1 **Title:** Micropropagation of *Juniperus navicularis*, an endemic and rare species from
2 Portugal SW coast
3
4 **Original research article**
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ABSTRACT

Juniperus navicularis Gand (= *J. oxycedrus* L. subsp. *transtagana* Franco) is an endemic dioecious shrub from the Plio-Plistocene transition sands of the west Portuguese coast line. It grows in sole or mixed stands along with *Pinus pinea* and *P. pinaster* and, more rarely, with *Quercus lusitanica*. In order to reduce fire incidence in these Mediterranean mixed stands, forest management practices of mechanical or chemical treatments, have drastically reduced *J. navicularis* populations. In addition, the fast expansion of touristic areas along with a low seed germination rate further endangers this species. In this study we developed a protocol for micropropagation of adult *J. navicularis*. Microcuttings were obtained from lateral and apical twigs of both female and male mature plants, and used as explants. Microcuttings with axillary buds were grown on different media and plant growth regulators combinations. Developmental parameters were evaluated to define the best medium and plant growth regulator concentration for shoot bud induction during the multiplication cycles. Olive Medium and Gupta and Durzan medium, both supplemented with 0.45 µM 6-benzyl-amino-purine, were the most favourable of all combinations tested achieving more than 3 new shoots per explant. The highest rooting (60%) was obtained in microshoots cultured in Olive Medium when supplemented with indole-3-butyric acid at 12.3 µM. With this multiplication protocol, it was possible to obtain more than 500 shoots from the initial 20, after three multiplication cycles in three months.

51 **Key words**: Conifer, conservation, Cupressaceae, endangered species, juniper,

52 microshoots, rooting, tissue culture

53

54 **Abbreviations**:

55 AA: Ascorbic acid

56 AE: von Arnold and Eriksson, 1981

57 BAP: 6-benzylamino purine

58 GD: Gupta and Durzan, 1985

59 IBA: Indole-3-butyric acid

60 ICN: Instituto da Conservação da Natureza e Biodiversidade, Portugal

61 KIN: Kinetin

62 MS: Murashige and Skoog, 1962

63 NAA: Naphthaleneacetic acid

64 OM: Olive medium; Rugini, 1984

65 PGR: Plant growth regulator

66 SH: Schenk and Hildebrandt, 1972

67 WH: White, 1942

68 WPM: Woody Plant Medium, Lloyd and McCown, 1981

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70

71 **INTRODUCTION**

72

73 The Portuguese Prickly Juniper, *Juniperus navicularis* Gand. (= *J. oxycedrus* L. subsp.

74 *transtagana* Franco), is a fastigiated shrub from the *Cupressaceae* family. This species

75 inhabits the coastal transition sands and is endemic to the Sado District (mainly to Sado

76 River Estuary) of South West Portugal (Rivas-Martínez et al. 1990). It belongs to the
77 xerophytic formations of the psammophil dry thermo-mediterranean bioclimatic area of
78 Portuguese southwest coast (Neto 2002). *J. navicularis* is a dioecious shrub reaching up
79 to 2-3 m in height. It has green needle-like leaves (5-20 mm x 1-2 mm) in whorls of
80 three and berry-like spherical seed cones, green at first and orange-red after ripening.

81 The urgency for conservation of *J. navicularis* is mainly due to the urban
82 expansion that has occurred in most of its native areas (Castro, Zavattieri, Afonso and
83 Belo; unpublished) and to mechanical or chemical treatments in forested areas to reduce
84 bush proliferation and prevent fire spread (Beja 2005). In addition to a low and irregular
85 seed production (Garcia et al. 1999), most junipers have seed germination problems
86 caused by embryo dormancy, seed coat impermeability to water and/or the presence of
87 germination inhibitors (Juan et al. 2006). It has been suggested that the germination
88 paucity might be related to climate changes, because warmer and drier summers can
89 influence seed viability due to water deficit stress (García et al. 1999). Taken together,
90 the insufficient seed production and germination have negative influence on
91 reproductive yield and, consequently, on population persistence.

92 Besides the inherent value as an endemic species, *J. navicularis* has a high
93 ecological value since it contributes to nutrient and water cycle regulation in these
94 particular soils, and acts as a refuge for fauna and as a nursery shrub for associated
95 flora. It is also pharmacologically interesting plant mainly due to the essential oils
96 contained in its leaves (Adams 1998; Velasco-Negueruela et al. 2002) and berries
97 (Cavaleiro et al. 2003).

98 Micropropagation of conifers through somatic embryogenesis has become a
99 method of choice for several species belonging to the *Pinaceae* family, however species
100 in the *Cupressaceae* family are recalcitrant (Bonga et al. 2010). To date, only two

101 reports showed that it is possible to induce somatic embryogenesis in *J. oxycedrus*
102 (Gómez and Segura 1995) and *J. communis* (Helmersson and von Arnold 2009).
103 However, in neither species mature somatic embryos nor plants were obtained.

