greater agronomical gains in polyploid plants by producing larger organs like flowers,

89   fruits, leaves, stems, roots, etc. The fruits of *Actinidia chinensis* tetraploid increase by 50-60% in

90   size compared with those of the diploid (Wu *et al.*, 2012), while in *Thymus vulgaris* tetraploid, an

91   increase in the essential oil extracted from the plants was observed (Shmeit *et al.*, 2020).

92   Polyploidization is vital in the creation of triploid plants either within species or between species.

93   Triploid plants are high in demand, especially in the fruit and vegetable industries where seedless

94   fruits are produced (Sattler *et al.*, 2016; Wang *et al.*, 2016; Hassan *et al.*, 2020). Furthermore,

95   triploids also exhibit heterosis or hybrid vigor (Fu *et al.*, 2014; Urwin, 2014).

96   Flow cytometry (FCM) analysis is employed in the polyploid identification stage to

97   determine the ploidy level status and DNA content of regenerants treated with colchicine. FCM is

98   an important procedure in polyploidization study where induced polyploids or mixoploids can be

99   segregated from plantlets with unchanged ploidy levels (Bicknell *et al.*, 2019; Huy *et al.*, 2019;

100   Julião *et al.*, 2020). Mixoploidy exists due to incomplete polyploidization resulting in chimeric

101   tissue comprising a mixture of tetraploid and octoploid cells. The cytometer used in FCM is a high

102   throughput and reliable instrument where a large number of cells can be analyzed in a single run.

103   The FCM, therefore, expedites the detection of representative population and subpopulation of the

104   sample (Doležel *et al.*, 2007; D'Hondt *et al.*, 2011). However, FCM is not efficient in detecting

105   aneuploidy. Therefore, a combination of FCM and karyotype examination is the best way to

106   determine the number of chromosomes and ploidy level. Chromosome count is a straightforward,

107   explicit and standard way of chromosome number determination. Chromosome count can also help

108   to detect the creation of double haploids (Kim *et al.*, 2019), interploids (de Alencar *et al.*, 2020)

109   and polyploids (Martin *et al.*, 2019) in plant breeding.

110   In this study, chromosome doubling or whole-genome duplication of *N. cadamba* was

111   attempted through *in vitro* mutagenesis using colchicine. The polyploid plants were identified

112   using FCM and validated using chromosome count followed by morphological characterization.

113   These newly induced polyploid plants may be beneficial for further genetic improvement of *N.*

114   *cadamba* to produce improved clones for planted forest development. To the best of our

115   knowledge, this is the first report of polyploidization in *N. cadamba.*

116

Materials and Methods

Nodal segments preparation and colchicine treatment

Nodal segments were obtained from shoots regenerated from a selected *N. cadamba* seedling on regeneration medium (B5 medium + 1.0 mgL^{-1} BAP + 2% sucrose). The nodal segments were treated with colchicine in different concentrations and durations, i.e., 0.1%, 0.3%, and 0.5% of colchicine for 24 h and 48 h, respectively. In the control treatment, sterile distilled water was used for 24 h and 48 h. The nodal segments in the colchicine solution were incubated with shaking using a platform shaker at 100 rpm to improve contact between colchicine and explants. The whole process of colchicine treatment was conducted in the dark at 25 ± 2 ºC to avoid deterioration of colchicine under light.

Flow cytometry analysis (FCM) and chromosome count

The colchicine treated nodal segments of *N. cadamba* were subcultured for five cycles (four weeks per cycle) on shoot regeneration medium (B5 + 1 mgL^{-1} BAP + 2% sucrose) before the ploidy level determination. The fully developed *in vitro* young leaves from the control and colchicine treated plantlets were collected and used to determine the ploidy level. Untreated plantlets were used as control while *Glycine max* cv. Polanka was used as an external standard in FCM analysis (Doležel *et al.*, 1989). About 1 cm^2 of an *in vitro* young leaf for each sample was chopped in 1 ml of lysis buffer or LB01 using a scalpel on the disposable petri dish. The LB01 buffer was made up of 80 mM KCl, 20 mM NaCl, 15 mM Tris, 15 mM mercaptoethanol, 2 mM Na$_2$EDTA, 0.5 mM spermidine, 0.1% Triton X-100, pH 7.5, 50 μg propidium iodide and 50 μg RNase (Doležel *et al.*, 1989). The finely chopped leaf pieces of each sample were filtered through a 50-μm cell strainer separately into a 5 mL flow cytometry tube. The filtrate of each sample was analyzed by a

140 FACSCalibur flow cytometer - Becton Dickinson equipped with an argon-ion laser (15 mW) at

141 488 nm. Histograms for each sample were collected over 1,024 channels. DNA content of each

142 sample can be estimated using this formula (Doležel *et al.*, 2007): $S_{PL} = (S_{(G0/G1)} / R_{(G0/G1)})$ x R_{PL},

143 where S_{PL} is 2C value of colchicine treated sample; R_{PL} is 2C of the control sample, i.e., *Glycine*

144 *max* Merr. 'Polanka' with a value 2.50; $S_{(G0/G1)}$ means the position of colchicine treated sample,

145 and $R_{(G0/G1)}$ means the position of control sample peak.

146 The 5-day-old *in vitro* root tips after transplantation were excised at 1 cm in length from

147 the tip of the roots. The root tips were washed with running tap water to eliminate the agar medium.

148 In pre-treatment, these root tips were first submerged in 0.002 M 8-hydroxyquionline for 2 h at 4

149 ℃ followed by another 2 h at room temperature. The pre-treated root tips were washed three times

150 with distilled water. In fixation, the root tips were dipped in freshly prepared cold Carnoy's

151 solution 1 (Ethanol 95% : glacial Acetic Acid; 3 : 1) for 24 h at 4 ℃. After fixation, the root tips

152 were washed with distilled water three times. The hydrolysis of the pre-treated and fixed root tips

153 took place using 1 N HCl for 15 min at 60 ℃. After HCl hydrolysis, the root tips were washed

154 with distilled water three times. The staining process was carried out at room temperature. Feulgen

155 stain was applied on the root tips for 30 min in dark condition before squashing with a metal bar

156 in a drop of aceto-orcein 1% stain followed by thumb squash (Eng *et al*., 2020).

157

158 ***In vitro* propagation and acclimatization**

159 *In vitro* nodal segments were excised from *N. cadamba* tetraploid, mixoploid and octoploid

160 plantlets and cultured on shoot regeneration medium (B5 + 0.8 mgL^{-1} BAP + 2% sucrose) for six

161 weeks with subculture once every three weeks. The number of shoots produced and the percentage

162 of nodal segments producing shoots were recorded. After regeneration, well-developed shoots,

163 about 1 cm in height, of the tetraploid, mixoploid and octoploid cultures were excised from the

164 shoot clumps and transferred to basal B5+ 2% sucrose medium for eight weeks with subculture

165 once every four weeks. Morphological characters of tetraploid, mixoploid and octoploid shoots

166 were recorded, namely plant height, number of leaves, number of nodes and stem diameter after

167 eight weeks of culture. The shoots of the tetraploid, mixoploid and octoploid cultures with the

168 height of 3.0 cm were excised and cultured on rooting medium, ½ B5 + 0.1 mgL^{-1} PBZ

169 (Paclobutrazol) + 2% sucrose. The number of roots and the percentage of rooting were recorded

170 after two weeks of culture. Well rooted *in vitro* plantlets were cleaned thoroughly under running

171 tap water before planted onto different acclimatization media. These acclimatization media are

172 Jiffy-7, loamy soil and soil mixture (loamy soil : compost = 3:1). The Jiffy-7 pellets were soaked

173 in tap water overnight before use. Both the loamy soil and the soil mixture were sterilized by

174 autoclaving at 121 °C for 20 min. All transferred plantlets were kept in air-tight containers for the

175 first week before the container's covers were removed gradually to regulate humidity and avoid

176 plantlets from wilting. Plantlets were maintained under room temperature. Fungicide was applied

177 weekly to prevent fungal contamination among the acclimatizing plantlets. The number of

178 surviving plantlets in different media was recorded after four weeks of acclimatization.

