

# 1       **Synthetic Seed Production as a Tool for the Conservation** 2               **and Domestication of *Celastrus paniculatus*; A Rare** 3                               **Medicinal Plant**

## 6       **ABSTRACT**

7       The black-oil tree (*Celastrus paniculatus* Willd) is a highly valued medicinal plant species  
8       belong to the Celastraceae family, known as Jyothishmathi in Ayurveda and Duhundu in Sri  
9       Lanka and grows as a perennial vine. It is an endangered medicinal plant species recorded  
10      in the red list of endangered fauna and flora of Sri Lanka in 1999. The seed oil of *Celastrus*  
11      *paniculatus* contains sesquiterpene alkaloids namely; celapagine, celapanigine, celapanine  
12      and celastrol, used in traditional system of medicine for various disorders and because of its  
13      high pharmaceutical value, plants are over exploited in natural habitats. Owing to poor seed  
14      germination and lack of successful vegetative propagation methods, domestication and  
15      commercial planting of this important medicinal plant species to meet the demand seems  
16      impossible. Therefore, it is of high importance to develop a reliable and efficient *in vitro*  
17      method to produce black oil plants for commercial use. In this study it was attempted to  
18      produce synthetic seeds of *Celestrus paniculatus* via *in vitro* multiple shoot proliferation.  
19      Nodal segment explants were collected from freshly emerged sprouts, surface sterilized and  
20      cultured in Murashige and Skoog medium supplemented with different BAP and TDZ  
21      concentrations for shoot induction. The highest soot proliferation rate; 25 shoot tips/explant  
22      was observed with 0.1 mg/L TDZ. Induced shoot tips were used for synthetic seed  
23      production after encapsulating with BAP and NAA enriched sodium alginate. Shoot tip  
24      encapsulated beads produced with 4% sodium alginate were firm, clear, round and uniform  
25      in size and easy to handle. The influence of growth regulators (BAP and NAA) and storage  
26      period on the germination of encapsulated shoot tips was studied to evaluate the success of  
27      encapsulated shoot tips as a propagule. The beads germinated with 2 mg/L BAP and 0.2  
28      mg/L NAA provided 80% *in vitro* germination percentage. Shoot tips of synthetic seeds  
29      remained green and healthy after storage at 5<sup>0</sup>C for a period of 8 weeks. Current findings  
30      suggest that encapsulated micro shoots (synthetic seeds) could be produced successfully,  
31      as the first step in domestication and conservation of *Celastrus paniculatus*. Further studies  
32      required on rooting of micro shoots, acclimatization and transferring of plantlets produced  
33      from synthetic seeds to *in vivo* conditions for domestication and conservation purposes.

34 *Key words: Celastrus paniculatus, conservation, germination, growth regulators, medicinal,*  
35 *synthetic seeds*

## 36 **1. INTRODUCTION**

37 The black-oil tree (*Celastrus paniculatus* Willd), locally known as Duhundu, Jyotishmati in  
38 India belongs to the Celastraceae family, is an endangered medicinal plant species recorded  
39 in the red list of endangered fauna and flora of Sri Lanka in 1999, as well as in Indian  
40 subcontinent (Martin et al., 2006; Raju and Prasad 2010). This plant is widely used in  
41 Ayurveda to cure many diseases such as leprosy, leucoderma, skin diseases, paralysis,  
42 depression, arthritis, asthma and fever (Sharma et al., 2001). The seed oil of *Celastrus*  
43 *paniculatus* Willd contain sesquiterpene alkaloids namely; celapagine, celapanigine,  
44 celapanine and celastrol, used in traditional system of medicine for various disorders. The  
45 plants exhibit varying degrees of therapeutic values, some of which are its use in the  
46 treatment of cognitive dysfunction, epilepsy, insomnia, rheumatism, gout, dyspepsia  
47 (Nadkarni et al., 1976).

48 Because of high pharmaceutical value and lack of domesticated or commercial plantings,  
49 plants in natural habitats are over exploited. Owing to poor seed germination and lack of  
50 successful vegetative propagation methods, mass propagation of this important medicinal  
51 plant species seems impossible. Therefore, it is of high importance to develop a reliable and  
52 efficient method for conservation and commercial cultivation of black oil tree (Arya et al.,  
53 2001).