104 Germination experiments with seeds of *J. navicularis* subjected to different
105 scarification treatments were unsuccessful due to the low seed viability (Castro, 2009).
106 Likewise, we were unable to root the cuttings (data not shown). Therefore we choose a
107 tissue culture based method (micropropagation) as an alternative means to conserve this
108 unique species.

109 The main objective of this study was to develop a micropropagation protocol for
110 adult *Juniperus navicularis*. We tested various plant culture media, plant growth
111 regulators (PGRs), and gelling agents with the material collected from adult male and
112 female plants. Emphasis was put on achieving the most satisfactory response from the
113 explants at each micropropagation stage.

114

115 **Materials and methods**

116

117 Plant material and aseptia

118

119 In autumn 2008, terminal young shoots approximately 30 cm in length were collected
120 from mature male and female plants from two populations in SW coast (38°24'8.30"N;
121 8°45'8.28"W and 38°18'19.17"N; 8°44'18.66"W). Shoots were tagged by location and
122 shrub sex and transported to the laboratory for further processing.

123 Shoot tips and nodal segments (not longer than 1 cm) were cut from the young
124 shoots and disinfected. The explants were washed in tap water for 5 minutes, then
125 surface-sterilized with 70% (v/v) ethanol for 2 minutes and rinsed 3 times with sterile

126 distilled water. Afterward, the explants were immersed in 3% (v/v) commercial bleach
127 (Domestos®, Unilever with ≤ 5% active chlorine) for 20 minutes, rinsed with sterile
128 water, washed in 1% (w/v) Benlate® (DuPont) solution for 10 minutes and rinsed 3
129 times with sterile water. Finally, the explants were immersed in 70% ethanol for 2 min
130 followed by 3 rinses in sterile water.
131
132 Shoot bud development from explants of adult plants (1st multiplication cycle) and
133 culture conditions
134
135 Decontaminated explants with at least one axillary or terminal bud were placed in
136 culture tubes with different semi-solid media and six different PGR combinations
137 (Table 1). Both the media and the PGRs were selected based on the previously
138 published results (Ragonezi et al. 2010) and other standard formulations described in
139 Gupta and Durzan (1985) (GD); Lloyd and McCown (1981) (WPM); Murashige and
140 Skoog (1962) (MS); Schenk and Hildebrandt (1972) (SH); von Arnold and Eriksson
141 (1981) (AE) and White (1942) (WH). All media were supplemented with 20 g l^{-1} of
142 sucrose and 8 g l^{-1} agar (Merck®) and the pH was adjusted to 5.8 before autoclaving.
143 Treatments consisted of three repetitions of five explants per medium x PGR
144 combination, one shoot per culture tube with 10 ml of medium, 450 explants in total.
145 Culture chamber conditions for all experiments were 16h photoperiod (Cool White
146 lamps, Philips Master LD36W/840, photosynthetic photon flux density 90 µmol m^{-2} s^{-1})
147 and 24/19 ºC day/night, respectively. The first multiplication cycle lasted 30 days, after
148 which the number of developed lateral and basal shoots, the length of the longest shoot
149 and the physiological state of the main shoot (dead or alive) were evaluated.
150 Additionally, some qualitative parameters were evaluated such as the colour of the

151 whole explants, basal leaves and the shoot bud general appearance (hyperhydric,
152 abnormal or normal).
153
154
155 Multiplication of microshoots (2nd and 3rd multiplication cycles)
156
157 Axillary shoots were excised from the original explants (see above) and transferred to
158 the glass culture vessels (V8630+B8648, Sigma®) with 30 ml of fresh culture medium
159 solidified with gellan gum (Phytagel, Sigma®). Subcultures were carried out every 30
160 days, at which time the number of shoots per explant was counted. Based on the results
161 from the 1st multiplication cycle, only two media were selected for further testing: MS
162 supplemented with 0.93 µM KIN and 3 or 6 g l^{-1} gellan gum and GD supplemented with
163 0.45 µM BAP and 3 or 6 g l^{-1} gellan gum (Table 1). For the 2nd multiplication cycle, 10
164 shoots derived from the initial male and female shrubs were tested in each medium and
165 PGR combination. Three repetitions of each combination were done with a total of 240
166 shoots.
167 The 3rd multiplication cycle was performed to compare MS and GD media with
168 OM medium that previously was used in micropropagation of *J. phoenicea* (Loureiro et
169 al. 2007). GD and OM, each containing 0.45 µM BAP, and MS containing 0.93 µM
170 KIN, all with 3 or 6 g l^{-1} gellan gum, were used for comparison. Twenty shoots per
171 medium per PGR combination per gellan gum concentration were placed in culture and
172 the experiment was repeated two times. In both multiplication cycles, culture conditions
173 were the same as used in the 1st multiplication cycle.
174
175

176 Rooting of microshoots

177

178 *Treatments with naphthaleneacetic acid*

179

180 Fifty microshoots were placed for elongation in GD medium with 2 g l^{-1} acid-washed
181 activated charcoal (Sigma-Aldrich®) and kept for two weeks under 16h photoperiod at
182 24/19 ºC day/night, respectively. The elongated microshoots (2 cm long separated from
183 any browning tissues) were then transferred to modified WPM root induction medium
184 with half concentration of macronutrients and 5.35 µM of naphthaleneacetic acid
185 (NAA), where they remained for two weeks. Culture conditions were 19ºC in darkness
186 during the first week and 19ºC with 16h photoperiod during the second week (Zavattieri
187 et al. 2009). Following the root induction treatment, the shoots were transferred to root
188 expression medium consisting of modified WPM (half concentration of macronutrients)
189 PGR-free medium.

