Overcoming sexual sterility in conservation of endangered species: the prominent role of biotechnology in the multiplication of *Zelkova sicula* (Ulmaceae), a relict tree at the brink of extinction

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Abstract

Biotechnology provides valuable tools to support conservation of plant species, especially in case of threatened taxa or when dealing with seed unavailability, low viability or sterility. However, plant cell culture methods have often to face problems associated with tissue recalcitrance to *in-vitro* systems. Recalcitrance can be related to a variety of triggering factors, involving many efforts and manipulations within one or more of the micropropagation stages before obtaining successful results.

An *in-vitro* propagation protocol was developed for *Zelkova sicula*, a very rare and endangered relict tree, endemic to Sicily (Southern Italy). The species revealed extremely recalcitrant to *in-vitro* culture approaches, but after many trials throughout a number of years an effective micropropagation protocol was completed. The rooting rate was about 84% of the treated explants, 8% of which were successfully acclimatized outdoor and reintroduced in the wild within a comprehensive conservation project.

The technique allowed to overcome the problems of sexual sterility of this species, hence contributing concretely to contrast the problems connected with its conservation. However, additional efforts need to be carried out in order to refine the acclimatization step and further improve the whole process effectiveness.

Keywords

Axillary buds, clonal species, *ex vitro* acclimatization, *in vitro* propagation, recalcitrance, threatened species

53 **1. Introduction**

54

55 Biotechnological techniques are essential in plant preservation programs in order to provide a
56 complementary approach to the traditional *in situ* and *ex situ* conservation strategies (Krishnan et al.
57 2011; Reed et al. 2011). Furthermore, the importance of *in vitro* culture of plant tissues has notably
58 increased in recent years, especially for the conservation of endemic, rare and threatened species
59 (Hummer, 1999; Sarasan et al. 2006; Mallón et al. 2010; Bunn et al. 2011; San José et al. 2017),
60 since it offers many advantages with respect to traditional methods, e.g.: i) no need for repeated
61 collecting of plant material from living trees in the field, ii) potential production of endless amounts
62 of plantlets from very small quantities of parental plant tissue, iii) theoretically not strict
63 dependence on season cycles, and iv) relatively rapid production of new plants, that may be difficult
64 to obtain using traditional approaches (Bayraktar et al. 2015). Accordingly, *in vitro* methods have a
65 major implication when seeds are unavailable, sterile or non-viable and/or when *in vivo* cuttings
66 have poor rootability (Fay, 1992; Hummer, 1999; Reed et al. 2011).
67 However, a number of issues mostly relying on the lack of specific knowledge about the optimal
68 conditions for *in vitro* growth can jeopardize the effectiveness of this method, giving rise to
69 problems of recalcitrance in many taxa. *In vitro* species recalcitrance is the inability of plants to
70 respond to tissue culture, often with genetic connotation (McCown, 2000) or depending on various
71 factors related for instance to plant physiology and/or *in vitro* culture stresses (Benson, 2000).
72 Recalcitrance in shoot formation as well as in root onset may be associated with problems such as
73 endogenous contamination (e.g. by endophytic bacteria or fungi), hyperhydricity or lethal tissue
74 browning (Keskitalo, 1999). Hence, the success could be strongly committed, and this could
75 represent a troubling issue especially when dealing with threatened taxa, for which the source
76 material is often very scarce and/or located in remote areas in the wild (Sarasan et al. 2006). In
77 addition, the stabilization stage of a shoot culture system, with uniform and continuous *in vitro*
78 shoot-growth, is usually most challenging in long-lived perennial woody plants (McCown, 2000).
79 As a result, the *in vitro* culture of such taxa may only be successful after a relatively long period of
80 in *vitro* 'domestication' (Keskitalo, 1999).
81 The relict tree *Zelkova sicula* Di Pasquale, Garfì and Quézel (Ulmaceae) is a very rare threatened
82 species, endemic to South-eastern Sicily (Italy). At present it is known to consist of only two single
83 populations restricted to the northern slopes of the Iblei Mountains, province of Siracusa, each
84 including only a few hundred individuals. One population, named ZS1, is located at Bosco Pisano
85 (Municipality of Buccheri); the other one, ZS2, lies in the countryside of Ciranna (Municipality of
86 Melilli) (Garfì et al. 2011; Garfì et al. 2017). Since no intra-population genetic variability coupled

