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SHORT COMMUNICATION

OPEN ACCESS

The effect of changing temperature and agar concentration at proliferation stage in the final success of Aleppo pine somatic embryogenesis

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Abstract

Aim of the study: The effect of physical and chemical conditions at proliferation stage was evaluated in order to elucidate if this stage is the determinant phase to induce a marked effect in *Pinus halepensis* somatic embryogenesis.

Area of study: The study was conducted in research laboratories of Neiker (Arkaute, Spain).

Material and methods: *Pinus halepensis* embryonal masses from ten embryogenic cell lines subjected to nine treatments (tissues cultured at three temperatures on media supplemented with three agar concentrations) at proliferation stage.

Main results: Significant differences were observed among different proliferation conditions months later at the end of maturation, germination and acclimatization stages.

Research highlights: Aleppo pine embryonal masses are cultured under standard conditions on a culture medium supplemented with 4.5 g/L Gelrite® at 23ºC. However, better results in terms of plantlet production can be obtained proliferating the embryonal masses at 18ºC in a culture media with significantly lower water availability.

Additional keywords: embryogenic cell lines; *Pinus halepensis*; somatic embryos; water availability.

Abbreviations used: ECL (embryogenic cell line); EM (embryonal mass); SE (somatic embryogenesis).

Authors´ contributions: All authors meet the criteria for authorship described in the instructions for authors. Specifically, conceived and designed the experiments: JMC, PM; performed the experiments: CP, IAM and SC; analyzed the data: TG, MDU; wrote the paper: PM and IAM.

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Introduction

In the last years, efforts have been carried out to propagate high-value trees through somatic embryogenesis (SE) in order to increase forest productivity. SE can capture the benefits of breeding or genetic engineering programs by multiplying trees with improved characteristics (Pullman & Bucalo, 2014).

In this sense, Aleppo pine (*Pinus halepensis* Miller), a species native to the Mediterranean basin, can thrive under semi-arid climatic conditions in a wide variety of soils (Maestre & Cortina, 2004) and is susceptible to be used in large afforestations in the future scenario of climate change (Jeddi *et al*., 2009).

It is generally known that adjustments of tissue culture media can result in higher success of SE initiation and proliferation frequencies (Montalbán *et al.,* 2012). However, few studies have focused on the impact of temperature (Kvaalen & Johnsen, 2008), and the effect of different concentrations of agar in the culture medium has been studied mostly at maturation stage (Teyssier *et al*., 2011; Morel *et al*., 2014). In this sense, in our laboratory García-Mendiguren *et al.* (2016) described in *Pinus radiata* (D. Don) that different temperatures and water availability conditions at initiation affected subsequent phases of SE; however, this effect on subsequent phases of the process disappeared when different environmental conditions were applied at proliferation. Then, Pereira *et al.* (2016) found out that

