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Investigación, gestión y valores del agua en el mundo actual

Miguel Angel Alvarez-Vázquez y Elena De Uña-Alvarez
(Coordinadores)

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Meléndez Valdés, 61 - 28015 Madrid
Teléfono (+34) 91544 28 46 - (+34) 91544 28 69
e-mail: info@dykinson.com
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IN VITRO PROPAGATION OF TOLERANT ALDERS FOR THE CONSERVATION OF RIVERBANKS. STUDY OF THEIR RESISTANCE TO PHYTOPHTHORA ALNI

M.C. San José [a], M.J. Cernadas [a], L.V. Janeiro [b], N. Blázquez [a], F. Mosteiro [a], A. De Anta [c], E. De Castro [c] y B. Cuenca [d]

[a] Instituto de Investigaciones Agrobiológicas de Galicia, CSIC, Avda. de Vigo s/n, 15705 Santiago;

[b] Diputación de Lugo. San Marcos 8, 27001 Lugo;

[c] Confederación Hidrográfica Miño-Sil (CHMS), El Progreso 6, 32005 Ourense;

[d] TRAGSA, Vivero de Maceda, 32700 Maceda, Ourense

Corresponding author: sanjose@iiag.csic.es

I. INTRODUCTION

The common alder [*Alnus glutinosa* (L.) Gaertn.] is a medium-sized tree that is widely distributed over the continent of Europe, north and north-east Asia, and north-east Africa, where it has considerable importance in the maintenance of the riparian ecosystems. In the last few years, its habitat has greatly deteriorated, not only due to deforestation, but also due to the effect of different diseases, among which should be highlighted is that known as alder blight disease caused by the *Phytophthora alni* oomycete (Brasier et al., 2004). The disease has caused the death of numerous specimens without there being, until now, any effective method for its control.

Biotechnology techniques, in particular micropropagation, offer the possibility to propagate, conserve, and improve selected specimens of different species. Within these methods, the *in vitro* propagation by means axillary buds culture is the most widely used, since it ensures the genetic stability of the micropropagated material, as well as the production of a large number of plants in a relatively short period of time. The first works on the *in vitro* regeneration of alder go back to the 1980's in which the importance of actinorhizal host plants in forestry were recognized.

However, the works carried out in this period were mainly performed with material of juvenile origin and, and thus, of unknown characteristics. More than twenty years later, when the damage caused by the disease had already been observed, works were started with adult material (San José et al., 2016).

The aim of this work was the micropropagation of specimens of *A. glutinosa* tolerant to *P. alni*, which were selected in areas affected by the disease, and the subsequent evaluation of their resistance to *Phytophthora* in order that they could be used for the restoration of the ecosystems affected.