179   This experiment consisted of tetraploid, mixoploid and octoploid plantlets; each genotype

180 consisted of six plantlets, and the experiment was repeated thrice. The data collected were

181 subjected to ANOVA using the IBM SPSS version 23 program. The ANOVA was carried out first,

182 followed by Duncan New Multiple Range Test (DNMRT) ($p < 0.05$).

183

184 **Morphological characteristics and soil plant analysis development (SPAD) value**

185 The well-developed third leaf from the shoot tip of one-month-old acclimatized seedlings was

186 obtained for leaf and midrib thickness studies. Using a razor blade, cut across the width of the leaf

187 sample about 1 mm wide. The leaf section was placed on a slide vertically with the cut section

188 facing upwards. The leaf section was observed using a LEICA DM500 compound light microscope

189 4x objective lens. The image of the leaf section was captured using a LEICA ICC50 HD camera.

190 LEICA Application Suite (LAS EZ) was used to measure the leaf and midrib thickness. A total of

191 10 leaves for each genotype was analyzed, namely tetraploid, mixoploid and octoploid to

192 determine leaf and midrib thickness.

193   The well-developed leaves of acclimatized seedlings were obtained for abaxial leaf study.

194 A layer of clear nail polish was painted on the abaxial leaf side and leave it to dry. Then a pair of

195 tweezers was used to peel the nail polish from the leaf surface. Place the nail polish peel with leaf

196 impression on a slide. The leaf surface was observed using a LEICA DM500 compound light

197 microscope 40x objective lens, and the image was captured using a LEICA ICC50 HD camera. A

198 total of 100 stomata was measured for each genotype, namely tetraploid, mixoploid and octoploid.

199 LEICA Application Suite (LAS EZ) was used to measure the length and width of the stomata. Ten

200 optical micrographs (0.33 x 0.25 mm) were used to determine the density of the stomata and

201 trichomes. ImageJ software was used to determine the number of stomata and trichomes per mm^2.

202   Scanning electron microscope (SEM) was used to examine the structure and other details

203 of the stoma and trichomes. Initially, six-month-old leaves were sectioned using a blade, and only

204 the middle part of the leaf sections was used for the SEM study. Leaf samples were prepared using

205 the protocol developed by Talbot and White (2013). The leaf sections were fixed in 100% methanol

206 for 10 min. After the fixation, they were dehydrated two times in 100% methanol for 30 min before

207 further dried using a critical point drying machine. The leaf sections were mounted on SEM stud

208   with the abaxial part facing upwards. The samples were then coated with gold coatings before they

209   were observed using SEM by JEOL model JSM-6390OLA.

210   A mature stem from a one-year-old plant was cut using a fine tooth saw to study the cross-

211   section of the 10-month-old stem. The thickness of bark, pith and wood of *N. cadamba* were

212   recorded with the aid of a pair of vernier calliper. A total of three stem section for each genotype

213   were analysed, namely tetraploid, mixoploid and octoploid.

214   The leaf SPAD values were measured using portable chlorophyll meter, model TYS-B

215   (Mindfull Technology Co. Ltd) that was equipped with two light sources, namely red light (650

216   nm) and infrared light (940 nm). To measure SPAD value, grip the leaf sample between the

217   pressure head and measuring head of the meter. The buzzer of this meter will buzz for less than 3

218   seconds signalling the measurement is completed and the reading can be recorded. A total of 30

219   trees consisting of tetraploid, mixoploid and octoploid plants were measured, and each genotype

220   consisted of 10 plants.

221   The data collected were subjected to ANOVA using the IBM SPSS version 23 program.

222   The ANOVA was carried out first, followed by Duncan New Multiple Range Test (DNMRT) (p

223   < 0.05).

224

225   **Results**

226   **Flow cytometry analysis (FCM) and chromosome count**

227   In all colchicine treatments (0.1%, 0.3% and 0.5%), the longer treatment duration at 48 h tend to

228   produce a higher number of octoploids and a lower number of mixoploids (Table 1). In total, 39

229   tetraploids, 14 mixoploids and 22 octoploids were produced in this study. Two treatments, namely

230   0.3% colchicine for 48 h and 0.5% colchicine for 48 h gave higher percentages (20%) of

231 octoploids. However, treatment with 0.3% colchicine for 48 h produced the best result that yielded

232 20.0% octoploids and 6.7% mixoploids. In the 0.3% colchicine treatment for 24 h, the highest

233 percentage of mixoploids was obtained. Generally, a combination of a higher concentration of

234 colchicine and a longer treatment duration yielded a higher percentage of octoploids in *N.*

235 *cadamba*. The ploidy level of regenerants of *N. cadamba* can be determined based on the histogram

236 generated using a flow cytometer (Figures 1A-1C). The histogram showed a single dominant peak

237 in control and tetraploid plants with the mean position of a peak for 4x (Figure 1A). For the

238 mixoploid plants, the histogram showed two separate dominant peaks with the mean position of

239 the first peak for 4x and the mean position of the second peak for 8x (Figure 1B). For the octoploid

240 plants, the histogram showed a single dominant peak with the mean position of a peak for 8x

241 (Figure 1C). It was also noted that the octoploid cells (Figure 1C) are two times greater in the mean

242 position of the peak than those of the tetraploid cells (Figure 1A). Our data showed that the

243 estimated DNA content for tetraploid (4C DNA) is 2.59 ± 0.09 pg, while for octoploids, there is a

244 two-fold increase in the estimated DNA content (8C DNA = 5.35 ± 0.24 pg).

245 The ploidy level of samples determined using FCM was verified using the optimized

246 chromosome count procedure. The micrograph of the somatic cells of *N. cadamba* root tips showed

247 that both the tetraploid and the control contain 44 chromosomes per nucleus (Figure 1D); the

248 octoploid (a.k.a *N. cadamba* "Kestamas-19") contains 88 chromosomes per nucleus (Figure 1E),

249 while the mixoploid contains either 44 or 88 chromosomes per nucleus. The ploidy level results

250 determined using the FCM procedure agree with results obtained using the optimized chromosome

251 count method.

252

253 ***In vitro* propagation and acclimatization**

254   The shoot regeneration ability between the tetraploid, mixoploid and octoploid was observed and

255   compared with each other (Table 2). The mean number of shoots produced was significantly higher

256   in the tetraploid (5.4) as compared to mixoploid (4.3) and octoploid (4.2) (Table 2). In terms of

257   the mean percentage of explants producing shoots, the tetraploid (94.4%) and mixoploid (100.0%)

258   showed significantly higher mean percentages of explants producing shoots compared to the

259   octoploid (83.3%). The new genotypes of *N. cadamba*, i.e., the mixoploid (Figure 2B) and

260   octoploid (Figure 2C) showed significantly lower achievement in terms of the number of shoots

261   produced as compared with the tetraploid (Figure 2A). The octoploid responded with the lowest

262   mean percentage of regeneration among the three genotypes (Table 2). The observation from this

263   study concluded that polyploidization resulted in higher mortality in the shoot regeneration

264   medium.