54 Conventionally, *C. paniculatus* is propagated mainly through the seeds. However, the  
55 viability and germination of the seeds (11.5 %) are poor (Rekha et al., 2005). Thus,  
56 alternative approaches such as *in vitro* techniques are highly desirable for large-scale  
57 propagation of this medicinally important plant. Polymerization of sodium alginate in calcium  
58 chloride ( $\text{CaCl}_2$ ) resulted in the formation of hydrogel capsules with a somatic embryo (SE)  
59 or with propagules inside, called artificial seeds (ASs) or synthetic seeds. Artificial seed  
60 technology is an implementation of modern plant biotechnology, which offers tremendous  
61 potential in micropropagation as well as germplasm conservation of rare, endangered plants  
62 along with its easy handling and transportation (Gantait et al., 2015). In this backdrop,  
63 present study was conducted to explore the possibility of multiple shoot proliferation and  
64 synthetic seed production of *Celestrus paniculatus* with the aim of initiating commercial  
65 cultivation through domestication.

66

## 67 **2. MATERIAL AND METHODS**

68

69 2.1. Plant material and surface sterilization

70 Mother plants of *Celestrus paniculatus* is maintained at the Department of Crop Science,  
71 Faculty of Agriculture, University of Ruhuna, Sri Lanka under protected house conditions and  
72 nodal segment explants (2-4 cm) were collected from freshly emerged sprouts. Explants  
73 were washed thoroughly in running tap water and washed with detergent 'Teepol' for 5 min  
74 and surface sterilized with 10% Clorox for 10 minutes. The disinfected explants are washed  
75 (3-4 washes) with sterile double distilled water to remove traces of sterilent. Nodal segments  
76 (3-5 mm) were cultured in Murashige and Skoog (1962) (MS) medium supplemented with  
77 different BAP and TDZ concentrations for shoot induction.

## 78 2.2 Media preparation and culture condition

79 All the experiments were carried out on Murashige and Skoog basal medium containing 3.0  
80 % (w/v) sucrose as a carbon source and, 0.8% (w/v) agar for gelling was adjusted to 5.8 pH,  
81 and sterilized in an autoclave under 15 psi at 121°C. All the equipment and reagents were  
82 autoclaved at 121 °C and 15 psi for 15 minutes. The medium was supplemented with  
83 various concentrations and combinations of auxin [ $\alpha$ -naphthalene acetic (NAA) (0.1 and 0.2  
84 mg/L)], cytokinin [6-benzylaminopurine (BAP) (0.5, 1.0, 1.5 and 2.0 mg/L) and thidiazuron  
85 (TDZ) (0.1 and 0.2 mg/L)] for shoot proliferation of nodal segments. All cultures were sub  
86 cultured to their respective fresh medium at every 3-week intervals. For the encapsulation of  
87 nodal segments, the encapsulation medium (EM) was prepared by slight modification of MS  
88 basal medium. The EM is devoid of calcium salt and agar but fortified with 3.0 % sucrose  
89 and sodium alginate. For optimizing encapsulation sodium alginate solution was prepared in  
90 liquid Murashige and Skoog's (MS) medium at different weight is to volume concentrations  
91 (2-5%).The pH of the medium was adjusted to 5.6 prior addition of sodium alginate. For  
92 polymerization calcium chloride ( $\text{CaCl}_2$ ) solutions of  $100 \text{ mmolL}^{-1}$  strength was prepared in  
93 double distilled water. To test regeneration and germination of artificial seeds combinations  
94 of NAA and BAP were used. All cultures were incubated at  $25 \pm 2^\circ \text{C}$  with 16/8h photoperiod  
95 under white fluorescent light.

## 96 2.3 Shoot induction and Proliferation

97 All the surface sterilized explants (3-5 mm) were cultured MS medium supplemented with  
98 two different concentrations (0.1 and 0.2 mg/L) of NAA in combination with 6-BAP (0.5-2.0  
99 mg/L) and TDZ (0.1 and 0.2 mg/L) as shown in table 1. All the cultures were incubated  
100 under  $25 \pm 2^\circ \text{C}$  with 16/8h photoperiod under white fluorescent light. The percentage of shoot  
101 induction, number of shoots per explant were obtained after 12 days and shoot proliferation  
102 rate was recorded at weekly intervals for ten weeks.