190

191

192 *Treatments with indole-3-butyric acid*

193

194 Five different approaches were followed to induce adventitious rooting using IBA
195 treatments: addition into the medium after filter sterilization (IBA1); quick-dip
196 treatment (IBA2); pulse treatment (IBA3); three-day contact (IBA4); induction in a
197 substrate (IBA5).

198 For adventitious root induction in IBA1, OM medium without L-glutamine was
199 supplemented with IBA at the following concentrations: 2.5 ($IBA1_1$); 12.3 ($IBA1_2$);
200 24.6 ($IBA1_3$) and 49.2 µM ($IBA1_4$). Twenty microshoots (approximately 2 cm long)

201 were used for each IBA concentration and maintained for two weeks in the root
202 induction medium: the first week in the dark and the second at a 16h photoperiod, both
203 weeks at 19ºC. Afterward, shoots were transferred to OM medium without PGRs for
204 root expression, placed in the culture chamber and periodically observed for new roots.
205 In the quick-dip treatment (IBA2) shoots were immersed for 5 seconds in sterile
206 solutions of IBA with the following concentrations: 12.3 ($IBA2_1$); 24.6 ($IBA2_2$); 36.9
207 ($IBA2_3$); and 49.2 ($IBA2_4$) x 10^3 µM. The thirty shoots used per IBA concentration
208 were thereafter dried at the laminar flow cabinet and immediately transferred to OM
209 medium without PGR's and placed in the culture chamber.
210 In pulse treatment (IBA3) shoots were placed in Eppendorf tubes for 5 minutes
211 in contact with different concentrations of IBA sterile solutions: 3.7 ($IBA3_1$); 4.9
212 ($IBA3_2$); 6.2 ($IBA3_3$) and 7.4 ($IBA3_4$) x 10^3 µM. The shoots were then transferred to
213 OM root expression medium and kept in the same conditions as those described above.
214 IBA4 (three days in solution) differed from IBA3 not only with respect to the
215 period of time each shoot was in contact with IBA solution, but also with respect to the
216 concentration: 1.2 ($IBA4_1$); 2.5 ($IBA4_2$); 3.7 ($IBA4_3$) and 4.9 ($IBA4_4$) x 10^3 µM. Each
217 microshoot was introduced into a test tube containing 3 ml of IBA, maintained in the
218 dark during three days, and subsequently transferred to the root expression medium
219 under the same conditions used for the other treatments.
220 In all the rooting experiments (with IBA and NAA), shoots were observed for
221 new roots during six weeks and discarded after this period if no roots were produced.
222
223 Rooting of plantlets in various substrates and acclimatization
224

225 To test if *J. navicularis* could produce roots directly in a substrate (IBA5), eight
226 substrates with different ratios of perlite, vermiculite and peat moss were tested with
227 each of the following five PGR combinations: 2.5 x 10^3 µM IBA + 5.7 x 10^3 µM AA
228 ($IBA5_1$); 5 x 10^3 µM IBA + 5.7 x 10^3 µM AA ($IBA5_2$); 10 x 10^3 µM IBA + 5.7 x 10^3
229 µM AA ($IBA5_3$); 5 x 10^3 µM IBA ($IBA5_4$); 5.7 x 10^3 µM AA ($IBA5_5$). These eight
230 substrates were 100% perlite; 100% vermiculite; two mixtures of vermiculite:peat moss
231 at the ratios of 3:7 and 7:3; two mixtures of vermiculite:perlite at the ratios of 3:7 and
232 7:3 and two mixtures of perlite:peat moss at the ratios of 3:7 and 7:3. Substrates were
233 distributed into glass flasks, sterilized, transferred to culture vessels (Combiness Full-
234 Gas Microboxes Model: O118/120; 870 ml with green Extra Gas Exchange Filters
235 Model: OD118) and soaked in liquid OM without sucrose after which the excess of OM
236 was drained. In addition, 3 ml of AA was added to the substrate in each flask to
237 minimize browning. Shoots were dipped for 5 seconds in the corresponding sterile IBA
238 solution, dried out in the active laminar flow unit and transferred to the matching culture
239 vessel. Fourteen shoots were cultured per vessel in the culture chamber.