265   The growth of *N. cadamba* tetraploid, mixoploid and octoploid shoot tips on the basal B5

266   medium was evaluated after they have been transferred from the regeneration medium. After eight

267   weeks of growth on the basal B5 medium, the tetraploid attained the highest mean plant height at

268   7.2 cm, followed by the mixoploid at 6.8 cm and the octoploid at 4.4 cm only (Table 2).

269   Polyploidization in *N. cadamba* has noticeably reduced the plant height (Table 2 and Figure 2D).

270   There was no significant difference between the tetraploid, mixoploid, and octoploid shoots in

271   terms of the mean number of leaves and the mean number of nodes. The mean number of leaves

272   ranges from 12.8 to 13.0, while the mean number of nodes ranges from 6.4 to 6.5 for tetraploid,

273   mixoploid and octoploid (Table 2). The octoploid plantlets have shorter stems making each of

274   them an interestingly compact architecture (Figure 2D). The mean stem thickness of the shoots in

275   tetraploid was 2.3 mm only, while the octoploid 2.89 cm and mixoploid 2.7 mm. The stem of the

276   two new polyploids was significantly thicker than the tetraploid (Table 2). From the *in vitro* growth

277   parameters recorded, polyploidization resulted in the reduction of stem height but has the opposite

278   effect of increment in stem diameter. The octoploids are sturdier than mixoploid and tetraploid

279   plantlets due to their shorter stems.

280   The excised shoots of tetraploid, mixoploid and octoploid of *N. cadamba* were cultured in

281   rooting medium for 14 days to compare the rooting capability of plantlets of different ploidy levels

282   (Table 2 and Figures 2E-2G). All shoots, regardless of ploidy level, produced roots after the 14

283   days on rooting medium. The tetraploid (13.8) and mixoploid (16.1) produced a higher mean

284   number of roots compared with the octoploid (11.1). In terms of mean root length, the tetraploid

285   produced the longest mean root length (1.8 cm), followed by the mixoploid (1.6 cm) and octoploid

286   (1.4 cm) (Table 2). In this study, the shoots were cultured on the rooting medium for no longer

287   than 14 days to avoid excessive rooting, leading to a high number of roots and longer root length.

288   The rooted plantlets were transferred to three different media types, namely Jiffy-7, loamy

289   soil, soil mix (loamy soil : compost; 3 : 1) to assess the percentage of acclimatization of the

290   plantlets (Figure 2H). The mean percentage of acclimatization of the tetraploid plantlets planted

291   on Jiffy-7 pellets and loamy soil was 94.4% and 88.9%, respectively. These two media types were

292   significantly superior to the soil mix that produced only 50.0% mean percentage of acclimatization.

293   A similar trend was observed in the mixoploid and octoploid plantlets (Table 2). Two factors that

294   caused mortality to the *N. cadamba* plantlets during acclimatization were water stress and fungal

295   contamination. Water stress can be controlled by placing plantlets in an enclosed container to

296   maintain higher humidity and prevent water loss. In this study, 5 gL^{-1} Benocide solutions were

297   sprayed with a mist sprayer. Incorporating compost in the soil mix should be avoided in this

298   experiment as it would encourage fungal contamination even after autoclaving before planting.

299

Morphological characteristics and SPAD value

300

301 The *N. cadamba* octoploid (247.2 μm) had thicker leaves than its tetraploid counterpart (160.8

302 μm). However, there was no significant difference in mean leaf thickness between the mixoploid

303 and tetraploid. The mean midrib vertical thickness of the octoploid (831.7 μm) was higher than

304 both the mixoploid (762.4 μm) and tetraploid (666.6 μm). The mean midrib horizontal thickness

305 of the octoploid (899.1 μm) and the mixoploid (793.0 μm) was higher than the tetraploid (616.2

306 μm). However, there was no significant difference between the mixoploid and the tetraploid in

307 terms of mean midrib vertical thickness (Table 3 and Figures 3A-3C).

308 The mean stomata density per mm^{-2} in octoploid (301.8) and mixoploid (298.2) were

309 significantly less than the tetraploid (426.7) of *N. cadamba* plants (Table 3 and Figures 3D-3F).

310 Similarly, the mean trichome density mm^{-2} was significantly less in octoploid (4.9) and mixoploid

311 (4.9) when compared with the tetraploid (33.9) of *N. cadamba* plants. The mean stomata length of

312 either the mixoploid (29.3 μm) or octoploid (30.7 μm) was higher than the tetraploid (25.3 μm).

313 The mean stomata width of either the mixoploid (16.1 μm) or octoploid (16.6 μm) was also higher

314 than the tetraploid (14.9 μm). Generally, the mixoploid and octoploid plants of *N. cadamba* possess

315 a bigger stomata size than the tetraploid plant (Table 3 and Figures 3G-3I). The stoma of *N.*

316 *cadamba* tetraploid, mixoploid and octoploid was then observed using SEM at 2000x. The

317 micrograph showed that regardless of ploidy level, the stoma is made up of two complementary

318 kidney-shaped guard cells arranged in opposite sites to form an aperture. Each guard cell is flanked

319 by a subsidiary cell that aligns parallel to its long axis. The trichomes of *N. cadamba* in this study

320 were observed using SEM at 2000x. The micrograph showed, regardless of ploidy level, the

321 trichomes were categorized as unicellular and non-glandular trichomes (Figures 3J-3L) based on

322 trichomes anatomical categorization by Werker (2000) study. The shape and other attributes of *N.*

323   *cadamba* trichome are categorized as ornithorhynchous based on the nomenclatures determined

324   by Payne (1978).

325   In the stem cross-section study, the bark, wood and pith layers of *N. cadamba* plants were

326   studied. There was no significant difference between the tetraploid, mixoploid and octoploid in the

327   bark and wood layers. However, the tetraploid (5.7 mm) and mixoploid (5.2 cm) have a bigger

328   pith than the octoploid (3.6 mm) (Table 3 and Figure 3M).

329   The mean SPAD value of the octoploid was the highest (39.7) when compared to the

330   mixoploid (37.2) and tetraploid (37.1). Hence, the chlorophyll content of the octoploid was also

331   higher than both the mixoploid and tetraploid (Table 3).

332

333   **Discussion**

334   **Flow cytometry analysis (FCM) and chromosome count**

335   Colchicine is a commonly used chromosome doubling agent. Its anti-mitotic properties result in

336   the disruption of microtubules to produce polyploids. Polyploids induced by using colchicine are

337   also termed as colchiploids. This term was widely used in 1960's (Sen & Marimuthu, 1960;

338   Raghuvanshi & Joshi, 1964; Das *et al.*, 1970). The optimum amount of colchicine used in

339   polyploid production is wide-ranging, with concentrations ranging from 0.001% (Thao *et al.*,

340   2003) to 1.0 % (Demtsu *et al.*, 2013). The difference is one hundred times between the highest and

341   the lowest colchicine concentration. The percentage of successful polyploidization also varies. The

342   success rate could be attributed to the species under investigation, the colchicine application

343   protocol (*in vitro* or *ex vitro*) and the explant used.