103

## 104 2.4 Synthetic Seed Preparation

105

106 *In vitro* produced nodal segments [(4±1) mm long]] were drenched for 10 minutes with  
107 autoclaved sterilized 2%, 3%, 4% and 5% (w/v) Sodium alginate (NA) gelling matrix  
108 dissolved in calcium free 1/2 MS liquid medium. For development of artificial seeds, the  
109 aliquots (0.2 mL approx.) of NA solution, each containing single micro shoot (nodal  
110 segment), were taken aseptically by a Pasteur pipette (5 mm diameter) and softly dropped  
111 one by one in 100 mmolL<sup>-1</sup> autoclaved CaCl<sub>2</sub>·2H<sub>2</sub>O (CC) liquid. For optimum polymerization  
112 the droplets were kept for 30 minutes in CC with constant agitation to allow absolute  
113 polymerization. Polymerization can be measured apparently as the artificial seeds become  
114 translucent instantly next to the formation and then displays an opaque nature as the  
115 polymerization proceeds (Javed et al., 2017). The CC solution was then decanted and the  
116 artificial seeds were double washed with sterile water. Immediately after, the beads were  
117 surface dried by blotting on filter paper. The bead formation was compared after a  
118 complexion time of 30 min for all the treatments. The artificial seeds were kept under  
119 appropriate incubating conditions.

120

## 121 2.5 Culture Conditions and Synthetic seed germination

122

123 Encapsulated and non-encapsulated nodal segments were kept in petri dishes sealed with  
124 paraffin film and kept in three different temperature regimes ((5 ± 1) °C, (15 ± 1) °C, and (25  
125 ± 1) °C) without illumination. For storing at (5 ± 1) °C, tubes were kept in refrigerator, for (15  
126 ± 1) °C in an incubator whereas for (25 ± 1) °C the tubes were maintained in culture room.

127 These were tested for germination competence on regeneration media in culture tube, after  
128 every two weeks (up to 8 weeks) on ½ MS medium having 3% sucrose (w/v) supplemented  
129 with two different concentrations (0.1 and 0.2 mg/L) of NAA in combination with 6-BAP (1.0-  
130 5.0 mg/L) and were maintained for 6 weeks at 24 ± 2 °C and 55–65% relative humidity with  
131 16/8 h light and dark period, light was provided by cool fluorescent white lamps (Table 2).

132

133

## 134 2.5. Statistical assessment

135

136 Experimentations were replicated twice comprising 5 samples for each replication following  
137 completely randomized design. Data were statistically assessed through One way analysis  
138 of variance (ANOVA). Treatment data (Means ± SE) were evaluated based on Duncan's  
139 multiple range test (DMRT) (Duncan, 1955) (P ≤ 0.05) through SAS 9.4 software.

140

141

142 **3. RESULTS AND DISCUSSION**

143

144 3.1 *In vitro* shoot induction and proliferation

145 A simple and effective protocol was developed for the *in vitro* micro propagation of *Celestrus*  
 146 *paniculatus*. Immature nodal segments were cultured on MS medium containing different  
 147 concentrations of BAP and NAA to evaluate their effects on shoot initiation. Explants showed  
 148 varying success in shoot initiation depending on the growth regulators added. The response  
 149 of explants cultured in MS media supplemented with BAP, NAA and TDZ are shown in Table  
 150 1.

151

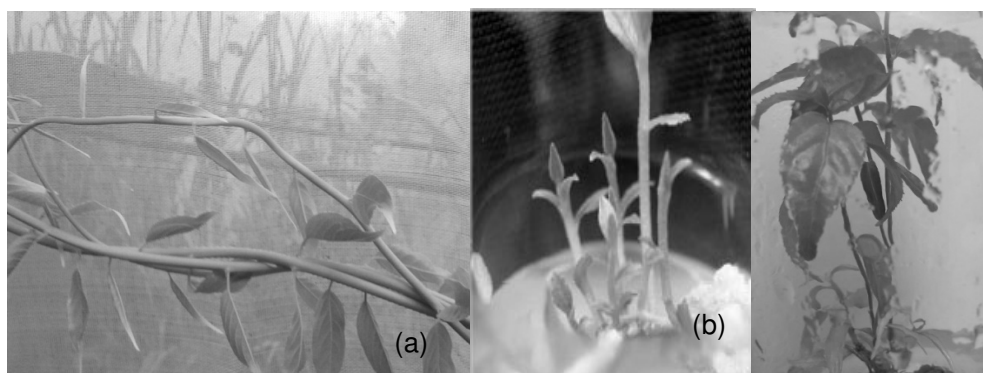
152 Table 1. Effect of plant growth regulators on shoot induction of explants (after 12 days) and  
 153 average no of shoots (after 9 weeks) of culture

