

Poster

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

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Abstract: *Austrocedrus chilensis* is a Cupressaceae native to Patagonia. *Phytophthora austrocedri* is a soil-borne pathogen that causes severe mortality of *A. chilensis*. Since factors associated with the spread of the disease are difficult to control, propagation and planting of tolerant individuals seems to be the best solution. At present, a micropropagation protocol for *Austrocedrus* has not been developed. The aim of this study was to contribute to the development of a somatic embryogenesis protocol. The effect of culture medium, collection date and seed family on embryogenic tissue initiation and proliferation in *Austrocedrus* was analyzed. Immature cones were collected from eleven selected trees from natural stands. Sampling was done every 14–21 days from the mid-December to mid-February 2020 and six media were used. The best percentages of extrusion were observed from material collected on January 3 and January 22, and with media EDM and SH supplemented with a mixture of aminoacids and without activated charcoal (AC). However, callus proliferated only when previously obtained in EDM or SH containing AC. Initiation and proliferation processes were genotype dependent, with four seed families showing the highest percentages. This work is the first report of success in obtaining embryogenic cell lines for *A. chilensis*.

Keywords: Patagonian cypress; somatic embryogenesis; plant disease resistance; *Phytophthora* diseases

1. Introduction

Austrocedrus chilensis; (D. Don) Pic. Serm. & Bizzarri (Patagonian 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 [1]. 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 [2]. *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 [3–4]. 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. The aim of this study was to contribute to the development of a somatic embryogenesis protocol.

2. Materials and Methods

2.1. 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, Argentina (43°11'39.7"S, 71°28'23.2"W, elevation: 543 m) during summer 2019–2020. Green cones were sampled every 14–21 days, from selected eleven healthy open pollinated trees, from the mid of December to the mid of February, and stored for a maximum of a week at 4°C. Cones are mature to be harvested on March.

2.2. Initiation and proliferation of potentially embryogenic tissue

Intact cones were surface sterilized with 3 % H₂O₂ 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 made it difficult to isolate the megagametophytes.

Six media treatments were tested: Embryo Development Medium (EDM) [5], SH [6] and DCR [7], supplemented 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). Before autoclaving, the pH of the medium was adjusted to 5.7 and then 3 g/l gellan gum was added. 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 L-glutamine, 525 mg/l asparagine, 175 mg/l arginine, 19.75 mg/l L-citrulline, 19 mg/l L-ornithine, 13.75 mg/l L-lysine, 10 mg/l L-alanine and 8.75 mg/l L-proline] were added to the cooled medium prior to dispensing into Petri dishes (90 x 15 mm) (named here as EDMaa, SHaa and DCRaa) [8]. Also the same media were used but instead of the addition of the mixture of amino acids, it was added 1 g/l L-glutamine, 0.5 g/l myoinositol and 1 g/l activated charcoal (AC) (named here as EDMglu, SHglu, DCRglu). The experiments consisted of three Petri dishes per treatment with ten immature seeds per Petri dish laid out randomly. Cultures were maintained in the dark at 21° ± 1°C. The percentage of extrusion (number of callus respect to the total number of explants) in each treatment was recorded.

After 8–12 weeks from the start of the experiment, proliferating potentially embryonal tissue (ET) with a size around 5 mm in diameter was separated from the immature seed. ET were sub-cultured to the proliferation media, with 2-week subculturing intervals. The proliferation media had the same composition of the initiation media but contained 4.5 g l⁻¹ gellan gum. Following four subculture periods, actively growing ET were recorded as established cell lines (ECL) and the number of ECL respect to the total number of callus per treatment was registered as the percentage of proliferation. Acetocarmine staining was performed to examine potentially embryogenic tissues.

3. Results and Discussion

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 1), 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.

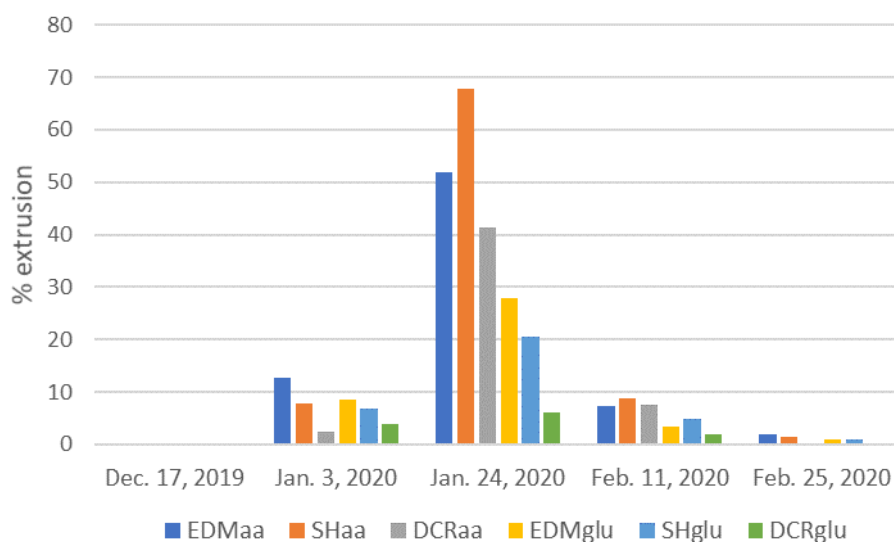


Figure 1. Percentages of extrusion of immature seeds of *A. chilensis* in different media respect to the collection date of the material.