87 to strong differences between the two populations has been detected (Christe et al. 2014), it is very
88 likely that each population is clonal, issuing from probably centuries-long sprouting of two single
89 surviving genetic individuals (Garfì and Buord, 2012; Gratzfeld et al. 2015). This condition is
90 consistent with the sexual sterility of this tree, probably related to the triploid karyotype (Garfì,
91 1997) and ascertained by unsuccessful natural regeneration and germination tests (authors' personal
92 observations).
93 According to a number of criteria, *Z. sicula* is ranked in the IUCN (International Union for
94 Conservation of Nature) Red List as Critically Endangered (Garfì et al. 2017). The main threatening
95 factors, besides the ineffective sexual reproduction, include environmental constraints (e.g. summer
96 water stress) and direct and indirect human disturbances, such as wildfires and climate change. The
97 latter is among the most critical ones since in the recent decades the current refugial area reveal to
98 be more and more unsuitable to the persistence of the species, whose diffusion in new locations
99 hence appears as the last chance to secure its future survival in the wild (Alkemade et al. 2009;
100 Brooker et al. 2011).
101 Based on these topics, *in vitro* propagation techniques become of crucial importance for the
102 conservation of such a troubled species. In order to design an effective protocol, we have carried out
103 many trials in the last years, differing by the collecting period of plant material throughout the year,
104 the culture medium, the type and concentration of growth regulators, the type of plant tissues
105 (woody or green apical explants, origin from branch shoots or root suckers, leaf explants, flower
106 buds). Such a huge effort impacted against the extreme recalcitrance of *Z. sicula* and on the whole
107 at least 24 diverse treatments (3 for sterilization, 14 for regeneration and 7 for rooting, including not
108 less than 4 repeated cycles each) had to be tested before successfully completing the entire
109 propagation system, from *in vitro* introduction to acclimatization outdoor.
110 In the present paper, we illustrate the results of the most effective protocol that, though still needing
111 to be refined in the final acclimatization step, nevertheless allowed obtaining for the first time some
112 new plantlets of *Z. sicula* to use within *in situ* and *ex situ* conservation programs.
113
114
115 **2. Material and methods**
116
117 *2.1 Plant material and axenic* culture establishment
118 Woody explants of *Z. sicula,* 10-15 cm long, were harvested from both known populations ZS1 and
119 ZS2. The collection was carried out on December 2014 since according to a number of preliminary
120 trials winter season revealed as the best period with regard to the low rate of explant contamination.

In the laboratory, explants were cut into nodal segments about 3.0-3.5 cm in length, rinsed two times in distilled water added with two drops of Tween 20 for 5 min, and finally dipped three times in sterile distilled water for 5 min.

After many adjusting trials that allowed overcoming the problems of endophytic fungi and/or bacterial contamination the following sterilization procedure was elaborated. Shoot segments were disinfected under laminar flow with ethanol 70% for 5 min, and then rinsed once with sterile distilled water for 5 min. Next, explants were soaked in a 0.05% solution of $HgCl_2$ for 10 min, followed by three rinses with sterile distilled water for 5 min each. After sterilization, explants were dipped in 2% Plant Preservative Mixture (PPM) over night and kept at room temperature under constant magnetic shaking.

Based on the percentage of contaminations, that differed according to the population of origin, for the next experimental steps we decided to use only plant material coming from ZS2 which showed a significantly lower microbial contamination rate compared with ZS1.

2.2. Media and culture conditions

For axenic culture establishment, shoot multiplication, plant development and rooting we used Petri dishes 10 cm in diameter by 2.5 cm in height and 25 ml capacity, sealed with Parafilm MTM.

Explants were incubated in Woody Plant Medium (WPM, Lloyd and McCown, 1980) solidified substrate (7 g/L Plantagar S1000, B&V, Italy), with 30 g/L sucrose as carbon source. The pH of the media was adjusted to 5.7 ± 0.1 with 0.5 M KOH before autoclaving at 121 °C and 1 atm for 20 minutes.

