Initiation of embryogenic suspensor masses in *Austrocedrus chilensis*, a vulnerable conifer

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INTROCUCTION

Austrocedrus chilensis; (D.Don) Pic.Serm. & Bizzarri (cypress) is an endemic tree belonging to the Cupressaceae family found in Southern Argentina and Chile, across 140, 000 ha in a wide variety of ecological niches and different soil types. In Argentina, it grows in a 60 to 80 km wide strip along the Andean foothills across a broad moisture gradient (Bran et al. 2002). Cypress is valued not only because of its ecological function, also is one of the few native tree species with high potential to be planted for timber production. It grows relatively fast and the wood has been widely used since it is quite stable and appealing (Aparicio *et al.,* 2009). *Phytophthora austrocedri* is a soil borne pathogen that causes severe mortality of *A. chilensis*. Mortality was first registered in 1948 and the cause remained unknown until few years ago, which generated a deleterious effect on the native forests, leading the species to a serious threat of conservation (Greslebin *et al.,* 2007; Greslebin *et al.,* 2010). Individuals with different degrees of susceptibility to the pathogen are generally observed in affected areas. Since factors associated with the spread of the disease are difficult to control, detection and reproduction of tolerant/resistant individuals seems to be the best solution to the problem. At present, little work with almost no success regarding vegetative propagation of the cypress was done.

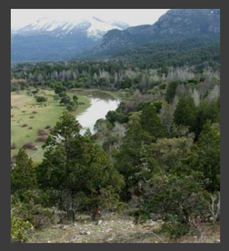

Fig 1 Sites with a high incidence of the disease were selected and in these sites they were identified with a permanent mark and dasometric data was taken from the selected candidate trees.

Materials and methods

THE AIM

of this study was to contribute to the development of a somatic embryogenesis protocol.

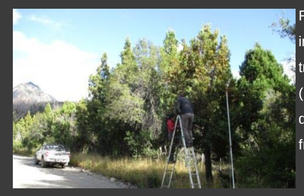

Fig 2: Plant material: One-year-old green female cones, enclosing immature zygotic embryos of A. chilensis, were collected from mature trees growing in a natural stand near Nant y Fall falls, Trevelin Chubut (latitude: 43°11′39.7″S, longitude: 71°28′23.2″W, elevation: 543 m) during summer 2019–2020, ten green cones were sampled fortnightly, from eleven OP families

Fig. 3: Intact cones were surface sterilized with H2O2 3% (v/v) for 5 min, submerged in 70% ethanol for 2 min, followed by 20% bleach treatment (58 g/l). Cones were washed three times with sterile distilled water between each treatment. Cones (<10 mm length, 4 seeds per cone) were opened and immature seeds were directly plated in the different media since their small size, making difficult to isolate the megagametophytes.

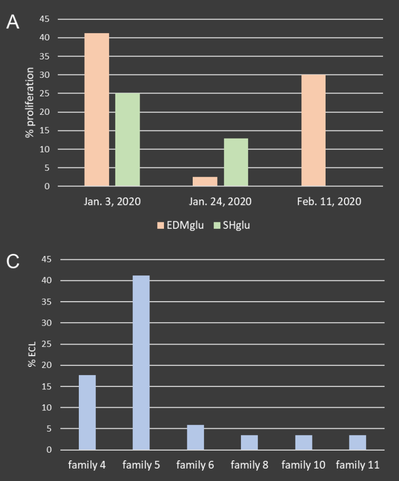

Figure 7. Proliferation of potentially embryogenic tissues (ET) of A. chilensis obtained in different media. A. Percentage of proliferation of selected ET in different media respect to the collection date of the immature seeds. B. Percentage of embryogenic cell lines (ECL) respect to different media. C. Percentage of ECL obtained from different seed families.

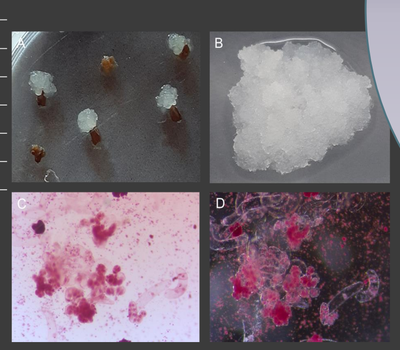

Figure 6. Initiation of the cultures from immature seeds in A. chilensis. A. Incipient callus formation. B. Potentially embryogenic tissue. C-D. Acetocarmine staining and microscope examination of induced tissues that were isolated as potentially embryogenic.

