**Improvement and assessment of a micropropagation protocol for walnut hybrid (*Juglans major* var. 209 (Torrey) Heller x *J. regia* L.)**

**Ricardo J. Licea-Moreno1,3, Yolanda González1, Ana Valeria Morales1, Ignacio Urbán2, Carlos Homar2, Angela Contreras3, Marcos Daquinta Gradaille4, Jordi Voltas5, Luis Gomez3**

1 Department of Biotechnology, Micropropagation Unit, Bosques Naturales S. A. Spain ricardolicea@bosquesnaturales.com

2 Department of Forestry, Bosques Naturales S. A., Spain

3 Department of Biotechnology, Center for Plant Biotechnology and Genomics, Universidad Politécnica de Madrid, Spain

4 Bioplantas Center, Universidad de Ciego de Avila, Cuba

5 Department of Crop and Forest Sciences, Universidad de Lleida, Spain

Abstract

*Juglans* is considered a genus highly recalcitrant to *in vitro* culture. Whereas a few genotypes have been successfully micropropagated, large scale production is impractical for most laboratories. In 2008, Bosques Naturales (Spain) set up a specialized unit with the main goal of micropropagating walnut hybrid clones selected for wood production. Once a clone is introduced in vitro, its multiplication behavior, rooting ability and survival under *ex vitro* conditions is largely genotype-dependent. Hardening success is typically linked to obtaining rooted microshoots before transplantation. Rooting ability and survival are influenced, in turn, by the quality of microshoots. Healthy and green microshoots of at least 20 mm in length are the most suitable for root pre-induction. We have demonstrated that the use of FeEDDHA as iron source, instead of FeEDTA, along with Phloroglucinol is pivotal to obtaining high quality microshoots under our experimental conditions. At the same time, we have found that rooting quality depends on subculture duration. Three sets of results are presented here: (1) an optimized protocol aimed to improve root formation during expression phase and survival during acclimation, after analyzing factors such as carbon source and concentration and the use of microshoots from Temporary Immersion System (TIS); (2) the choice of a set of 11 highly-polymorphic SSR markers to unambiguously define the genetic profile of selected plus trees, as well as to assess the genetic stability of micropropagated material; and (3) a detailed evaluation of the first field experiment with clonal material (7 clones) produced at Bosques Naturales using the aforementioned optimized protocol.

Key words: recalcitrance, acclimation, temporary immersion, microsatellites, genetic marker, rooting