PPM was supplemented to the medium at various percentages, according to the different steps: 0.2% in the *in-vitro* introduction step, 0.1% in the multiplication step and for some rooting treatments.

For the *in-vitro* introduction step, woody explants were cultivated on WPM medium without growth regulators. For the multiplication the following plant growth regulators (PGR), filter-sterilized through a 0.22 µm nylon filter after autoclaving, were added to the medium: 6-benzylaminopurine (BAP, Sigma B-4308), zeatin (ZEA, Sigma Z0163), N-(2-Chloro-4-pyridyl)-N'-phenylurea (4-CPPU, Sigma C-2791) and thidiazuron (TDZ, Sigma P-6186), whereas for rooting we used indol-3-butyric acid (IBA, Sigma I-5386) and 3-indoleacetic acid (IAA, Sigma I-2886).

Explants were maintained in a climate chamber at 25±1 °C under a 16 h day length, and a photosynthetic photon flux of 50 µmol $m^{-2}s^{1}$ provided by Osram cool-white 18 W fluorescent lamps.

155

156 *2.3. Shoot multiplication*

157 After about 4 weeks from culture establishment, two-node stem segments, approximately 2 cm in
158 length, were excised from initial woody explants and subcultured for multiplication under different
159 combinations of cytokinins of two different classes: the N6-substituted adenine derivative BAP and
160 ZEA, and the two synthetic phenylurea derivatives 4-CPPU and TDZ. Four hormonal combinations
161 were compared (Table 1): 2.2 µM BAP (treatment A), 8.0 µM 4-CPPU + 2.3 µM TDZ (treatment
162 B), 1.2 µM ZEA (treatment C) and 2.3 µM ZEA (treatment D).
163 Explants were subcultured at 30-days intervals and the effect of each treatment was checked 90
164 days after culture initiation by recording the percentage of responsive explants, the number of new
165 shoots per explant, and the average length of newly regenerated shoots.
166

167 *2.4. Plant rooting*

168 Actively growing bi-nodal shoots, obtained from the most performing treatment of the
169 multiplication step (treatment D, ZEA 2.3 µM), were used for rooting. Individual shoots were cut 4
170 weeks after culture initiation and cultured under different rooting treatments.
171 Initially, during preliminary tests (see Table 2), explants were plated on WPM medium
172 supplemented with two different rooting PGRs, IAA and IBA at either 5 µM or 10 µM, in light
173 conditions (treatments E, F, G, H). Moreover, to test the light effect and the influence of continuous
174 presence of IBA, an additional group of shoots was cultured with IBA at 5 or 10 µM for 6 days in
175 the dark and then transferred to growth regulator free (GRF) medium in the light (treatments I, J).
176 Results addressed the following experiments on discarding IBA and using only IAA (treatments E
177 and F), which provided the best performance in term of global quality of rooting. According to that,
178 six different treatments on root formation were newly tested (Table 3). Explants were plated on
179 WPM supplemented with IAA at 5 or 10 µM, with and without addition of 0.1% PPM (treatments
180 E, F, E1, F1). Two additional groups of explants were cultured for 7 days on WPM only
181 supplemented with 0.1% PPM; next, they were transferred on WPM added with IAA at 5 or 10 µM
182 and 0.1% PPM (treatments E2, F2). Cultures were incubated under the same light conditions
183 described above.
184

185 *2.5. Plant acclimatization*

186 Plantlets, 3-5 cm tall and with well-developed roots, were moved from the rooting medium to
187 acclimatization, including a preliminary phase under controlled conditions (pre-acclimatization).
188 The roots were first washed with distilled water to remove agar residues. The plantlets were then