240 Rooted shoots were washed carefully in water to eliminate residual medium and
241 planted in pots filled with non sterile substrate mix of perlite:vermiculite:peat moss
242 (1:1:1) (v/v) (Zavattieri et al. 2009). We used a special culture chamber equipped with
243 light and dry mist system to provide high relative humidity, and therefore it was not
244 necessary to cover the plantlets. The temperature of the chamber was maintained at 22 ±
245 2ºC. All the plantlets were maintained under the same environmental condition and
246 watered every week alternately with MS macronutrient solution and distilled water.
247 After two months, plants were transferred to the greenhouse.

248

249 Data analysis

Data analysis was done using generalized linear models to determine influence of media, PGR combinations and their interaction on several parameters (see below). In the 2nd multiplication cycle we analyzed the influence of media, gelling agent concentration, sex and their interactions. A log Poisson regression was used for the number of new lateral shoots and the number of new basal shoots.

Differences in general colour of the explants and in colour of basal leaves were analysed with a non parametric two-way ANOVA and multiple comparisons of mean orders was performed with LSD test (equivalent to Conover-Inman test). Mann-Whitney-Wilcoxon test was applied to evaluate differences in colour by sex. All statistical studies were performed using PASW Statistics (v.18, SPSS Inc., Chicago, IL).

Results and discussion

Axillary bud development from nodal explants of adult juniper (1st multiplication cycle)

After two weeks, explants on AE, SH and WH media started to change colour from green to yellow/brown, most likely caused by inappropriate medium x PGR combination for this species. One week later, these cultures were discarded due to necrosis of the explants (approximately 92%), and therefore were not included in the statistical analysis. The disinfection procedure was very effective and only as few as 1% of the initial explants were lost to contamination.

Explants on the other media (MS, WPM and GD) developed several lateral shoots. The final number of new lateral shoots depended on the medium composition

275 (Fig. 1). Explants in GD medium produced more new lateral shoots than those in MS
276 and WPM ($\chi^2 = 7.077$, df = 1, P < 0.008). No differences were observed between these
277 PGR combinations when GD was used as the basal medium. WPM medium was
278 generally less suitable for culture than the other media tested, especially when used
279 together with PGR combinations III ($\chi^2 = 8.224$, df = 1, P = 0.004), IV ($\chi^2 = 19.471$,
280 df = 1, P < 0.001) and V ($\chi^2 = 5.848$, df = 1, P = 0.016).

281 The longest shoot was observed in GD medium when supplemented with PGR
282 combination V (Fig. 2). In MS and WPM media the longest shoot was significantly
283 shorter (MS: $\chi^2 = 15.224$, df = 1, P < 0.001; WPM: $\chi^2 = 16.231$, df = 1, P < 0.001).
284 Shoots in MS medium were longer when PGR combination III was used ($\chi^2 = 4.698$,
285 df = 1, P ≤ 0.030). In WPM medium the longest shoot grew when supplemented with
286 PRG combination I ($\chi^2 = 8.3$, df = 1, P = 0.04) and II ($\chi^2 = 6.682$, df = 1, P = 0.10).

287 We evaluated the colour of the explants because this parameter could be
288 indicative of the general physiological status of the plant, and also because media x
289 PGR combination could have toxic effects or cause nutritional imbalances. This
290 combination have not significantly altered the colour of the explants ($\chi^2 = 6.39$, df = 10,
291 P < 0.001). There were visible differences in colour between explants by media
292 ($\chi^2 = 35.42$, df = 2, P < 0.001), with significant differences between WPM and MS
293 (P < 0.001) and WPM and GD (P < 0.010) media. In general, on MS and GD, the
294 explants maintained their natural green colour, rarely becoming yellow or brown.
295 Colour was affected by PGR ($\chi^2 = 38.6$, df = 5, P < 0.001).When BAP was added at
296 4.43 µM, significant differences were detected in the colour of the explants, which
297 became yellow or brown in comparison with shoots from all other PGR combinations
298 (all P < 0.015). No significant differences were detected in the colour with PGR
299 combinations III and IV (P = 0.396) nor with I, II and VI (P > 0.05). Most of the media

300 and PGR combinations also affected the colour of the basal leaves some of which
301 became yellow, brown or died. On WPM almost all basal leaves became brown and no
302 significant difference with PGR combination were detected ($F = 0.45$, $P = 0.816$).
303 Nevertheless, on GD medium with PGR combinations II, III and IV, most of the basal
304 leaves remained green and did not show any detrimental effect of the culture conditions.
305 Another analysed parameter was the number of basal buds developed on the
306 cultured shoots which showed that MS medium was more favourable than WPM
307 (($\chi^2 = 39.96$, df = 1, $P < 0.001$) or GD ($\chi^2 = 6.826$, df = 1, $P = 0.009$). There were more
308 buds developed in MS medium when used in combination with PGRs I, III and V. High
309 concentration of BAP had detrimental effect in all media tested ($\chi^2 = 6.759$, df = 1,
310 $P = 0.009$). Low concentrations of KIN or BAP induced high number of new shoots in
311 all media tested.
312 Lastly, we evaluated the appearance of the basal buds, namely hyperhydricity,
313 the presence of callus, abnormal development and necrosis (Figs. 3a and 3b, 3c, d, e,
314 respectively). Both PGR and medium influenced the phenotype of the basal buds in
315 culture. KIN caused fewer abnormalities than the other PGRs tested. BAP at 4.43μM or
316 higher increased the frequency of abnormal basal buds independently of the basal
317 medium used. Buds developed in GD medium had fewer abnormalities than those
318 developed in other media.
319
320 Shoot multiplication - 2nd cycle
321
322 In the 2nd multiplication cycle, GD was used either with 3 or 6 g l^{-1} gellan gum and
323 produced significantly more shoots (approximately 2.6 per explant) ($\chi^2 = 10.471$,
324 df = 1, $P = 0.001$) than MS (approximately 1.9 per explant) (Fig. 4 a). Concentration of