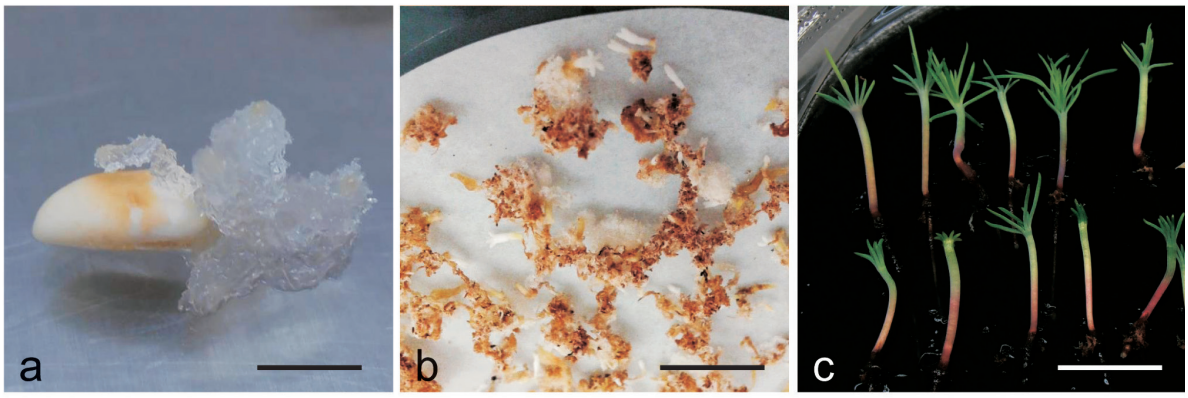

Figure 1. *Pinus halepensis* somatic embryogenesis process: a) Initiation embryonal mass carried out at 23ºC on initiation medium supplemented with 4 g/L Gelrite®, bar=5 mm; b) somatic embryos at the end of maturation stage, bar=15 mm; c) somatic plantlets after 6 weeks of germination medium, bar=12 mm.

different environmental conditions at initiation did not influence maturation and germination phases in *P. halepensis*. Taking all this information into account, our aim was to analyze the effect of physical and chemical conditions at proliferation stage of *P. halepensis* SE on the final success of the process including the obtention of somatic plants.

Material and methods

One-year-old cones of *Pinus halepensis* from five open-pollinated trees located in Berantevilla (Spain) were used. The cones were stored following Montalbán *et al.,* (2015). Initiation of embryonal masses (EMs, Fig. 1a) was performed as described in Montalbán *et al.* (2013). Ten embryogenic cell lines (ECLs) were randomly selected and four EMs with an approximate diameter of 16 mm from each ECL were transferred to different proliferation conditions (Fig. 2). Embryonal masses were subcultured after two weeks to the same proliferation condition. After one month in the different proliferation conditions described in Fig. 2, EMs were subjected to maturation. Maturation and germination were carried out according to Pereira *et al.* (2016). At maturation, four replicates per ten ECLs and nine proliferation treatments (a total of 360 Petri dishes) were performed. At germination, three Petri dishes (with 20 somatic embryos per Petri dish) per each ECL and proliferation treatment were maintained under 16 h photoperiod at 100 µmol/(m^2 s) provided by cool white fluorescent tubes (TFL 58 W/33; Philips, France). After 16 weeks on germination medium, the plantlets were transferred to sterile peat:perlite (70:30,

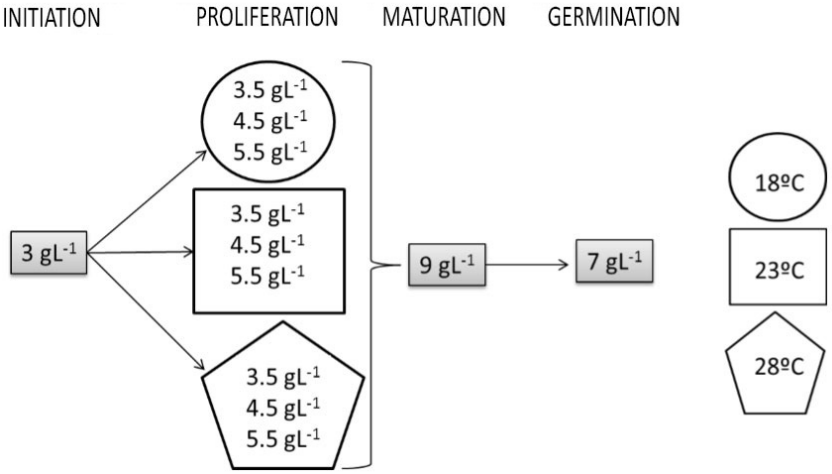

Figure 2. Graphic representation of *Pinus halepensis* somatic embryogenesis culture conditions for the experiment: at the proliferation stage, culture medium was supplemented with 3.5, 4.5, or 5.5 g/L Gelrite®. Explants were cultured at three different temperatures (circle 18ºC, rectangle 23ºC, and pentagon 28ºC). Initiation, maturation and germination were carried out at 23ºC.

v/v) and acclimatized in a controlled greenhouse, progressively decreasing the humidity from 99 to 70 %.