189 transferred into 10-cm diameter crystal-clear polypropylene pots equipped with a "breathing"
190 hermetic cover (vitro-vent pots), containing a 1:1 peat:loam sterilized mixture. The potted plants
191 were placed in a climate chamber at 18 ± 1 °C under a 12 h day length, and a photosynthetic photon
192 flux of 50 µmol m^{-2} s^{-1} provided by Osram cool-white 18 W fluorescent lamps.
193 After 8 weeks under these conditions, the pot cap was replaced by a transparent polyethylene bag,
194 which was gradually perforated. During the next two weeks, the plants were ventilated by removing
195 the bags for one hour once a week. Plants were watered as needed with diluted (1:10) WPM salts
196 supplemented with 1.2 µM ZEA. The acclimation bags were definitively removed after 4 weeks and
197 the plants were transferred outdoor under natural daylight conditions, but sheltered by a shadowing
198 net for the final acclimatization. The survival rates were recorded after 2 months.
199 Most of the acclimatized plants were reintroduced in the wild during the autumn seasons 2016 and
200 2017.
201
202 *2.6. Data analysis*
203 *In vitro* experiments were carried out in a completely randomized design and the data were
204 evaluated by analysis of variance. Differences within and between treatments were estimated by
205 average separation analysis, using the least significant difference test (LSD) with the significance
206 level set at 0.05%. Each treatment for shoot multiplication and rooting consisted of 50 uniform
207 explants equally divided into Petri dishes.
208 The percentage of responsive explants was first considered to evaluate the effects of the different
209 media. For statistical analysis, the following additional parameters were taken into account: length
210 of shoots and number of shoots per explant.
211 Response to rooting was assessed one month after the beginning of the rooting phase, and the
212 following parameters were considered: rooting percentage rate, mean root number per explant and
213 mean root length.
214 The percentage of successfully acclimatized plants was recorded two months after transplanting.
215 Prior to analysis, percentage data were arcsin-square root transformed. Statistical analysis was
216 performed using SigmaStat 3.5 for Windows.
217
218 **3. Results**
219 *3.1. Shoot multiplication*
220 During the phase of *in vitro* culture establishment (Fig. 1A), shoot regeneration generally started
221 within 15 days after culture initiation and the new shoots grew 2.8 to 4.0 cm in 4 weeks. (Fig. 1B).

222 Multiple shoot regeneration (Fig. 1C, D) was obtained under all tested treatments, but with variable
223 response according to the hormonal composition (Table 4). The percentage of responsive explants
224 ranged from 13.9% (treatment B) to 40% (treatment D), and the latter was significantly different
225 from all the others. The average number of new shoots per responsive explant varied from 1.5
226 (treatments A, B, C) to 2.1 (treatment D), but with no significant differences among treatments.
227 Finally, the best result in term of mean shoot length (2.8 cm) was obtained with explants cultured in
228 presence of 2.3 μM ZEA (treatment D), and the value was significantly different from all the others.
229
230 *3.2. Plant rooting and acclimatization*
231 On actively healthy growing explants obtained by using ZEA 2.3 μM, roots began to emerge
232 generally 10 days after culture initiation (Fig. 1E) under all tested combinations, with significant
233 differences among treatments.
234 Results of the preliminary tests, including the growth regulators IBA or IAA at various
235 concentrations and daylight conditions (Table 2), showed that the percentages of rooted shoots
236 ranged from 5% to 37.1% (treatments G and E, respectively) (Table 5). However, in terms of
237 rooting rate there had not been significant differences among treatments, whereas root elongation
238 had been significantly better (25%) in explants cultivated in IBA 10 μM for six days in the dark,
239 and then transferred in the light in WPM GRF medium (treatment J). Nevertheless, in the whole the
240 micro-cuttings cultivated under all IBA combinations produced a callus mass at the cut surface, that
241 is usually unsuitable for subsequent root development and plantlets growth. This is the reason why
242 any further attempts involving the use of IBA was discarded and only IAA treatments in various
243 conditions and combinations, as showed in Table 3, were preferred to refine the definitive rooting
244 protocol.
245 The percentages of rooted plants obtained from the new six IAA-based treatments (Table 6) ranged
246 from 37.5% to 65.4% (treatments F and E2, respectively). The highest root number per explant (2.1)
247 was instead obtained with treatment F2, but it was not significantly different as compared to most of
248 treatments. With regard to root elongation, the best performance was achieved with treatment E (3.6
249 cm), though the value did not differ statistically from the treatments E2 and F. Since in E the
250 percentage of rooted plants (42.5%) was not fully satisfactory, we retained as the best performing
251 (65.4%) the treatment E2, based on explants cultured for 7 days in WPM medium with 0.1% PPM
252 and then transferred in WPM supplemented with 5 μM IAA and 0.1% PPM.
253 All the rooted plantlets obtained from the different rooting trials (Tables 5 and 6) were moved as a
254 whole to the pre-acclimatization (Fig. 1F) and acclimatization (Fig. 1G) phases and, from the total