Growth regulator concentration (mg/L)			Shoot induction (%)	Average number of shoots/explant
NAA	BAP	TDZ		
0.1	0.5	-	70.4	16.0 ± 0.02 <sup>g</sup>
0.1	1.0	-	76.2	17.2 ± 0.01 <sup>f</sup>
0.1	1.5	-	80.8	18.0 ± 0.04 <sup>e</sup>
0.1	2.0	-	97.8	25.2 ± 0.02 <sup>a</sup>
0.2	0.5	-	66.8	13.2 ± 0.04 <sup>h</sup>
0.2	1.0	-	82.4	20.6 ± 0.07 <sup>d</sup>
0.2	1.5	-	85.2	22.8 ± 0.02 <sup>c</sup>
0.2	2.0	-	85.2	20.8 ± 0.06 <sup>d</sup>
0.0	0.0	0.1	88.2	24.4 ± 0.06 <sup>b</sup>
0.0	0.0	0.2	92.4	25.0 ± 0.01 <sup>a</sup>

154 Means in each column followed by the same superscript letters are not significantly different  
 155 according to DMRT at  $P < 0.05$ .

156 Bud break was observed for the explants cultured on MS medium containing cytokinins  
 157 (BAP or TDZ) after 12 days. The increased level of BAP resulted in significantly higher shoot  
 158 initiation and the number of shoots at the initiation stage, with constant level of NAA.  
 159 Although bud break was dependent on BAP supply, the synergistic combination of BAP and

160 NAA induced the optimum frequency of shoot formation as well as shoot number. The  
161 highest rate of shoot induction (97.8%) which was significantly different from all other  
162 hormonal combinations used at 5% significant level and highest number of shoots per  
163 explant (25.2) were obtained in MS medium supplemented with 2.0 mg/l BAP + 0.1 mg/L  
164 NAA after nine weeks of nodal culture (Table 1). Therefore 9<sup>th</sup> week is the best period to get  
165 optimum number of shoots from nodal segments. Furthermore, Seyoum & Mekbib (2014)  
166 reported that the cotyledonary node explants of Yeheb (*Cordeauxia edulis*) cultured on MS  
167 medium supplemented with 2.0 mg/L BAP resulted in the highest rate of shoot initiation (89  
168 %) and the highest number of shoots per culture after nine weeks. When consider about the  
169 effect of TDZ, the highest soot proliferation rate; 25.0 shoot tips/ explant was observed with  
170 0.2 mg/L TDZ which was not significantly different with the hormonal combination 2.0 mg/L  
171 BAP + 1.0 mg/L NAA after nine weeks of nodal culture at 5% significant level. Nodal  
172 explants of *R. tetraphylla* cultured on MS + 5  $\mu$ M TDZ has given optimal (90%) regeneration  
173 response for maximum ( $9.2 \pm 1.20$ ) shoot production (Ahmad & Faisal 2018). Repeated  
174 transfer of the cultures was suggested as an efficient technique for rejuvenation and  
175 reinvigoration of *in vitro* cultures (Sanchez et al., 1997), which was further supported by  
176 subsequent reports on different plants (Phulwaria et al., 2012b). The importance of plant  
177 growth regulators on shoot propagation has been highlighted in various studies. Consistent  
178 with this result, Daneshvar et al. (2013) reported that 2.5 mg/L BAP + 0.15 mg/L NAA in MS  
179 medium produced the highest number of Aloe vera plantlets (up to 28.47 plantlets per  
180 explants).



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182

183 Figure 1. Stages of multiple shoot induction of *C. panicaultus*. (a) Mother pant (b) Multiple shoot  
184 induction (c) proliferated shoots