325 gelling agent had no significant effect when MS or GD medium was analyzed
326 separately; however a higher concentration of gellan gum slightly reduced the
327 multiplication efficiency on both media as well as the abnormal appearance of the basal
328 buds.

329 Although female plants showed a small decrease in the multiplication efficiency
330 of shoots on media with 6 g l^{-1} of gellan gum (Fig. 4 b), there were no evident statistical
331 differences between male or female plants regardless of the gellan gum concentration or
332 their interaction ($P > 0.6$ for all). Shoots derived from female plant grew greener
333 ($U = 5739$, $W = 7884$, $P < 0.001$) compared with plants regenerated from male
334 counterparts. This could be attributed to a higher lignification of male plants most likely
335 being ontogenically older (George et al. 2008).

336

337 Shoot multiplication - 3rd cycle

338

339 In the 3rd cycle of multiplication, we tested OM medium and the best medium x
340 PGR combination that was selected from the previous multiplication cycles. It has been
341 shown that OM medium was superior for *J. phoenicia* when compared with other media
342 (Loureiro et al. 2007). In this multiplication cycle, explants on GD and MS had a higher
343 multiplication rate than in the previous cycles. These results are in agreement with
344 Loureiro et al. (2007) for *J. phoenicea* who also reported a higher shoot lengths and
345 number of branches per shoot during subsequent subcultures. The authors concluded
346 that during successive multiplication cycles there was a reduction in the level of stress
347 that ultimately occurs after the first adaptation period to the *in vitro* culture conditions.

348 In GD and OM media gelled with 3 g l^{-1} of gellan gum there were significantly
349 more new lateral shoots than on MS medium ($\chi^2 = 5.936$, df = 1, $P = 0.015$). However,

350 gellan gum concentration had no significant effect on the number of formed shoots
351 when analyzed in each medium formulation separately (Fig. 5).
352
353 Rooting of shoots in vitro
354
355 Rooting experiment using NAA in a culture medium for 6 wks was unsuccessful. On
356 the other hand, we observed the development of aerial parts, which reached almost 8
357 times the original size. These results were in agreement with the report of Gómez and
358 Segura (1995) on *J. oxycedrus* where 100% rooting was obtained in the presence of 2.5
359 μM NAA and 4 % sucrose from shoots regenerated from leaves, however when shoots
360 were collected directly from the field plants and multiplied *in vitro*, NAA was
361 ineffective. In that study, there was a correlation between the plant material source for
362 shoot production and the effectiveness of NAA in adventitious root induction.
363 In the present study, the best rooting treatment resulted in 60% shoots producing
364 adventitious roots after 3 months directly from the base of the shoot and without an
365 intermediate callus formation (Fig. 6). The treatment was with IBA ($IBA1_{2)}$, which was
366 added to the induction medium at 12.3 μM (Fig.7). Hence, the rooting frequency was
367 higher than that obtained with other Mediterranean junipers such as *J. phoenicea* (40%,
368 Loureiro et al. 2007), *J. excelsa* (31%, Negussie 1997) and *J. oxycedrus* (7-10%, Gómez
369 and Segura 1995). In all other treatments, the rooting percentage was less than 25%.
370
371 Rooting of shoots in a substrate
372
373 The best rooting results (42.9%) were obtained when shoots were directly transferred to
374 vermiculite after a 5 second immersion of their bases in 5 x 10^3 μM IBA alone ($IBA5_4$),