255 of 600 treated explants, we achieved the following results: rooted 202 (33.7%), pre-acclimatized
256 182 (30.3%), acclimatized 26 (4.3%).
257 Later, based exclusively on the most effective treatments selected for both shoot multiplication and
258 rooting steps (protocols D and E2, respectively), in 2016 we performed new complete cycles that
259 significantly improved both the rooting and pre-acclimatization rates. From 162 treated explants we
260 obtained 111 (68.5%) rooted plants, out of which 58 (35.8%) survived to pre-acclimatization and 13
261 (8.0%) successfully acclimatized. In 2017, after having entirely renewed the stock of plant material
262 and concentrated the activities in the assumed most suitable season for *in vitro* rooting (spring), we
263 carried out five additional trials including a total of 139 explants. The success rate further increased
264 in rooting and pre-acclimatization steps (84.2 and 69.8%, respectively) but remained almost
265 unchanged for the final amount of acclimatized plantlets (7.9%) (Fig. 1H).
266 The total stock of 51 acclimatized plants produced until 2016 were introduced in the wild (Fig. 1H)
267 in two plantation campaigns in 2016 and 2017, respectively. At the end of 2018 only two plants
268 died, with the exceptional survival rate of 96.1%.
269
270
271 **4. Discussion**
272
273 *4.1. Recalcitrance: a major hurdle*
274 Designing an effective micropropagation protocol for *Z. sicula* was a very challenging task and
275 involved a great effort that lasted several years. Finally the entire procedure was crowned with
276 success, but the required numerous trials, especially in the shoot multiplication and rooting steps,
277 highlighted the extreme recalcitrance of this species to both propagation and rooting. Hence, the
278 protocol we described represents the very first complete scheme to obtain new plantlets of such a
279 rare plant by tissue cultures.
280 Recalcitrance is a rather common hurdle *in vitro* culture of perennial crops and, within woody
281 plants, taxa characterized by seasonal shoot growth like *Z. sicula* are usually even more
282 problematic. Most often, recalcitrance is genetically driven (McCown, 2000), but as reported for
283 many species (Duhem et al. 1988; Cassells et al. 1988; Onay, 2000; Carimi and De Pasquale, 2003;
284 Bunn et al. 2011), major difficulties may also arise owed to bacterial or fungal contaminants that
285 hamper the initiation of axenic cultures. Fungal contamination during the *in-vitro* introduction step
286 was relatively difficult to be overcome in *Z. sicula*. Different species of endophytic fungi, such as
287 *Botryosphaeria* spp., *Neofusicoccum ribis* and *Diaporthe neotheicola* were already known in this
288 plant (Granata et al. 2002; Campo et al. 2018). Despite it is generally recognized a positive