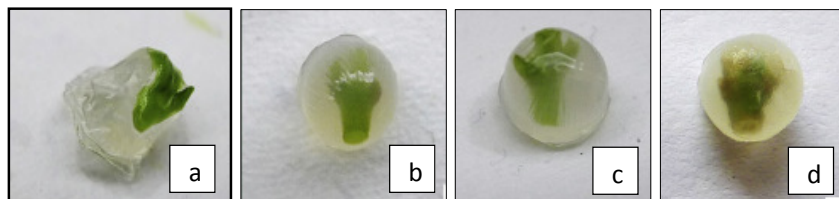
185

186 3.2 Effect of alginate and calcium chloride concentration on Bead formation

187

188 Formation of beads with appropriate texture is the key for producing effective synthetic  
189 seeds. The stability and hardness are main two factors affecting the effectiveness of these  
190 seeds, because very hard beads limit the germination ability while fragile beads limit the  
191 handling. NA was chosen for encapsulation because of its minimal toxicity to micro  
192 propagules and its rapid gelatinization. The hardness and firmness of the beads depends on  
193 the ion exchange of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  during complexing. Thus, to obtain effective synthetic  
194 seeds, concentrations of sodium alginate and calcium chloride and the complexing time  
195 needs to be standardized (Rai et al., 2009). The present investigation shows that the  
196 polymerizing ability of synthetic seeds varied markedly at different concentrations (2-5%) of  
197 NA when used to encapsulate the nodal segments. Out of the four different concentrations of  
198 NA (2–5 %), evaluated to develop an encapsulation matrix most appropriate for short term  
199 storage and maximum regeneration.

200



201

202

203 Figure 2. Influence of  $\text{CaCl}_2$  and Na-alginate on shape and consistency of artificial seeds  
204 (encapsulated *in vitro*-derived nodal segments) of *Celastrus paniculatus* Willd. a: with 2% NA; b: with  
205 3% NA; c: 4% NA; d: with 5% NA

206 Lower concentrations resulted in weak structures with no definite shape and disintegrated  
207 while handling. Very firm, clear, isodiametric beads of viable, uniform size and shape, were  
208 obtained at and above the concentration of 3% NA upon complexation with  $100 \text{ mmolL}^{-1}$  CC.  
209 It was reported by several researchers that 3 % sodium alginate with  $100 \text{ mmolL}^{-1}$  CC was  
210 the most ideal combination for synthetic seed production (Sharma et al. 2013). The beads  
211 produced with 4% NA concentration were firm, clear, round and uniform in size and easy to  
212 handle. (Figure 2.C). The results obtained are in agreement with earlier reports in other  
213 species (Javed et al., 2017) & (Gantait et al., 2017).

214

### 215 3.2. Regeneration and Germination of Synthetic Seeds

216

217 Synthetic seeds developed using 2, 3, 4, and 5% NA were tested for their regenerating  
218 ability on the regeneration medium as mentioned above. Maximum regeneration was  
219 recorded in synthetic seeds developed using 4% NA, while the regeneration was significantly

220 less in seeds encapsulated with 5% NA solutions respectively. Similar observations have  
 221 also been reported by (Alatar et al., 2017). In our observations, levels on NA lower or higher  
 222 than 4% resulted in either too pliable or too firm artificial seeds or subsequent reduced  
 223 conversion frequencies. The combination of BAP (3.0 mg/L) and NAA (0.1 mg/L and 0.2  
 224 mg/L) produced maximum regeneration percentage and maximum number of shoots (Table  
 225 2). However, only shoots immersed and rooting was not observed.

226

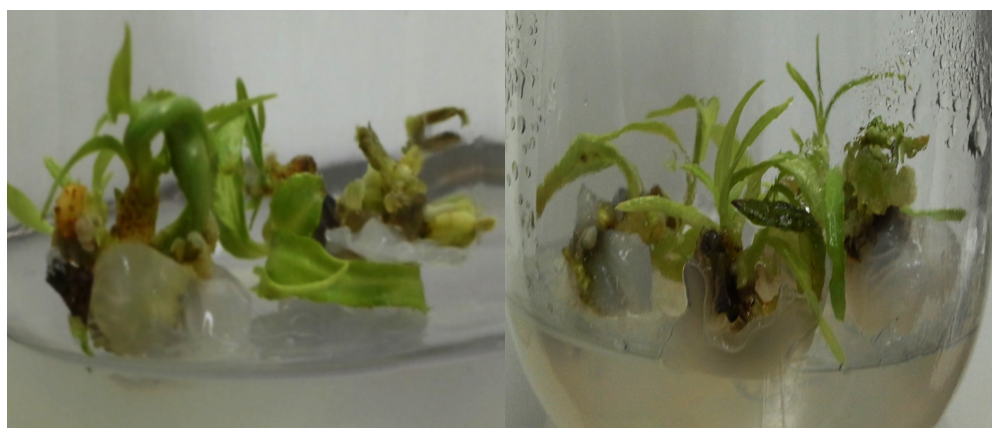
227 Table 2. Germination percentage and number of shoots produced from synthetic seeds  
 228 produced from *Celastrus paniculatus* Willd