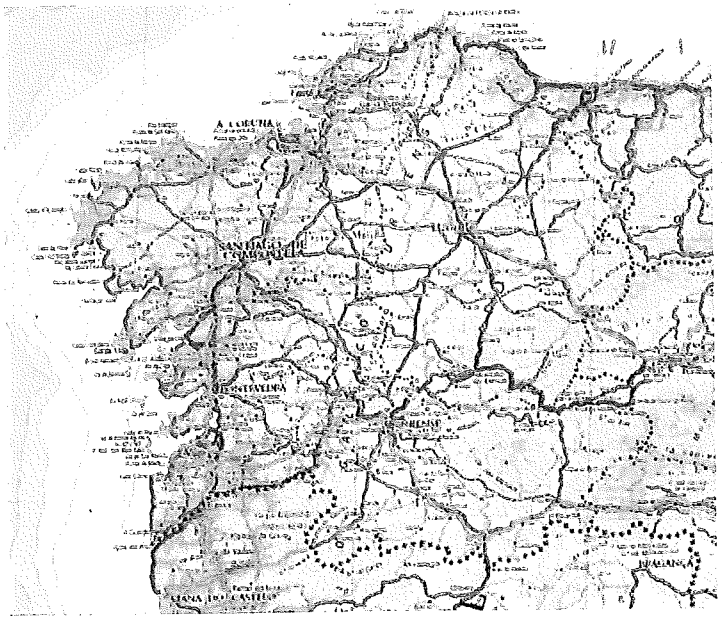
II. MATERIAL AND METHODS

Branches of the crown or basal shoots of trees between 25 and 40 years-old were collected in different riverside locations of the Communities of Galicia and Castilla-Leon. Within the Community of Galicia, material was collected in the river basins of: Orille (5 trees), Avia (5), Arnoia (5), Limia (4), Arenteiro (2), arroyo Polígono (4) (Ourense province); Azumaga (3), Pequeño (2), Miño (4) (Lugo province); Louro (5) (Pontevedra province); Sarela (3, in this case they were established from seeds), and Galanas (1) (A Coruña province). In the Community of Castilla-Leon, 2 tree specimens were collected in the River Burbia, and 3 trees in the River Selmo (Leon province) (Figure 1).

The branches (0.5-3 cm thick) were cut into segments of 20-25 cm in length and then treated with an anti-fungal solution for 2 hours, after which they were left to dry in the air. The cuttings were subsequently placed in a vertical position in a tray with perlite dampened with water and placed in a growth chamber with light (90-100 $\mu mol.m^{-2}.s^{-1}$ and 16h light/8h darkness photoperiod), controlled temperature (25°C) and humidity (80-90%). Ten to 40 cuttings were used per genotype. After 2-3 weeks, growth was observed from the axillary buds. The new shoots (2-5 cm) were cut, leaves removed, washed in running water for 5-10 minutes, and superficially sterilized with a solution of 0.6% hypochlorite (Millipore® chlorine tablets) with 2-3 drops of Tween 80® for 5 minutes. They were

then cleared with 3 washes of sterile water, ten minutes each. Apices and nodal segments (5-7 mm) were used as initial explants. The explants were placed in a vertical position in culture tubes containing 20 ml de Woody Plant Medium (WPM; Lloyd and McCown, 1980), supplemented with 30 g /l glucose, 7 g/l agar, 0.5 mg/l benzyl adenine (BA) and 0.5 mg/l indole acetic acid (IAA).

Figure 1. GALICIAN MAP SHOWING THE DIFFERENT LOCALIZATIONS FROM WHICH THE ALDER SAMPLES WERE TAKEN.



Due to their better behaviour *in vitro*, 15 genotypes were selected for their subsequent multiplication. In this stage the same mineral medium is used as in the establishment, but with the concentration of BA reduced to 0.1 mg/l. The cultures are transferred to fresh medium every 3 weeks until completing a cycle of 9 weeks. Shoots of 9 weeks (1.5-3 cm) were employed for the rooting stage, in which WPM medium was used with the macronutrients reduced to half (½WPM) supplemented with 0.1 mg/l of indole-butyric acid (IBA). The shoots remained in this medium for 7 days, subsequently being transferred to a medium of equal composition without auxin.

All the cultures were kept in a growth chamber with a photoperiod of 16 h/8 h, a light intensity of 50-60 $\mu mol.m^{-2}.s^{-1}$, and a temperature of 25°C (day) and 20°C (night).

The evaluation of the resistance to was made using the inoculation methods in leaves, twigs, and stems, and by supplementing the substrate with mycelium (Haque et al. 2015; Chandelier et al. 2016).