289 interaction between endophytes and host plants (Schulz and Boyle, 2005), recent investigations on
290 the Sicilian *Zelkova* (Campo et al. 2018) reported that the identified fungal species usually live in a
291 latent state in plant tissues, but under stress conditions (e.g. drought stress) they can become
292 responsible of several diseases by inducing bark canker on branches and stems. According to that,
293 *in-vitro* techniques can have the additional value to yield plants with a lower or null presence of
294 potential parasitic endophytes, allowing a higher success of reintroduction efforts in the wild.
295 As a matter of fact, temperate plants show a clear seasonal pattern in their response to tissue culture;
296 as a consequence, the explants have to be collected in the most suitable time of the year (Benson,
297 2000; Kartsonas and Papafotiou, 2007). Commonly, to avoid loss of material due to culture
298 contamination, the use of young and actively growing spring shoots is preferred (Carra et al. 2012).
299 In contrast to that, however, in our case it was very difficult to introduce *in vitro* plant material
300 harvested in spring and summer due to lethal browning of shoots and high contamination rates,
301 while plant material collected in winter reacted more positively, with a high percentage of
302 successful establishment. This result is consistent with those described for other woody taxa such as
303 *Acacia sinuata* (Vengadesan et al. 2003) and *Fagus sylvatica*, for which tissue culture could be
304 started only with dormant buds harvested in February and March (Nadel et al. 1991; Vieitez et al.
305 2003).
306 As already found for the congener species *Zelkova sinica* (Gao et al. 1996) and *Z. schneideriana*
307 (Jin et al. 2009), WPM confirmed as an effective medium for callus induction. However, as a
308 whole, a crucial factor in micropropagation is the type and the concentration of cytokinin applied in
309 the multiplication phase. BAP and ZEA are the most widely used cytokinins, with satisfactory
310 results for shoot proliferation in woody trees (Haddad et al. 2018; Grigoriadou et al. 2002; Martinez
311 et al. 2017). BAP was effective in shoot regeneration of *Z. schneideriana* (Jin et al. 2009), while for
312 *Z. sicula* the best results in terms of shoot proliferation were provided by ZEA, consistently to data
313 obtained for other woody plants like *Olea europaea* (Lambardi and Rugini, 2003), *Arbutus unedo*
314 (Papagianni et al. 2017), *Arbutus andrachne* (Bertsouklis and Papafotiou, 2009) and *Cinnamomum*
315 *camphora* (Babu et al. 2003). ZEA is also known to have additional positive and useful effects on
316 micropropagation processes. In fact, it has been reported to enhance shoot elongation (Debnath,
317 2005), which in turn is a promoting feature for the following rooting step (Tao and Sugiura, 1992),
318 and to induce low callus formation at the base of explants (Marks and Simpson, 1994). This last one
319 is a desirable trait ensuring the genetic stability of regenerated plantlets especially devoted to
320 germplasm conservation and reinforcement of natural populations (Giri et al. 2004). Our data
321 confirm these findings, since the shoots obtained under this culture conditions were the longest ones
322 among the different treatments.

323 Successful root initiation is a key step in clonal propagation of woody trees for which rooting rates
324 are usually low, especially when adult material is used. Without an effective and well-structured
325 root system, plant acclimatization is prone to failure and the success of the entire process will be
326 poor. According to our previous experiences, as well as to definitions from literature, *Z. sicula*
327 could be defined as “recalcitrant to root” (Benson, 2000), so root induction required the adoption of
328 different strategies and manipulations. Initially, rooting of *Z. sicula* was attempted using as auxins
329 IBA and IAA. Rooting was obtained with both auxins but with significant differences. The best
330 results, globally achieved with IAA whatever the concentration, actually contrasted with those
331 reported for other taxa, e.g. *Z. schneideriana* (Jin et al. 2009), *Liquidambar orientalis* (Bayraktar et
332 al. 2015), *Quercus ilex* (Martinez et al. 2017) and *Olea europaea* subsp. *laperrinei* (Haddad et al.
333 2018), whose adventitious root formation was more effectively induced by IBA. Therefore, the
334 promising results obtained with IAA prompted a deeper investigation on its effectiveness, involving
335 changes in standard cultural conditions and time of exposure to auxin.
336 As described above, already in the shoot propagation step the use of ZEA not only induced higher
337 amounts of new shoots production, but also favoured internode elongation, which is essential to
338 obtain a good rooting percentage, especially in woody plants (Kevers et al. 2009). Also changes in
339 medium composition during the first phase of the process revealed fruitful. After the propagation
340 phase, shoots were transferred in a medium deprived of growth regulators for seven days, before
341 rhizogenic treatment. This approach was preferred for two different reasons: i) rooting aptitude is
342 favoured by a preliminary accumulation of endogenous IAA, a situation that could be obtained only
343 when the levels of cytokinins absorbed from the multiplication medium decrease (Bouza et al.
344 1994) and ii) the seven-day period in PGRs free medium is probably needed to decrease the
345 peroxidase concentrations which is induced by wounding and is responsible for IAA oxidation and
346 consequent inactivation (De Klerk et al. 1999). Moreover, before root induction, cells must become
347 competent and, during this phase, an auxin treatment is not necessarily required (Kevers et al.
348 2009). Thanks to such adjustments, the success rate in terms of rooted plants of more than 84% is to
349 be considered quite exceptional as referred to a species initially classified as “recalcitrant to
350 rooting”.
351
352 *4.2. Acclimatization: the last challenge*
353 The broad success in any process of plant propagation is strictly dependent on the quantity of newly
354 produced plants able to survive in field conditions after acclimatization (Ziv, 1986). Contrarily to
355 the excellent results obtained for the congener *Z. schneideriana* (Jin et al. 2009), the total number of
356 acclimatized plants of *Z. sicula* is actually rather modest as compared to the amount of rooted