NAA (mg/L)	BAP (mg/L)	Germination Percentage	Shoot No.
0.1	0.5	85.57± 0.59 <sup>de</sup>	1.26± 0.01 <sup>e</sup>
0.1	1.0	87.83± 1.00 <sup>cd</sup>	1.86± 0.05 <sup>c</sup>
0.1	1.5	89.46± 0.69 <sup>bc</sup>	1.48± 0.02 <sup>d</sup>
0.1	2.0	94.43± 0.74 <sup>a</sup>	2.38± 0.10 <sup>a</sup>
0.1	2.5	85.00± 1.00 <sup>e</sup>	1.13± 0.06 <sup>ef</sup>
0.1	3.0	71.18± 1.04 <sup>h</sup>	0.99± 0.01 <sup>f</sup>
0.2	0.5	74.13± 0.42 <sup>g</sup>	1.20± 0.05 <sup>e</sup>
0.2	1.0	80.53± 0.57 <sup>f</sup>	1.25± 0.02 <sup>e</sup>
0.2	1.5	68.57± 0.39 <sup>i</sup>	1.00± 0.02 <sup>f</sup>
0.2	2.0	62.08± 1.01 <sup>j</sup>	0.97± 0.01 <sup>f</sup>
0.2	2.5	89.27± 1.10 <sup>c</sup>	1.29± 0.01 <sup>e</sup>
0.2	3.0	91.87± 0.90 <sup>b</sup>	2.20± 0.1 <sup>b</sup>

229 Means in each column followed by the same superscript letters are not significantly different  
 230 according to DMRT at  $P < 0.05$ .

231

232 BAP has been documented as a one of the most potent cytokinins among others (Arab et  
 233 al., 2014), effectively induced organogenesis in several species like *Euphorbia cotinifolia*  
 234 (Perveen et al., 2013). Combination of BAP with different auxins evoked different response  
 235 from the tissues, NAA assisted in enhancing adventitious shoots. The difference in response  
 236 could be attributed to different levels of NAA has been frequently reported to augment  
 237 adventitious shoot generation along with cytokinins.



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239

240 Figure 3. *In vitro* germination percentage of *Celestrus paniculatus* synthetic seeds.

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## 242 3.4 Effect of encapsulation on storage time

