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Research article

Physiological characterization and true-to-typeness evaluation of *in vitro* and *ex vitro* seedlings of *Pinus elliottii*: A contribution to breeding programs

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ABSTRACT

Pinus elliottii var. *elliottii* is a pine species with enormous economic value particularly for timber and resin industries, and is subject of high pressure for genetic improvement and cloning elite genotypes. We have recently developed a robust micropropagation protocol for this species. Plantlets performance needs to be evaluated to validate this protocol for further mass propagation. Micropropagated plantlets and seed-derived plants with similar age and shoot length were compared regarding photosynthesis, carbohydrates and pigments content, water status, DNA content and cell cycle dynamics. Micropropagated plantlets had an overall physiological performance similar to seed-derived plants. In particular, except for the transpiration rate (E), CO_2 assimilation rate (A) and total soluble sugars (TSS) content, no major differences between plantlets and seedlings in terms of relative water content (RWC), chlorophyll *a* fluorescence and pigments content were found. Genetic fidelity analyses support that the micropropagation protocol neither induce DNA content changes nor alterations in cell cycle dynamics.

1. Introduction

Slash Pine (*Pinus elliottii* var. *elliottii*) has particular economical value to timber industry due to its wood quality, and to its fast growth rates compared with other conifers. Moreover, this species is also characterized by the high quality resin production (Jain and Gupta, 2005). *P. elliottii* var. *elliottii* was already introduced in reforestation programs and industrial plantations of this species can now be found worldwide, particularly in North America, Brazil or Australia.

A breeding program of a selected species must include cloning strategies (e.g. Pascoe, 2002) of the elite genotypes. Vegetative propagation techniques have played a crucial role in the establishment of clonal seed orchards. In the last decades *in vitro* plant propagation, namely micropropagation, has been successfully applied to forestry species for the mass propagation of important and economically valuable forest species (e.g. Conde et al., 2008), including pine species (e.g. Marum et al., 2009; Klimaszewska et al., 2011). Micropropagation allows the conservation of forest species, as it makes possible an acceleration of plant physiological rejuvenation, and a germplasm rescue, regardless of the availability of

Abbreviations: Φ_{PSII}, effective photochemical efficiency of PSII; A, CO_2 assimilation rate; BAP, 6 benzylaminopurine; Chl, Chlorophyll; DW, dry weight; E, transpiration rate; FCM, Flow cytometry; FW, fresh weight; IBA, indole-3-butyric acid; NPQ, non-photochemical quenching; PE, *Pinus elliottii var. elliottii*; *Pi*, propidium iodide; PSII, photosystem II; qP, photochemical quenching; RH, relative humidity; RWC, relative water content; TSS, total soluble sugars; TW, turgid weight; WPB, Woody Plant Buffer.

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seed (Aguiar et al., 2011). This is particularly important for forest elite genotypes with long life cycles, as is the case of most *Pinus* species. In particular, for *Pinus*, the acceleration of growth rates and biomass production may have a crucial economical gain.

The successful application of micropropagation techniques on a commercial scale requires that this technique provide genetically stable plantlets (e.g. Lopes et al., 2006; Fernandes et al., 2008) with good physiological performance (Santos et al., 2003) obtained with low cost and high survival rates. Also major constraints may arise during acclimatization (often associated with, e.g. root dysfunctions, stomatal and gas exchange disorders), which may impact carbon metabolism and ultimately plant growth. In particular, typical *in vitro* propagation conditions such as high humidity, low illumination and CO_2 levels, high carbon source levels and the presence of growth regulators, provide minimal stress and optimum conditions for shoot/plant multiplication, but may result in the development of morphologically, anatomically and physiologically abnormal plantlets (Osório et al., 2012; Dias et al., 2013). The heterotrophism promoted during *in vitro* conditions and the poorly developed mechanisms to control water loss (e.g. poor stomatal control, cuticular abnormalities) render micropropagated plants vulnerable to the transplantation shocks when directly placed in a greenhouse or field. During acclimatization these abnormalities are corrected and plants adapt to the new growth conditions (Hazarika, 2006; Dias et al., 2014b). The ability to modify the phenotype and its underlying metabolism in response to environmental changes is known as phenotypic plasticity (Osório et al., 2013). So, to validate a micropropagation protocol there is a need to compare micropropagated and seed-derived plants in the same ambient conditions to evaluate the phenotypic plasticity of micropropagated plants and ensure that they can adapt to survive in their natural environment.