The water availability of the proliferation culture media was determined following Garcia-Mendiguren *et al.* (2016), performing five replicates per culture condition.

At the end of maturation stage, the percentages of maturation were calculated. Logistic regression and the corresponding analysis of deviance were conducted to assess the effect of the considered variables on maturation and germination rates. A heteroscedastic linear mixed model (and its analysis of variance) was conducted with the square root of the number of somatic embryos per gram of EM as the response variable. Temperature and agar concentration were considered as fixed effects and the ECL as a random effect.

A two-way analysis of variance was carried out to assess the effect of temperature and agar concentration on the water availability of the media and on *ex vitro* survival of somatic plantlets.

Results and discussion

All EMs grew properly in the different environmental conditions assayed. The medium solidified with 5.5 g/L agar showed significantly lower water availability than the media solidified with 4.5 g/L and 3.5 g/L agar. Neither the temperature nor the interaction between the temperature and the agar concentration were statistically significant. These results are in agreement with previous studies on initiation stage (Pereira *et al*., 2016) where only significant differences were found for agar concentration.

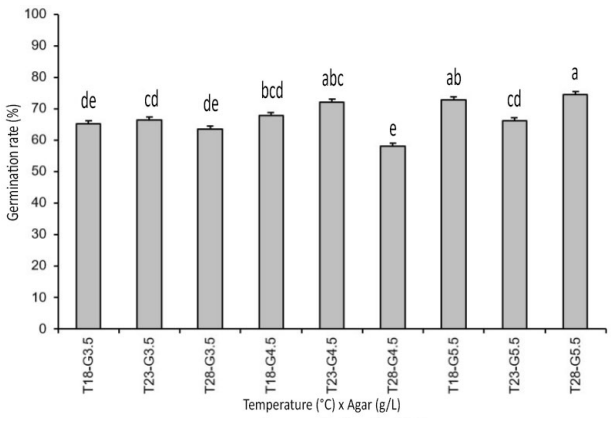

Figure 3. Germination rate (mean ± standard error) in *Pinus halepensis* plantlets from embryonal masses proliferated at three temperatures (18, 23 or 28 ºC) and three agar concentration (3.5, 4.5, and 5.5 g/L). Different letters show significant differences by Benjamini-Yekutieli´s post hoc test.

As almost all ECLs and treatments produced somatic embryos (89 out of 90, Fig. 1b), no statistically significant differences in the percentages of maturation were found among temperatures nor among agar concentrations nor double interactions between the variables. With respect to the number of somatic embryos per gram of EM, statistically significant differences were found for the interaction between the temperature and the agar concentration. However, these differences were only observed between EMs proliferated at 23ºC in a culture medium containing 3.5 g/L agar (860 somatic embryos per gram of EM) and those proliferated at 28ºC with 3.5 g/L agar (1212 somatic embryos per gram of EM). Our results are in agreement with results obtained by Kvaalen & Johnsen (2008) in *Picea abies*, and opposite to findings reported by García-Mendiguren *et al.* (2016) in *P. radiata* where they did not find differences among proliferation treatments for somatic embryo production. Furthermore, different environmental conditions at initiation stage of *P. halepensis* SE, did not lead to significant differences in the number of somatic embryos obtained (Pereira *et al.,* 2016). So, for this species, it appears that different environmental conditions have a greater influence when applied during proliferation than during initiation.

Statistical analysis of germination rates showed a significant interaction between the temperature and the agar. The highest germination rate (74%) was achieved in somatic embryos coming from EMs proliferated at 28ºC in a culture media supplemented with 5.5 g/L agar (Fig. 1c). However, embryos generated from EMs proliferated at lower temperatures (18ºC-5.5 g/L agar and 23ºC-4.5 g/L agar) did not show statistical differences in terms of germination with the abovementioned treatment (Fig. 3). The plantlets from EMs proliferated at 18ºC (69.5%) showed a significantly higher *ex vitro* survival than those proliferated at 28ºC (49.8%); plantlets from standard proliferation temperature (23ºC) did not show significant differences with the abovementioned temperatures (54.2%). The agar concentration at proliferation did not have a significant effect on *ex vitro* survival.