375 or in 2.5 x 10^3 µM IBA + 5.7 x 10^3 µM AA ($IBA5_1$) (Fig. 8). Other substrates were not
376 suitable.
377
378 Acclimatization of plantlets
379
380 Plants rooted in vitro or directly in a substrate were acclimatized, but those which
381 rooted directly in the substrate (IBA5) had healthier appearance and grew faster than
382 those rooted *in vitro*. This observation is not surprising because shoots that rooted in
383 substrates became autotrophic sooner than the in vitro rooted shoots.
384
385 **Conclusions**
386
387 We were successful in micropropagation of *J. navicularis* using OM, GD and MS media
388 supplemented with KIN (0.93 µM) and gelled with 3 or 6 g l^{-1} of gellan gum. However,
389 the appearance of the shoots in culture (evaluated through different qualitative
390 parameters such as the colour of the explants, the basal shoots hyperhydricity and the
391 morphological abnormalities) showed that GD medium was the most suitable regardless
392 of the PGRs used. However, more research must be done in relation to the gelling agent
393 concentration, because contradictory results were observed in the 2nd and 3rd
394 multiplication cycles. BAP at 0.45µM was the best PGR to obtain high multiplication
395 rates of lateral shoots. With this multiplication protocol, it was possible to obtain more
396 than 500 shoots from the initial 20, after 3 multiplication cycles (3 months).
397 Rooting is the main bottle-neck in micropropagation of most conifers (Oliveira
398 et al. 2003) limiting the possibilities to establish commercial protocols. However, in our
399 study we achieved 60% rooting of micropropagated shoots when IBA was added to the

400 medium as rooting inducer. The long rooting period (3 months), although not unusual
401 in conifers, also needs further studies because it is possible that other factors, such as
402 the stress caused by diminished nutrient availability during the prolonged culture period
403 that shoots remained in the medium could be separate from the PGR effect.
404 Simultaneous rooting and weaning of microshoots in substrates was also promising,
405 because it increased the survival rate of plants in the nursery. Light substrates
406 resembling the sandy soils where the species naturally grows should also be included in
407 the future research.

408

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410

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414

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Fig 1 Mean new lateral shoots formed (±SE) after one month in the first multiplication cycle with different media (WPM, MS and GD) and PGR combination

Fig 2 Mean longest shoot growth (±SE) after one month in the first multiplication cycle with different media (WPM, MS and GD) and PGR combination

Fig 3 Abnormal shoots in the first multiplication cycle (a) hyperhydricity of the basal buds and long internodes; b) hyperhydricity of the basal buds and different length of the leaves, c) calli development at the base of the explants; d) yellowish colour of the explant; e) death of the explant.

Fig 4 Number of new lateral shoots produced in the 2nd multiplication cycle according to a) the medium (MSI and GDIII, both with 3g/l or 6g/l gellan gum) and b) the plant sex.

Fig 5 Number of new lateral shoots formed during the 3rd multiplication cycle on MS I, GD III and OM III, gelled with 3g/l or 6g/l gellan gum.

Fig 6 Rooting percentages obtained with OM medium supplemented with IBA1 (included in a medium) at 2.5 ($IBA1_1$); 12.3 ($IBA1_2$); 24.6 ($IBA1_3$) and 49.2 μM ($IBA1_4$) ; IBA2 (quick-dip) at 12.3 ($IBA2_1$); 24.6 ($IBA2_2$); 36.9 ($IBA2_3$); and 49.2 ($IBA2_4$) x 10^3 μM ; IBA3 (pulse treatment) (IBA3) at 3.7 ($IBA3_1$); 4.9 ($IBA3_2$); 6.2 ($IBA3_3$) and 7.4 ($IBA3_4$) x 10^3 μM; IBA4 (3 days immersion) at 1.2 ($IBA4_1$); 2.5 ($IBA4_2$); 3.7 ($IBA4_3$) and 4.9 ($IBA4_4$) x 10^3 μM .

Fig 7 Rooted plant obtained in the expression medium derived from $IBA1_2$ without callus formation at the base of the shoot

Fig 8 Rooting percentages obtained in vermiculite after IBA5 treatments at 2.5 x 10^3 μM IBA + 5.7 x 10^3 μM AA ($IBA5_1$); 5 x 10^3 μM IBA + 5.7 x 10^3 μM AA ($IBA5_2$); 10 x 10^3 μM IBA + 5.7 x 10^3 μM AA ($IBA5_3$); 5 x 10^3 μM IBA ($IBA5_4$); 5.7 x 10^3 μM AA ($IBA5_5$)