Physiological performance of micropropagated plants has been followed in several species, such as *Tuberaria major* (Osório et al., 2013), *Olea maderensis* (Santos et al., 2003) and *Ceratonia siliqua* (Osório et al., 2012). In a few cases, differences in the performance of micropropagated plants were identified, but after a period of adaptation to the natural environment performances were mostly identical to those from plants originating from seed. For *Pinus taeda* no differences in the physiological or morphological performance between somatic *vs*. zygotic seedlings were found (Becwar and Pullman, 1995). However, when plants were grown in drought-prone sites, significant differences were found between the performances of seedlings versus micropropagated plantlets, but their magnitude was small and decreased with time (sixth and seventh growing seasons) (Rahman et al., 2003). These authors supported that plantations on regions prone to drought periods need to use more developed/elder loblolly pine plants when these have an *in vitro* origin (Rahman et al., 2003).

It has been described that the stability of plant genomes can be affected by the *in vitro* conditions to which the plants are subjected during the propagation process. Due to the possible occurrence of somaclonal variation, the analysis of the ploidy stability of micropropagated plants is of particular importance. Flow cytometry (FCM) is one of the most reliable techniques to estimate the DNA ploidy level and nuclear DNA content in plants (e.g. Loureiro et al., 2007a; Marum et al., 2009). In comparison with other methods, as Feulgen microdensitometry and chromosome counting, flow cytometry (FCM) provides unsurpassed ease, speed and accuracy (Doležel and Bartoš, 2005). In recent years, this technique has been successfully applied in the analysis of somaclonal variation in a vast number of woody species, including conifers (Loureiro et al., 2007a; Fernandes et al., 2008; Marum et al., 2009).

We have recently developed a protocol for micropropagation of *P. elliottii* var. *elliotti* (Nunes et al., 2012) (Fig. 1) suitable for mass propagation of elite genotypes. Despite protocols have been developed for other conifers and *Pinus* in particular (e.g. Kalia et al., 2007; Oliveira et al., 2012), no published work is available on the functional performance of micropropagated plantlets under *ex vitro* conditions in comparison with plants derived from seedlings. The aim of this work was to verify that the micropropagation protocol developed for slash pine did not compromise the performance of the micropropagated plants, compared to zygotic seedlings. For that, leaf gas exchange, chlorophyll fluorescence characteristics, relative water content and pigments and carbohydrates quantification in micropropagated and seed source plants were analyzed. The ploidy stability of plantlets and seedlings was assessed using FCM.

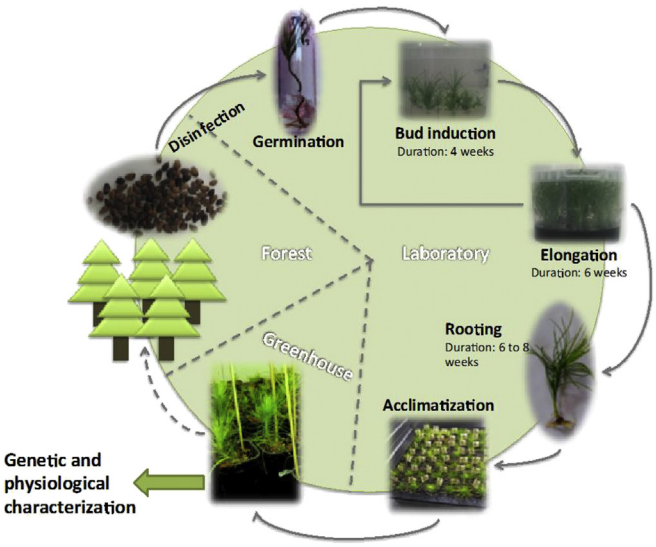

Fig. 1. Summary of micropropagation protocol developed for *Pinus elliottii* var. *elliotti*: Seeds were germinated and after 4 weeks shoot apices of seedlings were inoculated in WV5 medium enriched with 10 μM BAP. After 4 weeks, plants were transferred to an elongation medium (WV5+0.2% activated charcoal). Rooting was achieved after 6–8 weeks in WV5 medium +10 μM IBA (Nunes et al., 2012).

2. Materials and methods

2.1. Plant material and experimental conditions

Seeds obtained from open pollinated trees, of *Pinus elliotti* var *eliiotti* (PE), were provided by the company Resisul Fortaleza, Ltda. Seeds were collected in São Paulo region, Brazil at 2009 and conserved at 4 °C until utilization. These seeds were used to the control (seedlings germinated *ex vitro* on the greenhouse) and as explants for the production of micropropagated plantlets. Plantlets used in this study were obtained from micropropagated *in vitro* culture collection of PE previously established from shoot apices of seedlings as described by Nunes et al. (2012) and summarized in Fig. 1.

Groups of micropropagated plantlets and seedlings (each group with seven individuals) were used in this study. Both seedlings and plantlets had approximately 8 cm of shoot height and average age around 6 ± 1 months and were maintained in a growth chamber with a day/night temperature of 27/22 °C, a 16-h photoperiod, a relative humidity of 50% and a light intensity of 300 μmol m^{-2} s^{-1}.

2.2. Plant water status

The fresh and dry weight of needle segments were determined.