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 2A-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 2C-D).

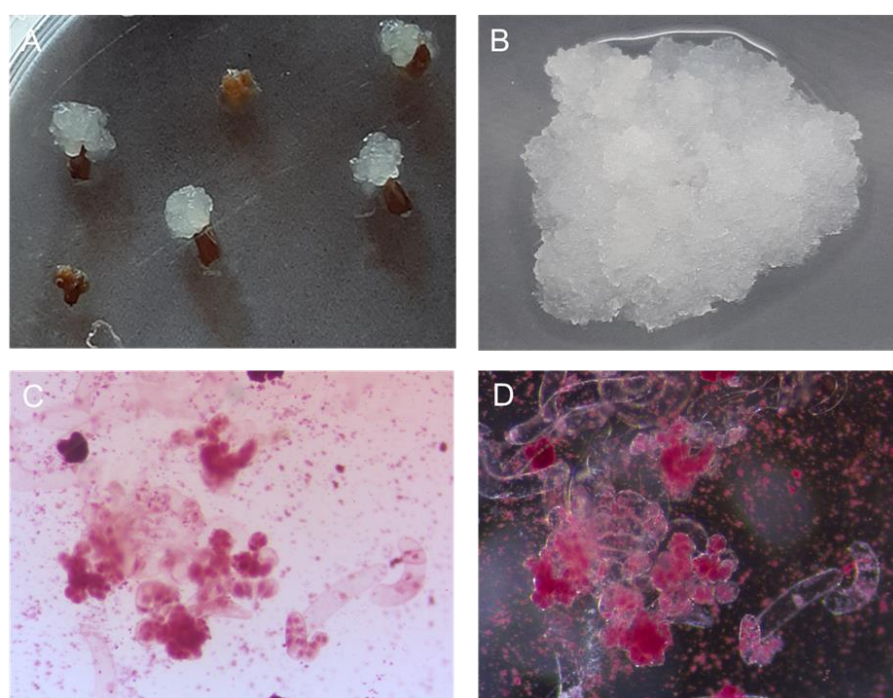


Figure 2. Initiation of the cultures from immature seeds in *A. chilensis*. A. Incipient callus formation. B. Potentially embryogenic tissue. C-D. Acetocarmine staining and microscope examination of induced tissues that were isolated as potentially embryogenic.

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 3A). 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 3A). 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 3B). Considering all ECL, approximately half of them were obtained in each media (Figure 3B). Approximately 59% of the ECL derived from the seed families 4 and 5 (Figure 3C).

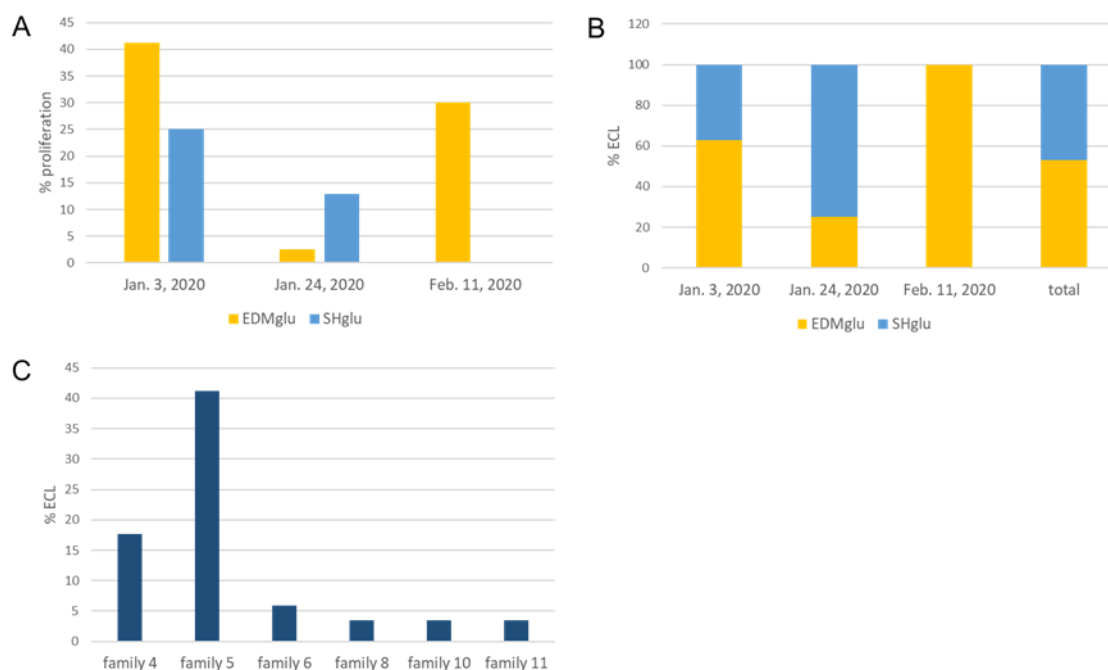


Figure 3. Proliferation of potentially embryogenic tissues (ET) of *A. chilensis* obtained in different media. A. Percentage of proliferation of selected ET in different media respect to the collection date of the immature seeds. B. Percentage of embryogenic cell lines (ECL) respect to different media. C. Percentage of ECL obtained from different seed families.

4. Conclusions

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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Conflicts of Interest: The authors declare no conflict of interest.

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