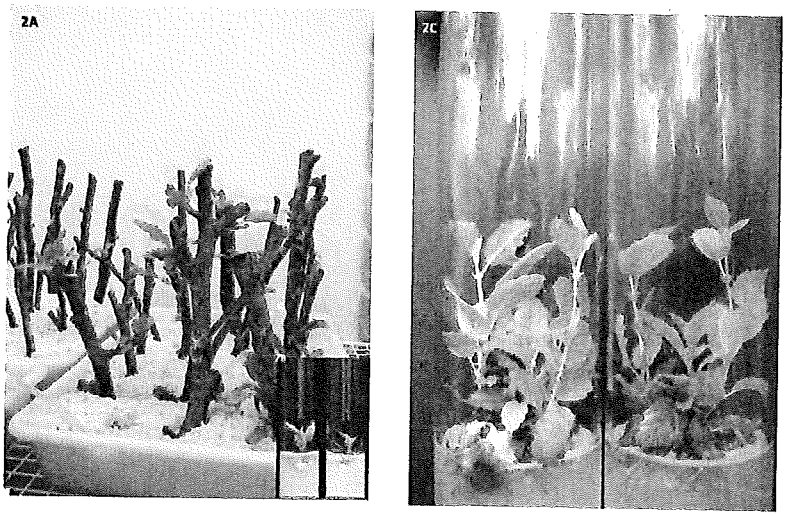
III. RESULTS AND DISCUSSION

The sprouting rate varied between 15% and 94% and, although all the clones selected produced sprouting, large differences were observed in their *in vitro* behaviour. As regards the genotype, the differences found could also be attributed to the type of material used for the establishment of the cultures and the age of the tree (Bonga et al., 2010). The branches obtained in the basal areas were more reactive than those coming from the crown. The development of the shoots in a growth chamber increases the vigor of the material that is going to be introduced *in vitro* and reduces the initial contamination (Figure 2A). The genotypes: Orille 3 and 5, Arnoia 1, Louro 4, Arenteiro 1 and 2, Polígono 1, 3 and 4, Rábade 1 and 4, Galanas 1, and Sarela 1, 2 and 3, developed better *in vitro*, and so were selected for micropropagation.

The stabilizing of the cultures was achieved between 5 and 12 months after their establishment. The cultures were considered to be stable when they had a uniform and predictable growth (Figure 2B). This is when the multiplication phase begins. The apical and nodal segments (1 cm) are sub-cultivated in a vertical position in multiplication media in which it is important to add a cytokinin (BA 0.1 mg/l) (Figure 2C). The results are dependent on the genotype and are also influenced by the type of explant, with a better response being obtained with nodal segments. In previous works it was shown that there was a need to establish a multiplication cycle of 9 weeks with 3 transfers of 3 weeks each one. The response percentages, the number and length of the shoots increased greatly when shoots were transferred to fresh medium every 3 weeks in a 9-week multiplication period. Frequent transfer to fresh medium was important

to prevent apical senescence, to increase multiplication rates and to reinvigorate the cultures (San José et al. 2013). At the end of each cycle, the apical and nodal segments of the new shoots may be sub-cultivated for a new multiplication cycle or used in the rooting stage.

Figure 2. ESTABLISHMENT AND MULTIPLICATION STAGES FOR ALDER GENOTYPES. A) BRANCHES FROM POLIGONO 4 GENOTYPE WERE FORCED TO FLUSH IN THE GROWTH CHAMBER; B) IN VITRO ESTABLISHMENT OF THE FORCING SHOOTS; C) MULTIPLICATION OF AXILLARY SHOOTS FROM ORILLE 3 GENOTYPE IN WPM MEDIUM SUPPLEMENTED WITH 0.1 MG/L OF BA AND 0.5 MG/L AIA.