344   FCM can detect the existence of mixoploids and it can analyze a huge population of cells

345   in a relatively short duration. In the histogram generated from FCM for *N. cadamba*, Figure 1c,

346 two distinct populations of cells were detected, namely 4x and 8x, and this finding can be

347 interpreted from the two dominant peaks in the histogram. Polyploidization studies conducted

348 without the FCM procedure often yielded very high polyploidization percentage but no occurrence

349 of mixoploids, such as 100% for *Pogostemon cablin* leaves (Widoretno, 2016), 42.3% for

350 *Gossypium arboretum* seeds (Yang *et al.*, 2015) and 41.7% for *Vitis* sp. (Sinski *et al.*, 2014) shoot

351 tips. These studies determined polyploidization through manual chromosome counting, which is

352 difficult because the researcher has to analyze a huge number of cells needed to represent an

353 accurate cell composition of the plant. Studies on polyploidization using seeds, seedlings, shoot

354 tips, and nodal segments are predominantly prone to the production of mixoploids which is an

355 unwelcome result (Eng & Ho, 2019). Studies using these sources of explants should adopt FCM

356 to avoid mixoploids being pooled into induced polyploids or the original ploidy category. FCM

357 will help the researcher to identify the mixoploids. The existence of mixoploids will often mislead

358 the analysis leading to a possible inaccurate conclusion.

359   According to Ohri *et al.* (2004), the estimated 4C DNA for *A. cadamba* was 2.77 ± 0.27 pg

360 using M86 Vickers microdensitometer. The data have been published and archived in the C-value

361 Kew Garden database (http://www.kew.org/cval/homepage.html), where the data are conveyed in

362 estimated 1C DNA, that is 0.69 pg. These data showed that *N. cadamba* is a tetraploid. Based on

363 these data, our estimated 4C DNA of *N. cadamba* was 2.59 ± 0.09 pg, which falls within the

364 published data range, while the octoploids estimated 8C DNA was 5.35 ± 0.24 pg. This study

365 indicated that the DNA content had doubled in *N. cadamba* tetraploid to form *N. cadamba*

366 octoploid. There are many uses of estimated DNA content apart from the indication of ploidy level

367 change. According to Bennett *et al.* (2000), estimated DNA content could be used in the molecular

368   investigation, evolution, variation, constancy, phylogenetic, phenotypic, phenological, ecological,

369   environmental indicators, and paleobiological trends.

370   According to Regalado *et al.* (2017), mixoploid plants are unstable as competition occurs

371   between the original cells and the polyploid cells leading to the elimination of the latter. As a

372   result, mixoploids status will be reversed to its original ploidy level. In citrus breeding, mixoploids

373   were discarded due to stability concern and low fertility if crossed with other species (Grosser *et*

374   *al.*, 2014). For this study, *N. cadamba* mixoploids produced were maintained and further evaluated

375   together with tetraploids and octoploids. Mixoploids are often regarded as a failure or undesirable

376   by-products of polyploidization studies so that efforts are made to eliminate them through

377   deliberate means, namely mechanical isolation of putative polyploids (Aleza *et al.*, 2009; Regalado

378   *et al.*, 2015); shoot regeneration using nodal segments (Zhou *et al.*, 2017) and repeated subcultures

379   of apical buds (Jiang *et al.*, 2020).

380   The chromosome count enables the verification of samples determined using FCM. In the

381   present study, the FCM results using leaf samples aligned with our findings through the

382   chromosome count using root tip samples. The control, which is a tetraploid (2n = 4x) contains 44

383   chromosomes per nucleus, the octoploid (4n = 8x) contains 88 chromosomes per nucleus and the

384   mixoploid (2n + 4n = 4x + 8x) contains either 44 or 88 chromosomes per nucleus. This study,

385   based on the chromosome count, has verified the ploidy level data obtained from FCM. The

386   polyploidization of *N. cadamba* using colchicine has successfully induced octoploids and

387   mixoploids from tetraploid explants. The chromosome number of *N. cadamba* (2n = 4x) has

388   doubled (4n = 8x) after colchicine treatment (Figures 1D and 1E). This is a two-fold increment of

389   chromosome number from the original *N. cadamba* explant. This is the first establishment of *N.*

390   *cadamba* octoploid ever reported, where the chromosome number is 88.

391

***In vitro* propagation and acclimatization**

In vitro technique was used in this study to examine the effects of *N. cadamba* polyploid plants at different stages of micropropagation following the established tissue culture protocol of *N. cadamba* (Mok & Ho, 2019). With these advantages, mass phenotyping and characterizing of new plants are now a possible task. Our study is in agreement with that of the induced polyploid plants of *Cichorium intybus* (Ravandi *et al.*, 2014) and *Dendrobium officinale* (Pham *et al.*, 2019). The explants of induced polyploid of *D. officinal* produced a higher protocorm induction rate but required a longer time for protocorm initiation. According to Ravandi *et al.* (2014), the ability to regenerate shoots from callus or organogenesis in induced polyploid explants was significantly reduced. Tsukaya (2008) reported that *Arabidopsis thaliana* with more than eight sets of homologous chromosomes will display high ploidy syndrome, exhibiting contrasting effects by producing bigger cell in volume but smaller leaves. Several recent studies have employed the *in vitro* system to study the effects of plant ploidy level on morphological traits (Javadian *et al.*, 2017; Pham *et al.*, 2019; Shmeit *et al.*, 2020). The present study is in agreement with Pham *et al.* (2019), where colchicine-induced tetraploids of *D. officinale* showed shorter stem length and thicker stem. The thicker stem was also found in the colchicine-induced tetraploid of *Linum album* (Javadian *et al.*, 2017). However, oryzalin-induced tetraploid of *Thymus vulgaris* showed longer stem length and thicker stem (Shmeit *et al.*, 2020).

The induced octoploid of *N. cadamba* exhibited slower root formation. The root growth was also slower in the rooting medium (Table 2 and Figures 2E-2G). According to Mok and Ho (2019), the rooting medium 1/2 B5 + 0.1 mgL^{-1} PBZ is an optimum medium in inducing a sufficient number of roots in *N. cadamba*. The PBZ concentration does not affect growth during the rooting,

414   and there is no callus formation on the shoot. The protocol has improved the success rate of

415   acclimatization. PBZ derived from an aromatic compound, trizol is a plant growth regulator with

416   many uses in agriculture. Among others, it is used to induce flowering, fruiting and prevent fungal

417   infections (Wang *et al.*, 2019). PBZ has many uses in *in vitro* culture for different plants, such as

418   shoot multiplication medium MS + 5 mgL^{-1} in *Phoenix dactylifera* (Awadh *et al.*, 2019), rooting

419   medium 1/2 B5 + 0.1 mgL^{-1} in *N. cadamba* (Mok & Ho, 2019) and rooting medium MS + 1.5

420   mgL^{-1} in *Zygopetalum crinitum* (Gimenes *et al.*, 2018).