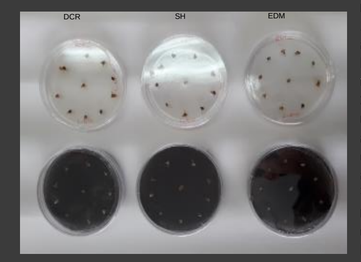

Fig. 4: Six media treatment were tested: Embryo development medium (EDM) (Walter et al. 1998), SH (Schenk and Hildebrandt 1972) and DCR Gupta and Durzan 1985) with 3% (w/v) sucrose and a combination of 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.7 µM benzyladenine (BA) was used. Before autoclaving, the pH of the medium was adjusted to 5.7 and then between 3 and 3.5 g of gellan gum L^{-1} were added depending on the medium used. The medium was autoclaved at 121°C for 20 min. After autoclaving, filter-sterilized solutions with the pH adjusted to 5.7 containing amino acid mix: 550 mg L^{-1} L-glutamine, 525 mg L^{-1} asparagine, 175 mg L^{-1} arginine, 19.75 mg L^{-1} L-citrulline, 19 mg L^{-1} L-ornithine, 13.75 mg L^{-1} L-lysine, 10 mg L^{-1} L-alanine and 8.75 mg L^{-1}L-proline were added to the cooled medium prior to dispensing into Petri dishes (90 x 15 mm)(EDMaa, SHaa and DCRaa) and the same media but instead of amino acid mix, it was added 1g/l L-glutamine, 0.5g/l Myoinositol and 1g/l activated charcoal(AC) (EDMglu, SHglu, DCRglu) (Montalbán et al. 2012).

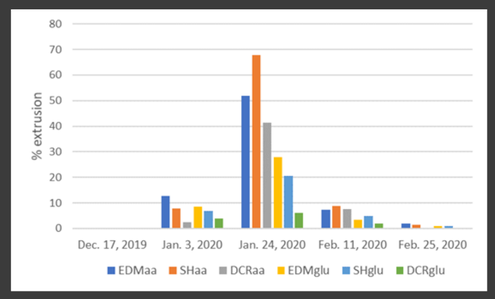

Figure 5. Percentages of extrusion of immature seeds of A. chilensis in different media respect to the collection date of the material.

Results

The best percentages of extrusion were observed from material collected on January 22 in all treatments, followed by the ones obtained from cones collected on January 3 (Figure 5), indicating seed collection time is critical for obtaining a high embryogenic mass initiation. Among media, in EDMaa and SHaa it was obtained the higher percentages of extrusion, denoting the influence of media composition. The highest initiation percentages were obtained with seed families 4, 5 and 11 (12, 18.5 and 21.3%, respectively), indicating the initiation process was genotype dependent. Only the tissues considered potentially embryogenic, i.e., whitish or light in color, translucent and soft, were separated from the explants and sub-cultured in proliferation media (Figure 6A-B). Acetocarmine staining of isolated tissues showed the presence of clusters consisting of both small cells with dense cytoplasm and bigger cells having distinguishable nucleus and light cytoplasm (Figure 6C-D). Considering all treatments, 324 potentially embryogenic callus were obtained and sub-cultured in proliferation media. Potentially embryogenic tissue (ET) derived from material collected on January 3 showed better percentages of proliferation than those collected on January 24, or after (Figure 7A). However, ET proliferated only when previously obtained in EDMglu or SHglu, denoting the importance of the presence of AC in the initiation and proliferation processes (Figure 7A). Taken into account all the embryogenic cell lines (ECL) obtained from material collected in each sampling date, 63% were obtained in EDMglu from material sampled on January 3, while 75% were obtained in SHglu from cones collected on January 24. From the sampling date of February 11, only one embryogenic cell line (ECL) (out of three initial callus) was obtained in EDMglu (Figure 7B). Considering all ECL, approximately half of them were obtained in each media (Figure 7B). Approximately 59% of the ECL derived from the seed families 4 and 5 (Figure 7C).

In conclusion,

it has been determined the most suitable media to achieve the initiation of cultures and obtention of embryogenic cell lines in A. *chilensis* (EDMglu and SHglu). It was demonstrated that the period that covers the month of January is the most suitable for sampling green cones to obtain embryogenic cell lines. Seed families of *A. chilensis* able to produce a greater number of embryogenic cell lines have been also identified. This work is the first report of success in obtaining embryogenic cell lines in *A. chilensis.*

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