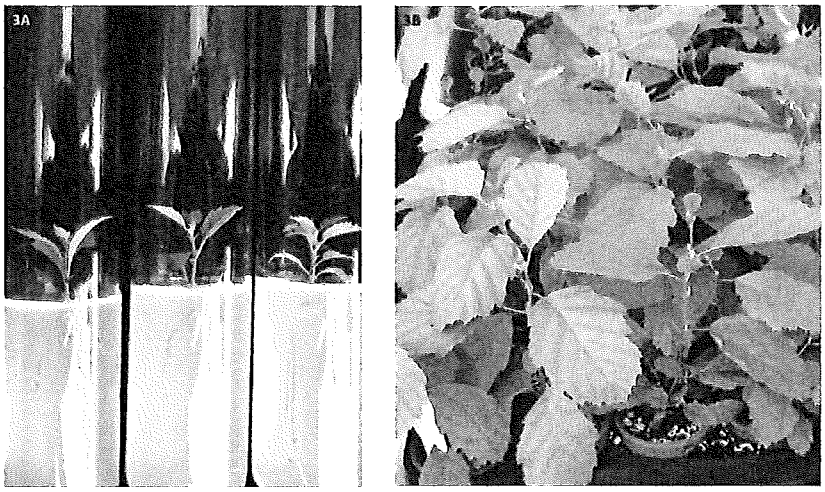


Although in some genotypes (R1, R4, G1, Sarela 1, 2 and 3) the rooting could be produced without the addition of IBA, the incorporation of the auxin to the rooting medium speeds up the formation of the roots and increases the number of roots per shoot (Figure 3A). As with the multiplication rates, the rooting percentages are highly influenced by the genotype. The response rate varies between 63%, in the case of Arnoia, to 99%, for Rábade 4. It increases to 100% in the case of the Sarela 1, 2, and 3 genotypes. It has to be taken into account that this material is established from zygotic embryos, and therefore is material of juvenile origin.

After 1 month in the rooting medium, shoots that had formed roots were successfully acclimatized in a growth chamber. After 6 weeks in the

greenhouse the survival rates ranged between 80–95% among the different genotypes (Figure 3B).

Figure 3. ROOTING AND ACCLIMATIZATION STAGES FOR ALDER GENOTYPES. A) ROOTING OF THE SHOOTS FROM R1 GENOTYPE IN ½ WPM CONTAINING 0.1 MG/L IBA FOR 7 DAYS. B) ACCLIMATIZATION OF THE IN VITRO PLANTS (GENOTYPE G1).



The genotypes R1, Orille 5, Sarela 3, Arenteiro 2, Louro 4, and Arnoia 1, gave the best results in the tolerance to *Phytophthora* test.

IV. CONCLUDING REMARK

This protocol provides a simple and efficient method for the selection of tolerant alders, which can be micropropagated in vitro, tested for their resistance to *P. alni* and used for the recuperation of the threatened ecosystems.

REFERENCES

BONGA, J.M., KLIMASZEWSKA, K.K. and VON ADERKAS P. (2010), «Recalcitrance in clonal propagation, in particular in conifers», *Plant Cell Tissue and Organ Culture*, n.º 100, pp. 241-254.

BRASIER, C.M., KIRK, S.A., DELCAN, J., COOKE, D.E.L., JUNG, T., and MAN IN'T VELD, W.A. (2004), «*Phytophthora alni* sp. *nova* and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees», *Mycological Research*, n.º 108, pp. 1172-1184.

CHANDELIER, A., HUSSON, C., DRUART, P., and MARÇAIS, B. (2016), Assessment of inoculation methods for screening black alder resistance to *Phytophthora* × *alni*. *Plant Pathology*, n.º 65, pp. 441-450.
HAQUE, M. M. U., MARTÍN-GARCÍA, J., and DIEZ, J. J. (2015), Variation in pathogenicity among the three subspecies of *Phytophthora alni* on detached leaves, twigs and branches of *Alnus glutinosa*, *Forest Pathology*, n.º 45, pp. 484-491.

LLOYD, G., and MCCOWN, B.H. (1980), «Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture», *Proceedings of the International Plant Propagators Society*, n.º 30, pp. 421-427.

SAN JOSÉ, M.C., JANEIRO, L.V., and CORREDOIRA, C. (2013), «Micropropagation of threatened black alder», *Silva Fennica*, n.º 47, pp. 1-12.

SAN JOSÉ, M.C., JANEIRO, L.V, MARTÍNEZ, M.T., VALLADARES, S., CERNADAS, M.J., MONTENEGRO, R., and CORREDOIRA, E. (2016), «Biotechnological approaches for the improvement and conservation of *Alnus glutinosa* (L.) Gaertner», ANIS, N., AHMADA, N. (eds.): *Plant Tissue Culture: Propagation, Conservation and Crop Improvement*, Springer Science+Business Media, Singapore, pp. 467-486.