421   Acclimatization is the final stage of *in vitro* system that will determine the production

422   number of the plantlets. *In vitro* acclimatization depends on the capability of the plantlets to

423   transform from being either mixotrophic or heterotrophic to photoautotrophic before they can

424   adapt to the harsh *ex vitro* conditions. Plantlets in *in vitro* system are mixotrophic or heterotrophic

425   where complete nutrients are provided to sustain growth, while plantlets under *ex vitro* conditions

426   need to become photoautotrophic where foods can be synthesized through photosynthesis

427   (Debergh, 1991; Pospíšilová *et al.*, 1999; Chandra *et al.*, 2010). With this principle in mind, *in*

428   *vitro* plantlets of *N. cadamba* of different ploidy levels were acclimatized using different media

429   types while controlling humidity and preventing fungal contamination. There was no significant

430   difference between Jiffy-7 pellet and loamy soil to produce higher numbers of acclimatized

431   plantlets than soil mix of loamy soil : compost; 3 : 1 (Table 2 and Figure 2H). As the loamy soil

432   can be locally sourced, it is more plentiful and cheaper than the Jiffy-7 pellet and, therefore, should

433   be used as the main medium for acclimatization. Loamy soil provides adequate nutrients to sustain

434   the growth of the plantlets apart from acclimatizing. The addition of compost into the loamy soil

435   does not contribute to better growth or more successful acclimatization of the plantlets at the

436   acclimatization stage. This study agrees with Mok and Ho (2019), where Jiffy-7 provides a high

437   percentage of successful acclimatization. Jiffy-7 pellet is only made of compressed peat moss and

438   coco fibers in the fine net that provides adequate aeration water. Mengesha *et al.* (2013) obtained

439   a similar result where Jiffy-7 pellet was found to improve not only acclimatization of *in vitro*

440   plantlets of *Ananas comosuss* but also the growth during acclimatization.

441   The approach to humidity control in this study during acclimatization was similar to the

442   method described by Ahmed *et al.* (2012). Newly deflasked plantlets of *N. cadamba* are

443   susceptible to water stress and low humidity. The newly transplanted plantlets were placed in a

444   clear, air-tight container to maintain high humidity. The container cover was removed slowly to

445   expose the plantlets progressively to *ex vitro* conditions. Adapting the plantlets slowly to *ex vitro*

446   conditions can prevent abiotic stress to the plantlets, which is the main cause of mortality.

447   According to Chandra *et al.* (2010), high humidity in *in vitro* culture vessels can impair the

448   stomatal apparatus function and retard the cuticle and epicuticular layer formation. This will lead

449   to excessive transpiration when the plantlets are transferred to *ex vitro* environment with low

450   humidity, high light intensity and wide temperature fluctuation. In nature, the *N. cadamba* trees

451   thrive near to the river banks, which experience intermittent submerges in river water due to tidal

452   phenomenon or rainfall (Mansur & Tuheteru, 2010). Therefore, regular watering is essential as the

453   leaves tend to wilt during water deficiency. Plantlet loss can be caused by fungal infections found

454   on either the roots, leaves or stems of the plantlets. The plantlets were misted with 5 gL^{-1} Benocide

455   solution weekly using a mist sprayer to reduce fungal infections.

456

457   **Morphological characteristics and SPAD value**

458   This study observed that the octoploid and mixoploid plants of *N. cadamba* produced thicker leaf

459   than the tetraploid plants (Table 3 and Figures 3A-3C). This characteristic is in line with several

460 other induced polyploid species, such as A*nthurium andraeanum* (Chen *et al.*, 2011); *Lobularia*

461 *maritima* (Huang *et al.*, 2015*)*; *Lycium rhuthenicum* (Rao *et al.*, 2019); *Manihot esculenta* (Zhou

462 *et al.*, 2017); *Bacopa monnieri* (Inthima & Sujipuli, 2019); *Plumbago auriculata* (Jiang *et al.*,

463 2020) and *Solanum lycopersicum* (de Alencar *et al.*, 2020). Detailed histological study on *S.*

464 *lycopersicum* leaf blade revealed that thicker leaf in the induced polyploid was the result of greater

465 thickness in the epidermis (adaxial and abaxial) and the parenchyma (spongy and palisade) layers

466 (de Alencar *et al.*, 2020). In the induced polyploid of *Plumbago auriculata*, the histological study

467 showed that thicker spongy tissue contributed to thicker leaf (Jiang *et al.*, 2020). These histological

468 studies reveal the thicker leaf compositions but do not provide further information regarding the

469 function of the thicker leaf in plants.

470   There is much empirical evidence showing that plants with thicker leaves have better

471 adaptability to a drier environment. According to Afzal *et al.* (2017), plant species with thicker

472 leaves can withstand greater drought and salinity. They also stated that leaf thickness assessment

473 is a promising technique to predict water status in plants. In another study, under arid conditions,

474 plants with thicker leaves can adapt by maintaining water potential when the water source is

475 limited (Ogburn & Edwards, 2010). A thicker leaf is associated with the plant ability to inhabit

476 and adapt to challenging environments like arid conditions and high irradiance (Li *et al.*, 2014;

477 Coneva & Chitwoo, 2018). A plant can adapt to a harsh environment inflicted by climate change

478 by modifying leaf morphological and anatomical traits (Dar *et al.*, 2013; Soudzilovskaia *et al.*,

479 2013; Tian *et al.*, 2016; Ribeiro *et al.*, 2016; Souza *et al.*, 2018). Inferring from the evidence as

480 stated, thicker leaf of *N. cadamba* plants acquired through polyploidization may similarly be more

481 adaptable to a drier condition, such as in locations with lower rainfall. This improvement is

482 essential to *N. cadamba* due to looming climate change exacerbated by global warming.

483   This study observed that the leaf midrib of the octoploid of *N. cadamba* is thicker than its

484   progenitor (Table 3 and Figures 3A-3C). This is in line with the finding of the polyploidization

485   study of *Salix viminalis* by Dudits *et al.* (2016), where induced tetraploid plants were reported to

486   have thicker midrib. This could increase water volume transported to the leaf blade where more

487   water can be stored to prevent the plant from excessive water loss during drought. Furthermore,

488   thicker leaf midrib provides a stronger leaf structure of *N. cadamba* that may prevent the leaf blade

489   from wind damage.

490   Stomata morphology is commonly used to determine how polyploidization has modified

491   the characteristics of the polyploid due to its ubiquity, homology across distantly related relatives

492   and uniform shape (Doyle & Coate, 2019). This makes stomatal study reliable and repeatable when

493   different leaf samples are used, apart from the relatively easier and faster (Xie *et al.*, 2015; Eng &

494   Ho, 2019). Polyploidization of plants always produces bigger stomata size and lesser stomata

495   density (number of stomata per unit area) at the abaxial side of the leaf of different species such as

496   *Impatiens walleriana* (Ghanbari *et al.*, 2019); *Dendrobium officinale* (Pham *et al.*, 2019); *Lycium*

497   *ruthernicum* (Rao *et al.*, 2019); *Paphiopedilum villosum* (Huy *et al.*, 2019); and *Plumbago*

498   *auriculata* (Jiang *et al.*, 2020). These characteristics can also be observed in *N. cadamba* in this

499   study, where octoploid and mixoploid plants pose bigger stomata in terms of mean length and

500   mean width and lower mean stomata density compared to their tetraploid progenitor (Table 3 and

501   Figures 3D-3F). A negative correlation between stomata size and stomata density is common in

502   the leaves (Camargo & Marenco, 2011; Doheny-Adams *et al.*, 2012; Bertolino *et al.*, 2019). This

503   contrasting characteristic is called plastic developmental responses to evolutionary adaptation and

504   environmental changes (de Boer *et al.*, 2016; Dittberner *et al.*, 2018).