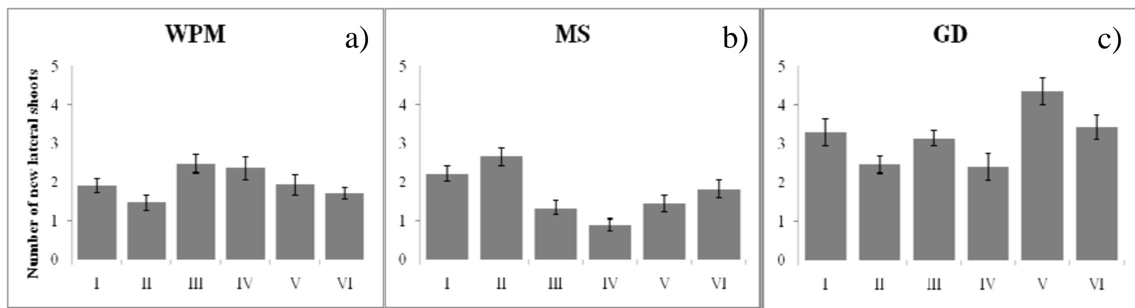


Fig. 1

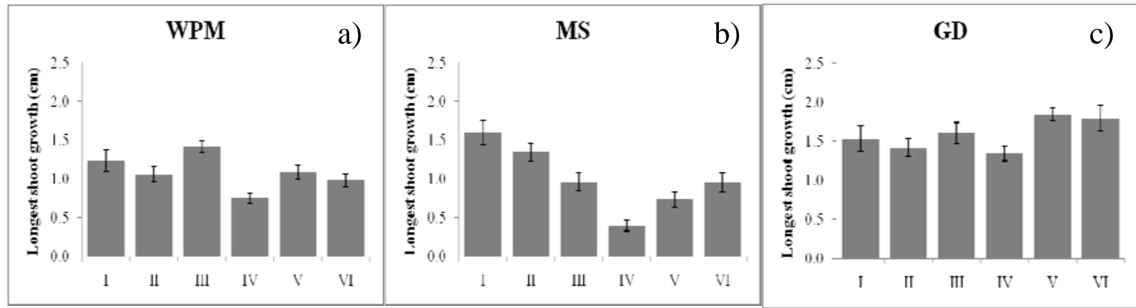


Fig. 2

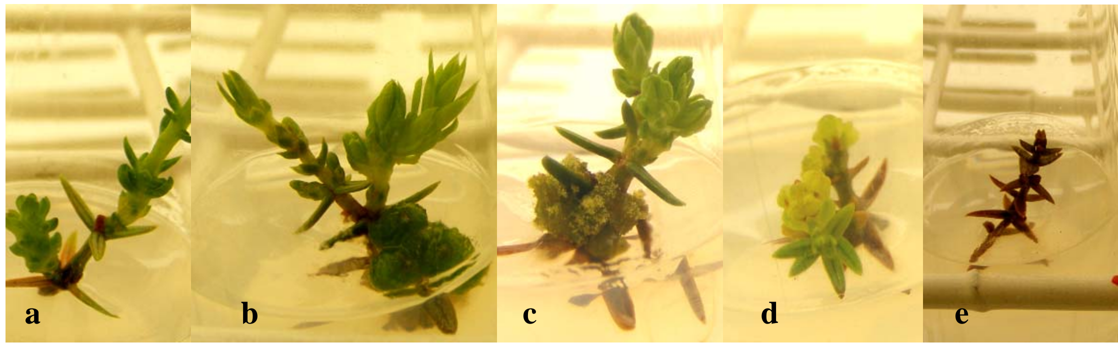


Fig. 3

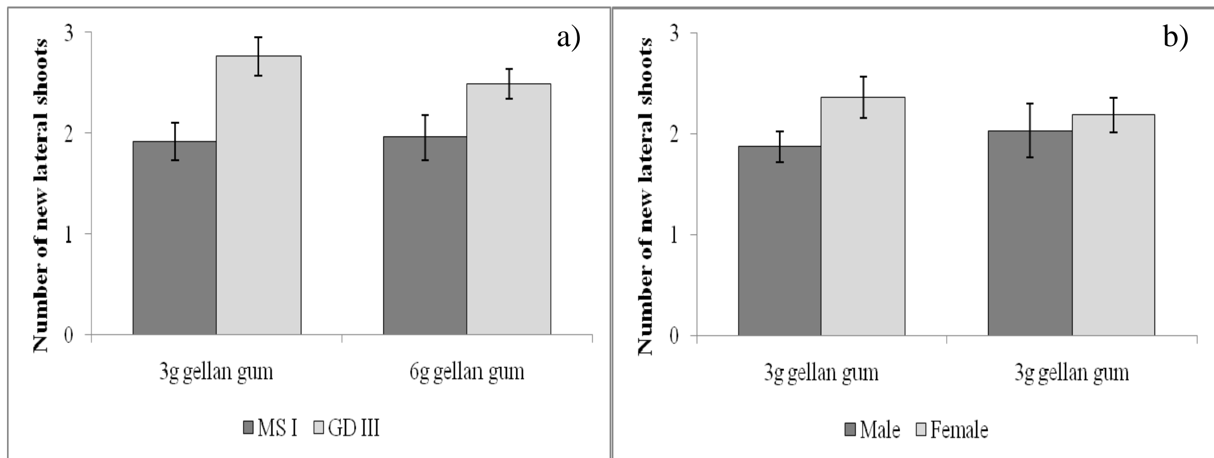
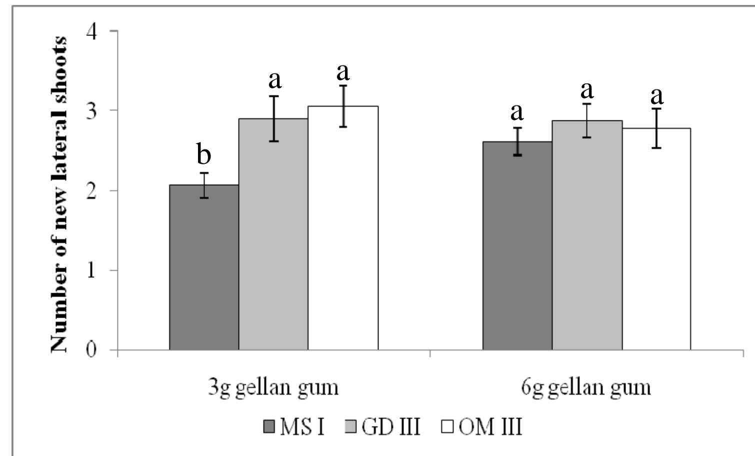