In summary, this work shows that manipulation of environmental conditions (temperature and water availability) during proliferation of EMs provokes an effect after several months in the production somatic plants. The best results are obtained proliferating the EMs at the lowest temperature assayed (18ºC) and reducing the water availability of the culture medium.

References

García-Mendiguren O, Montalbán IA, Goicoa T, Ugarte D, Moncaleán P, 2016. Environmental conditions at the initial stages of *Pinus radiata* somatic embryogenesis affect the production of somatic embryos. Trees-Struct Funct 30: 949-958. https://doi.org/10.1007/s00468-015-1336-7

Jeddi K, Cortina J, Chaieb M, 2009. *Acacia salicina*, *Pinus halepensis* and *Eucalyptus occidentalis* improve soil surface conditions in arid southern Tunisia. J Arid Environ 73: 1005-1013. https://doi.org/10.1016/j.jaridenv.2009.05.005

Kvaalen H, Johnsen Ø, 2008. Timing of bud set in *Picea abies* is regulated by a memory of temperature during zygotic and somatic embryogenesis. New Phytol 177: 49-59.

Maestre FT, Cortina J. 2004. Are *Pinus halepensis* plantations useful as a restoration tool in semiarid Mediterranean areas? Forest Ecol Manag 198: 303-317. https://doi.org/10.1016/j.foreco.2004.05.040

Montalbán IA, De Diego N, Moncaleán P, 2012. Enhancing initiation and proliferation in radiata pine (*Pinus radiata* D. Don) somatic embryogenesis through seed family screening, zygotic embryo staging and media adjustments. Acta Physiol Plant 34: 451-460. https://doi.org/10.1007/s11738-011-0841-6

Montalbán IA, Setién-Olarra A, Hargreaves CL, Moncaleán P, 2013. Somatic embryogenesis in *Pinus halepensis* Mill.: an important ecological species from the Mediterranean forest. Trees-Struct Funct 27: 1339-1351. https://doi.org/10.1007/s00468-013-0882-0

Montalbán IA, García-Mendiguren O, Goicoa T, Ugarte MD, Moncaleán P, 2015. Cold storage of initial plant material affects positively somatic embryogenesis in *Pinus radiata*. New Forest 46: 157-165. https://doi.org/10.1007/s11056-014-9457-1

Morel A, Teyssier C, Trontin JF, Eliásová K, Pešěk B, Beaufour M, Morabito D, Boizot N, Le Metté C, Belal-Bessai L *et al*., 2014. Early molecular events involved in *Pinus pinaster* Ait. somatic embryo development under reduced water availability: transcriptomic and proteomic analyses. Physiol Plantarum 152: 184-201. https://doi.org/10.1111/ppl.12158

Pereira C, Montalbán IA, García-Mendiguren O, Goicoa T, Ugarte D, Correia S, Canhoto JM, Moncaleán P. 2016. *Pinus halepensis* somatic embryogenesis is affected by the physical and chemical conditions at the initial stages of the process. J For Res-Jpn 21: 143-150. https://doi.org/10.1007/s10310-016-0524-7

Pullman GS, Bucalo K, 2014. Pine somatic embryogenesis: analyses of seed tissue and medium to improve protocol development. New Forest 45: 353-377. https://doi.org/10.1007/s11056-014-9407-y

Teyssier C, Grondin C, Bonhomme L, Lomenech AM, Vallance M, Morabito D, Label P, Lelu-Walter MA, 2011. Increased gelling agent concentration promotes somatic embryo maturation in hybrid larch (*Larix x eurolepsis*): a 2-DE proteomic analysis. Physiol Plantarum 141:152-165. https://doi.org/10.1111/j.1399-3054.2010.01423.x