505   There is no structural difference of stomata among the tetraploid, mixoploid and octoploid

506   plants of *N. cadamba* (Figures 3G-3I). The *N. cadamba* stomata belong to the paracytic type, where

507   each guard cell is accompanied by a subsidiary cell to its long axis (Willmer & Fricker, 1996).

508   This stomata type is typical to members of the Rubiaceae family. Most water obtained in plants is

509   transpired through stomatal apertures before being utilized for plant growth. The ability to

510   conserve water and use it efficiently will improve plant growth. Stomata characteristics, such as

511   size and density, play an essential role in determining water-use-efficiency in the crop, thus making

512   these characteristics essential to crop improvement target (Bertolino *et al.*, 2019). Under a

513   controlled atmosphere, *Arabidopsis thaliana* mutant plants with lower stomatal density are better

514   adapted to the higher concentration of atmospheric CO_2 and water-scarce environment (Doheny-

515   Adams *et al.*, 2012). This finding suggested that genetically modified plants with lower stomatal

516   density can be better adapted to predicted future climate change where elevated atmospheric CO_2

517   and more arid land become more prevalent. Recent research has also shown that genetically

518   engineered crops with reduced stomatal density may contribute to better water usage efficiency

519   and drought tolerance without productivity decline (Bertolino *et al.*, 2019). Given much evidence,

520   induced octoploid and mixoploid of *N. cadamba* with reduced stomata density and increased

521   stomata size are likely to attain a greater advantage in utilizing water resources in the field.

522   Polyploidization of *N. cadamba* caused significantly reduced trichome density of

523   mixoploid and octoploid plants than their tetraploid progenitor plants. However, the trichome size

524   of mixoploid and octoploid plants were relatively bigger than tetraploid plants (Table 3, Figures

525   3J-3L). This finding is in line with Zhang and Gao (2020), where trichomes density on leaf sheath

526   of *Dendrobium cariniferum* has reduced in the induced tetraploid plant compared with the diploid

527   progenitor plant. The trichomes play a vital role in protecting the plant from biological and

528 environmental hazards, namely herbivores, pathogens, excessive ultraviolet irradiation and over

529 transpiration (Xiao *et al.*, 2017). The characterization and classification of trichomes are

530 challenging due to the immense diversity of trichomes in morphology, origin, size, location,

531 surface microstructure, secretory organ, function and timing of activity. However, the main

532 characteristic is glandular or non-glandular (Werker, 2000). The trichomes of the tetraploid,

533 mixoploid and octoploid of *N. cadamba* are categorized as non-glandular and unicell trichomes.

534 The shapes and attributes of the trichomes have been described in detail by Payne (1978) and the

535 trichomes of *N. cadamba* polyploid plants are termed as ornithorhynchous, which resembles a

536 bird's bill (Figures 3J-3L).

537   Stem cross-section of *N. cadamba* showed that the pith of either the tetraploid or mixoploid

538 was significantly bigger than that of the octoploid (Table 3 and Figure 3M). However, there is no

539 significant difference for wood and bark layers of tetraploid, mixoploid and octoploid plants. The

540 smaller pith in the octoploid is an improvement to the stem of *N. cadamba*, which may be of

541 importance to wood production. The pith in *N. cadamba* will eventually dry up as the tree ages or

542 after harvesting. The stem cross-section study is destructive as the plant has to be sacrificed to

543 reveal the stem composition. Stem cross-section has rarely been studied in the polyploidisation of

544 plants. The stem cross-section study is important to those trees that are used for timber production

545 and where the quality and quality of the timber are desired. In the *Salix viminalis* polyploidization

546 study, the induced tetraploid plants have a significantly greater composition of bark and wood

547 layers than the tetraploid plants (Dudits *et al.*, 2016).

548   The SPAD value of induced polyploid plants is often studied to determine their chlorophyll

549 content (Wang *et al.*, 2017; Rao *et al.*, 2019). Our study is in line with both studies showing

550 induced polyploid plants of *N. cadamba* gain a higher mean SPAD value than their progenitor

551 (Table 3). SPAD value correlates positively to the chlorophyll content and greenness of the leaf

552 measured (Limantara *et al.*, 2015). Increased chlorophyll content in induced polyploids may show

553 increased photosynthesis rate (Wang *et al.*, 2017; Rao *et al.*, 2019). From these findings, the

554 mixoploid and octoploid leaves are predicted to have higher photosynthetic capacity due to their

555 higher SPAD value or higher content of chlorophyll. The improvement in SPAD value in polyploid

556 of *N. cadamba* signifies the importance of polyploidization as a plant breeding tool. The SPAD

557 value is not the sole factor that results in greater growth or yield of a crop. In wheat, the relationship

558 between SPAD value and grain productivities was studied (Monostori *et al.*, 2016). They found

559 that other factors also affect the grain productivity, such as soil nitrogen content, cultivar and

560 environment. According to Kandel (2020), SPAD value and yield are not often collinearly related

561 as it may result from genetic variation, differential absorption strength in nitrogen, chlorophyll

562 biosynthesis and chlorophyll degradation.

563

564 **Conclusions**

565 To the best of our knowledge, this is the first report of polyploidization in *N. cadamba*. Our study

566 lays the foundation for producing octoploid and mixoploid plants of *N. cadamba* using an anti-

567 mitotic agent – colchicine. Both colchicine-induced polyploid plants are growing slower than

568 tetraploids under *in vitro* conditions. However, the leaves of octoploid plants have thicker leaf

569 blades, thicker midrib, lower stomata density and bigger stomata size. This indicates that octoploid

570 plants could respond better to environmental changes. These novel polyploid plants could be

571 beneficial for future genetic improvement of *N. cadamba*; for instance, octoploid could backcross

572 with tetraploid to produce a novel hexaploid plant (2n = 6x = 66) or interploid. Hence, the *in vitro*

573 colchicine-induced mutation could be a valuable breeding strategy of *N. cadamba* to produce

574 improved clones for planted forest development.

575

576

Acknowledgements

578 This study was supported by funding from the Sarawak Timber Association (STA) to Universiti

579 Malaysia Sarawak (UNIMAS). The Ministry of Education Malaysia is also acknowledged for

580 granting financial assistance to the first author. The authors would like to acknowledge WTK for

581 providing the plant materials for this research project.

582

Funding

584 The funding from the Sarawak Timber Association (STA) was awarded to the second author with

585 grant numbers GL(F07)/06/2013/STA-UNIMAS(06) and GL/F07/STA01/2019.

586

Competing Interests

588 The authors declare there are no competing interests.

589

Author Contributions

591 Eng, WH and Ho, WS designed the research project. Eng, WH performed the experiments and

592 analyzed data. Eng, WH and Ho, WS wrote the manuscript. Ho, WS and Ling, KH modified the

593 manuscript. All authors read and endorsed the final manuscript.

594

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Table 1 Polyploidization rate of nodal segments of *N. cadamba* using different colchicine concentrations and treatment durations.

Figures in parentheses represent the percentage of corresponding figures.

2
3
4 **Table 1** Polyploidization rate of nodal segments of *N. cadamba* using different colchicine concentrations and treatment durations.