Fig. 4



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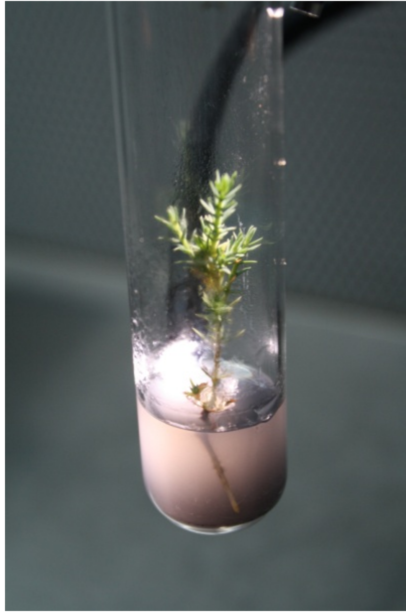

Fig.6

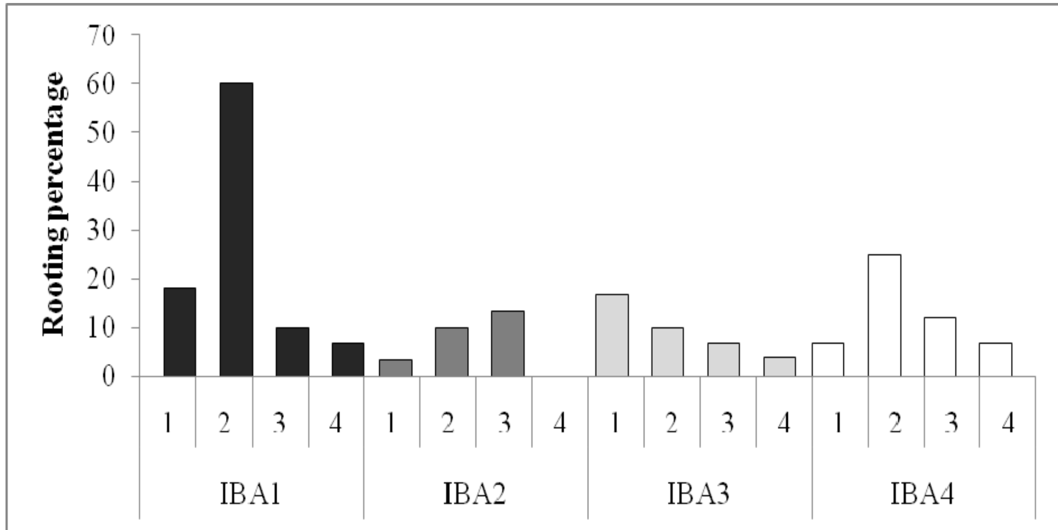

Fig 7

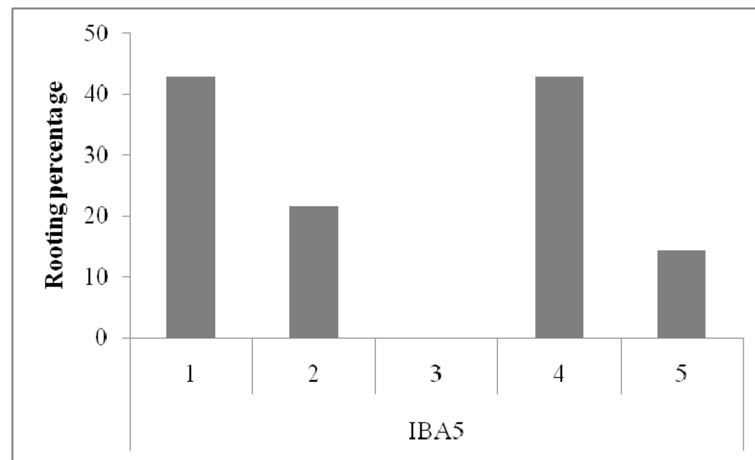

Fig. 8

Table 1 Plant growth regulator combinations (PGR_c) used on *Juniperus navicularis* shoot multiplication treatments

PGR_c	Growth Regulators (μM)		
	KIN	BAP	NAA
I	0.93	-	-
II	2.32	-	-
III	-	0.45	-
IV	-	4.43	-
V	-	0.90	2.74
VI	0.93	0.45	2.74