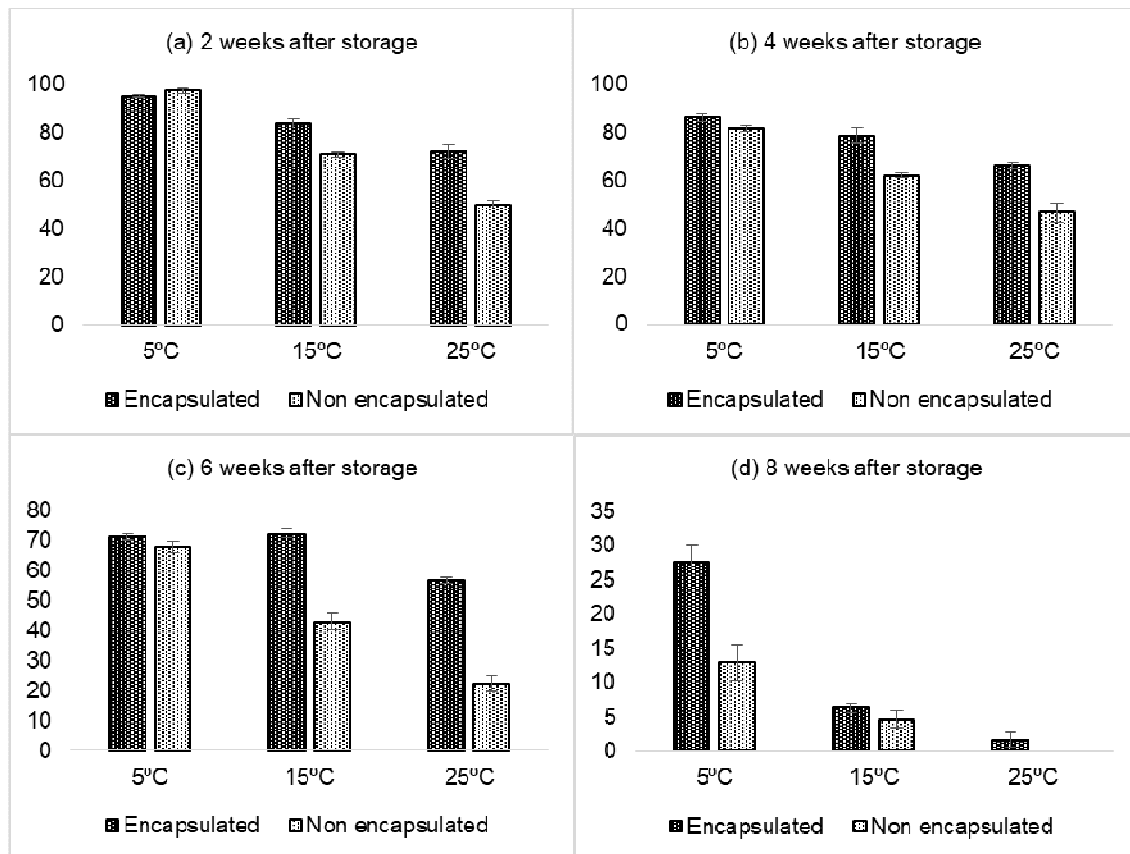
243

244 One of the most important objectives of synthetic seed formation is to increase the storability  
245 of the propagules. Therefore, the regeneration ability of the somatic tissues of *Celestrus*  
246 *paniculatus* stored in encapsulated and non-encapsulated forms at different storage  
247 temperatures and storage periods were tested. It was evident from the Figure 4, that the  
248 conversion of artificial seeds after each every two weeks (up to 8 weeks) of storage duration  
249 at temperatures ( $5 \pm 1$ ) °C, ( $15 \pm 1$ ) °C and ( $25 \pm 1$ ) °C varied markedly relying on the  
250 storage environment. Encapsulation was found very effective in maintaining the regeneration  
251 potential of the tissue. The regeneration potential remained unaffected up to 4 weeks of  
252 storage in  $5 \pm 1$ °C with 86% of germination frequency, beyond which a decrease in  
253 conversion rate was, recorded (Figure 4). However, even after 8 weeks of cold storage at  $5$   
254  $\pm 1$ °C, 27.6% of synthetic seeds showed regeneration while non-encapsulation nodal  
255 segments could not produce any shoots. Similar observations were obtained for the artificial  
256 seeds of *Erythrina variegata* which could be stored up to 8 weeks without lowering the  
257 germination ability(Javed et al., 2017). There are reports supporting higher conversion  
258 efficiency of synthetic seeds when stored at  $5 \pm 1$ °C. Ikhlaq et al., (2010) reported a  
259 promising degree of interaction between cold storage at  $5 \pm 1$ °C and storage up to 45 days  
260 for regrowth percentage as well as root and shoot development in olive. After 8 weeks of  
261 storage browning of seeds were observed and did not observe any germination. It was  
262 observed that the longer storage drastically decreases the conversion frequency, regardless  
263 of stored temperature, mainly might be due to the continuation of metabolic events in  
264 encapsulated micro shoots and accumulation of secondary metabolites in the matrix causing  
265 browning there by limiting the storage potential of the artificial seeds. Encapsulation matrix

266 reduces the dehydration of the tissue and limits oxidation of phenolic compounds, thus,  
 267 reducing cell death and sustaining the regeneration potential of the tissue up to a longer  
 268 duration than the non-encapsulated tissues. Our results agreed the findings of Alatar et al.,  
 269 (2016).

270

271 Figure 4. Conversion responses of encapsulated and non-encapsulated nodal segments of  
 272 *Celastrus paniculatus* Willd at two weeks intervals



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274

#### 275 4. CONCLUSION

276 Current findings suggested that nodal segments cultured on MS medium with 0.1 mg/L TDZ  
 277 showed highest multiple shoot induction in *Celastrus paniculatus*. Firm, round shaped and  
 278 easy to handle beads produced with 4% Na Alginate with 100 mmolL<sup>-1</sup> CC. Shoot tips of  
 279 synthetic seeds remained green and healthy after storage at 5°C for a period of 8 weeks.  
 280 Current findings suggest that encapsulated micro shoots (synthetic seeds) could be  
 281 produced successfully, as the first step in domestication and conservation of *Celastrus*  
 282 *paniculatus*. Further studies required on rooting of micro shoots and acclimatization of  
 283 plantlets produced from synthetic seeds to *in vivo* conditions for domestication and  
 284 conservation purposes.

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