Colchicine (%, w/v)	Treatment duration (h)	Number of explants	Number of surviving explants	Number of tetraploids (%)	Number of mixoploids (%)	Number of octoploids (%)
0.0	24	30	27 (90.0)	27 (90.0)	0 (0.0)	0 (0.0)
	48	30	20 (66.7)	20 (66.7)	0 (0.0)	0 (0.0)
0.1	24	30	18 (60.0)	16 (53.3)	2 (6.7)	0 (0.0)
	48	30	16 (53.3)	11 (36.7)	2 (6.7)	3 (10.0)
0.3	24	30	14 (46.7)	6 (20.0)	6 (20.0)	2 (6.7)
	48	30	12 (40.0)	4 (13.3)	2 (6.7)	6 (20.0)
0.5	24	30	8 (26.7)	2 (6.7)	1 (3.3)	5 (16.7)
	48	30	7 (23.3)	0 (0.0)	1 (3.3)	6 (20.0)

6 *Figures in parentheses represent the percentage of corresponding figures.*
7

Table 2 *In vitro* shoot regeneration, growth, rooting and acclimatization of tetraploids, mixoploids and octoploids of *N. cadamba*.

For each parameter, means with different letters indicate significantly different at 5% level of probability using Duncan's test (p<0.05).

2
3
4 **Table 2** *In vitro* shoot regeneration, growth, rooting and acclimatization of tetraploids, mixoploids
5 and octoploids of *N. cadamba*.

Morphological characteristics	Tetraploid	Mixoploid	Octoploid
Regeneration			
Number of shoots $\pm$ SE	5.4 ± 0.1^b	4.3 ± 0.2^a	4.2 ± 0.3^a
Regeneration (%) $\pm$ SE	94.4 ± 5.6^b	100.0 ± 0.0^b	83.3 ± 0.0^a
Growth			
Stem height (cm) $\pm$ SE	7.2 ± 0.0^c	6.8 ± 0.0^b	4.4 ± 0.4^a
Number of leaves $\pm$ SE	12.8 ± 0.2^a	12.9 ± 0.2^a	13.0 ± 0.3^a
Number of nodes $\pm$ SE	6.4 ± 0.1^a	6.4 ± 0.1^a	6.5 ± 0.0^a
Stem diameter (mm) $\pm$ SE	2.3 ± 0.1^a	2.7 ± 0.1^b	2.9 ± 0.1^b
Rooting			
Root length (cm) $\pm$ SE	1.8 ± 0.1^c	1.6 ± 0.0^b	1.4 ± 0.0^a
Number of roots $\pm$ SE	13.8 ± 0.9^b	16.1 ± 0.5^b	11.1 ± 0.7^a
Rooting (%) $\pm$ SE	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a
Acclimatization percentage			
Jiffy-7 $\pm$ SE	94.4 ± 5.6^b	94.4 ± 5.6^b	94.4 ± 5.6^b
Loamy soil $\pm$ SE	88.9 ± 5.6^b	94.4 ± 5.6^b	88.9 ± 5.6^b
Loamy soil : Compost 1:3 $\pm$ SE	50.0 ± 9.6^a	61.1 ± 5.6^a	66.7 ± 0.0^a

7   For each parameter, means with different letters indicate significantly different at 5% level of
8   probability using Duncan's test ($p<0.05$).
9
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Table 3 Morphological characteristics and SPAD values.

For each parameter, means with different letters indicate significantly different at 5% level of probability using Duncan's test (p<0.05).

4 **Table 3** Morphological characteristics and SPAD values.

Characteristics	Tetraploid	Mixoploid	Octoploid
Leaf thickness			
Leaf thickness (µm) ± SE	160.8 ± 38.5[a]	197.0 ± 8.7[ab]	247.2 ± 15.0[b]
Midrib vertical thickness (µm) ± SE	666.6 ± 25.0[a]	762.4 ± 38.1[ab]	831.7 ± 61.7[c]
Midrib horizontal thickness (µm) ± SE	616.2 ± 42.1[a]	793.0 ± 48.5[b]	899.1 ± 56.0[b]
Stomata and trichome			
Stomata density (mm^{-2}) ± SE	426.7 ± 9.4[b]	298.2 ± 6.3[a]	301.8 ± 10.9[a]
Trichome density (mm^{-2}) ± SE	33.9 ± 4.0[b]	4.9 ± 2.0[a]	4.9 ± 2.7[a]
Stomata length (µm) ± SE	25.3 ± 0.2[a]	29.3 ± 0.3[b]	30.7 ± 0.3[c]
Stomata width (µm) ± SE	14.9 ± 0.2[a]	16.1 ± 0.2[b]	16.6 ± 0.2[b]
Stem cross-section			
Bark (mm) ± SE	0.4 ± 0.1[a]	0.4 ± 0.1[a]	0.3 ± 0.0[a]
Wood (mm) ± SE	3.1 ± 0.3[a]	3.7 ± 0.3[a]	3.8 ± 0.3[a]
Pith (mm) ± SE	5.7 ± 0.3[b]	5.2 ± 0.1 [b]	3.6 ± 0.2[a]
SPAD value			
SPAD ± SE	37.1 ± 1.0[a]	37.2 ± 0.4[a]	39.7 ± 0.6[b]

5 For each parameter, means with different letters indicate significantly different at 5% level of
6 probability using Duncan's test (p<0.05).
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Figure 1

Figure 1 Polyploid plants detection using FCM and chromosome count of *N. cadamba*.