357 plants. The low success of the acclimatization stage could be due to several factors, resulting in the
358 interruption of growth and a progressive decline and death: i) the unsuitable soil substrate to which
359 plants were transferred; ii) the *in-vitro* regenerated roots could be incompletely functional owed to
360 low connection with the conduction systems; iii) a weak structure of the root system, yet
361 inappropriate for transplanting (Amoo et al. 2011), iv) water stress, due to deficient root system,
362 and photoinhibition that may promote production of noxious reactive oxygen species (Bunn et al.
363 2011).
364 Whatever the reason of such yet unsatisfactory results, nonetheless the first 51 micropropagated
365 plantlets of this very challenging species have been reintroduced in the wild within the framework
366 of activities foreseen by the conservation project Zelkov@azione (http://www.zelkovazione.eu/).
367 Plantations, carried out according to the principles of "Assisted Colonization" (Booker et al. 2011),
368 were fully successful (Fig. 1H), with an exceptionally high survival rate. These promising results
369 can also represent a starting point to test the effectiveness of this protocol for near relatives with
370 similar conservation problems, such as *Z. abelicea* from Crete, whose most populations suffer for
371 insufficient or null sexual regeneration (Kozlowski et al. 2013).
372 However, additional experiments need to be done in order to overcome the failures in the
373 acclimatization phase and improve the efficiency of the entire process. New trials could address
374 some changes in cultural conditions during the multiplication step such as the use of meta-topolin, a
375 cytokinin that was recently found to have a positive interference with rooting (Aremu et al. 2012),
376 hence assumed to promote plant acclimatization. Also, understanding through anatomical studies if
377 the adventitious roots formed *in vitro* are functional and effectively connected with the micro-
378 cutting conduction tissues is decisive in order to optimize acclimatization.
379
380
381 **5. Conclusions**
382 In this work, an effective *in vitro* propagation protocol was developed for the first time from
383 axillary buds collected from mature plants of *Z. sicula*, a threatened relict tree at the brink of
384 extinction. Despite some refinements are still needed, the accomplishment of the entire reproduction
385 process allowed to overcome the problems of seed sterility of this rare species that were a major
386 obstacle to regenerate new plantlets for programs of reintroduction or *ex situ* conservation. After the
387 initial difficulties with sterilization, which in turn caused numerous failures in the multiplication
388 and rooting stages, the global result may be deemed rather satisfactory and the recalcitrance may be
389 considered partially overcome.

390 Concerning the problems associated to the acclimatization step, for sure they do not depend on a
391 single factor. Accordingly, in the next foreseen experiments some additional aspects specifically
392 addressed to the acclimatization will be investigated, but in order to finally fulfil our expectations
393 also the previous steps will be taken into account at some extent. In any case, the achievements
394 obtained so far already allowed the creation of four new small viable populations and the diffusion
395 of a few trees in plant repositories, hence significantly contributing to reduce the risk of extinction
396 of this very peculiar relict species.
397

398 **Figure legends**

399

400 **Fig. 1:** *In vitro* procedure for *Z. sicula* plant regeneration from nodal explants. **(A)** *In vitro*
401 introduction of woody explants. **(B)** *In vitro* shoot regeneration. **(C, D)** Multiple axillary shoots
402 regeneration, 10 and 20 days after treatment, respectively. **(E)** *In vitro* rooting obtained according to
403 protocol I, 15 days after treatment. **(F)** Developed plantlet in a vitro-vent pot. **(G)** Acclimatized
404 plantlets in outdoor condition. **(H)** An *in-vitro* regenerated plantlet of *Z. sicula* 15 months after
405 reintroduction in the wild. A Bars: A-E = 1 cm; F = 5 cm; G = 3 cm; H = 5 cm.

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407 **Fig. 2:** Efficiency of rooting and acclimatization recorded in trials performed in 2016 and in 2017.

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