(A) FCM histogram for control or tetraploid plantlet (2n = 4x), (B) FCM histogram for mixoploid plantlet (2n + 4n = 4x + 8x), (C) FCM histogram for octoploid plantlet (4n = 8x), (D) Tetraploid cell with 44 chromosomes (Bar = 5 μm) and (E) Octoploid cell of *N. cadamba* "Kestamas-19" with 88 chromosomes (Bar = 5 μm).
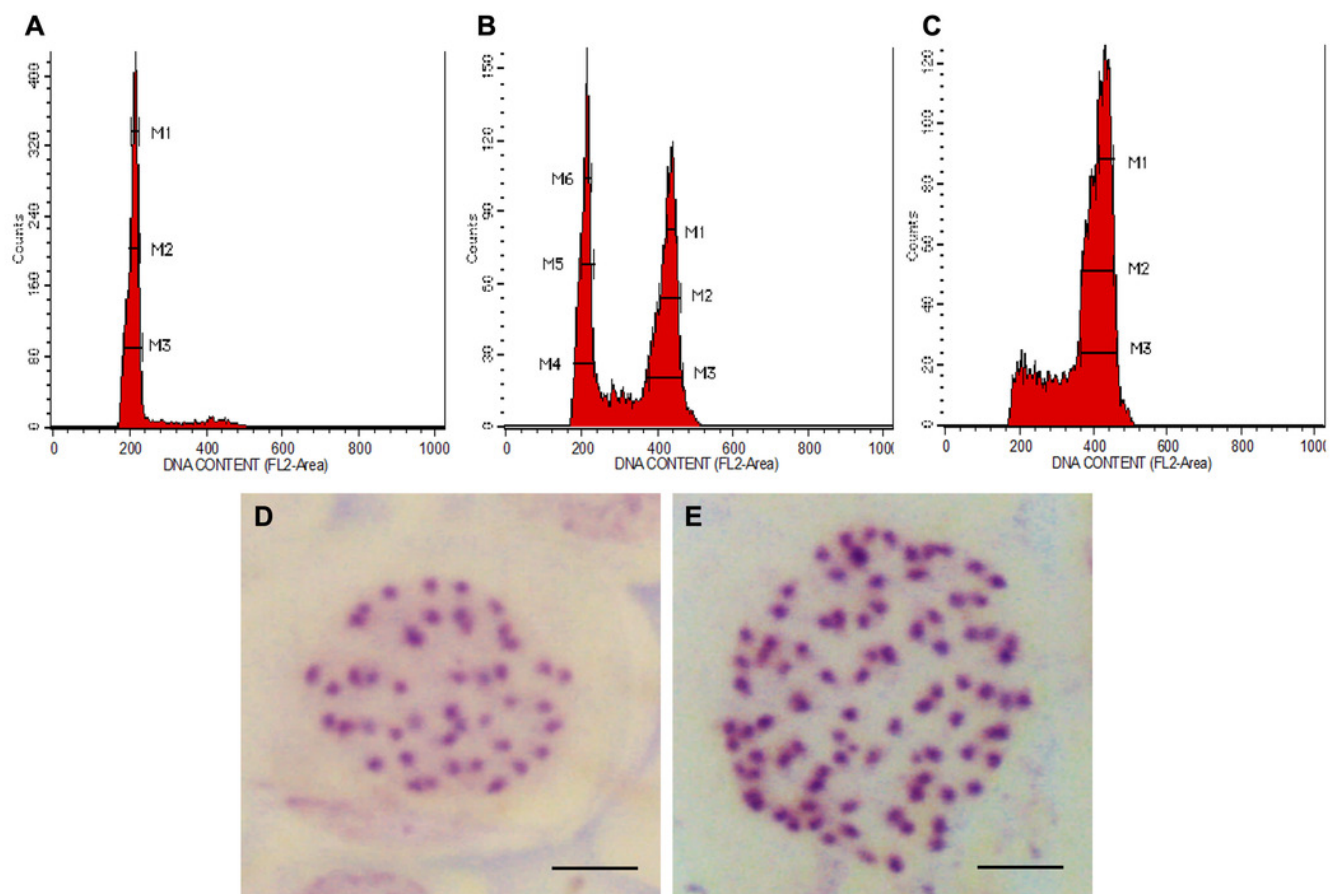
Figure 2

Figure 2 *In vitro* culture and acclimatization of *N. cadamba* polyploid plants.

Shoot regeneration of tetraploids (A), mixoploids (B) and octoploids (C). (D) Shoot tip growth of tetraploids (left), mixoploids (middle) and octoploids (right). Rooting of tetraploids (E), mixoploids (F) and octoploids (G). (H) Acclimatization of plantlets (T = Tetraploid, M = Mixoploid, O = Octoploid) on different media (J = Jiffy-7, S = Soil, C = Soil : Sompost 3 : 1), (Bar = 1 cm).
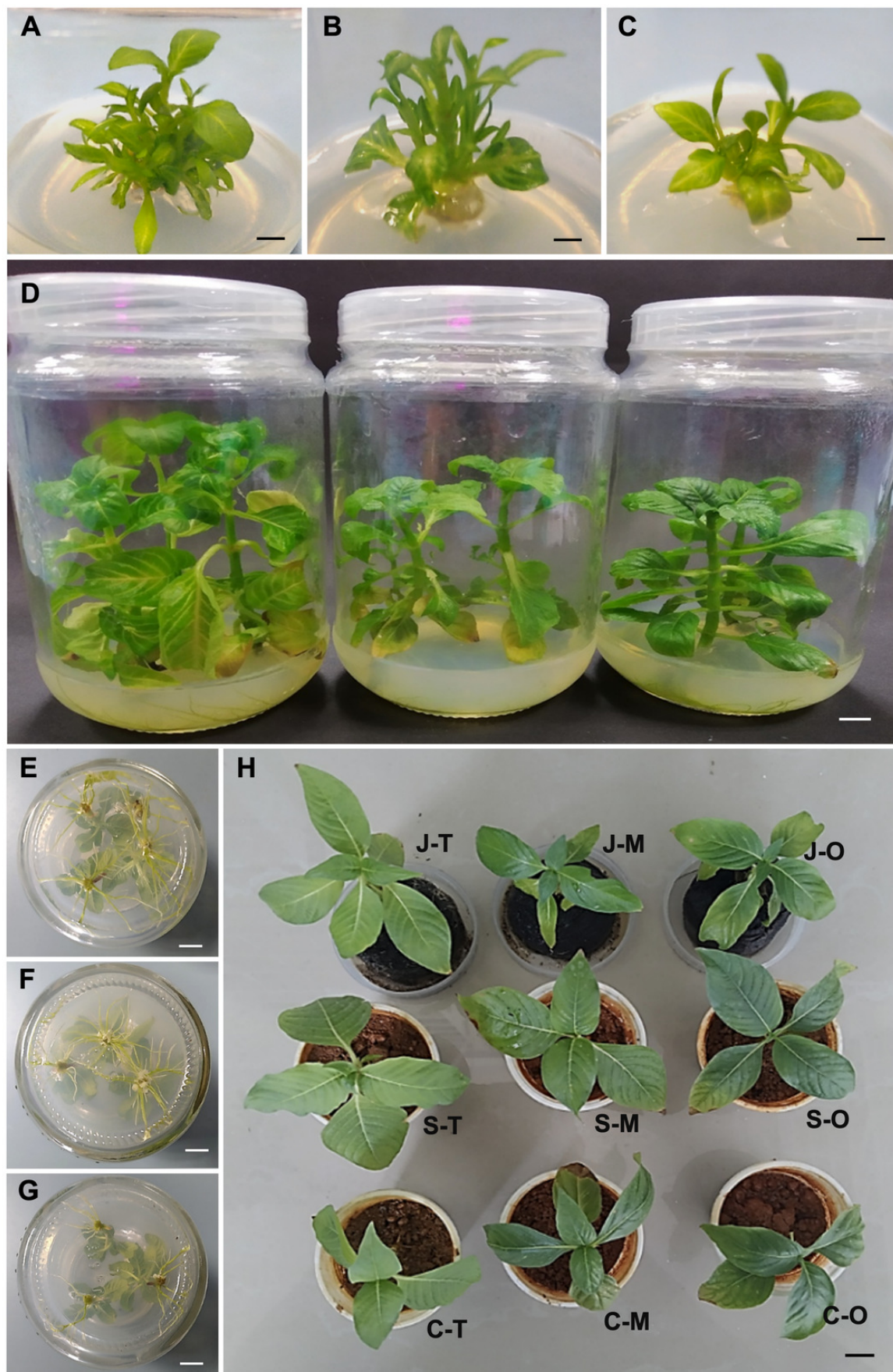
Figure 3

Figure 3 Morphological characteristics of *N. cadamba* polyploid plants.

Leaf sections of tetraploids (A), mixoploids (B) and octoploids (C). Stomata imprints of tetraploids (D), mixoploids (E) and octoploids (F). Stoma micrographs of tetraploids (G), mixoploids (H) and octoploids (I) under SEM. Trichome micrographs of tetraploids (J), mixoploids (K) and octoploids (L) under SEM. (M) Stem cross-section of tetraploids (left), mixoploids (middle) and octoploids (right) (b = bark; w = wood; p = pith). (T = Leaf thickness, V = Midrib vertical thickness, H = Midrib horizontal thickness) (Bar A-C = 0.5 mm) (Bar D-F = 0.05 mm).