The Fifth International Conference of the
IUFRO Unit 2.09.02:
Somatic Embryogenesis and Other Vegetative Propagation Technologies

Proceedings

Clonal Trees in the Bioeconomy Age: Opportunities and Challenges

September 10-15, 2018
University of Coimbra
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CENTRE FOR FUNCTIONAL ECOLOGY

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IUFRO Unit 2.09.02
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Micropropagation of willow shoots under photomixotrophic and photoautotrophic conditions: proliferation in liquid medium and acclimation in different soils

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Abstract
Salix viminalis is a species with a high capacity for micropropagation and acclimation, and could therefore be used to develop emergent techniques in the field of plant propagation. The aims of this study were to propagate willow in liquid medium, both by temporary and continuous systems, to explore the application of photoautotrophic conditions and to investigate the acclimation of willow plantlets in different types of soils that could be used as alternatives for commercial peat. The results indicate that liquid medium under photomixotrophic and under photoautotrophic conditions can be satisfactorily used to culture willow axillary shoots. Regarding acclimation, although there were some differences in aerial growth, the willow plants were successfully acclimated in all soils.

Keywords: bioreactors, continuous immersion, enzymes, rhizosphere, temporary immersion

Introduction

Salix viminalis L., also known as basket willow, is a promising candidate for bioenergy and phytoremediation applications (Mleczek et al. 2010; Touceda-González et al. 2017). Some micropropagation protocols in semisolid medium have been developed for *Salix* species (Bhojwani 1980; Bergman et al. 1985; Read et al. 1989; Amo-Marco and Lledo 1996; Park et al. 2008; Mashkina et al. 2010; Skálová et al. 2012; Palomo-Ríos et al. 2015). Its feasibility for propagation and acclimation make it a useful material to develop emergent techniques in the field of plant propagation. Recently, a protocol for culture in liquid medium by temporary immersion was reported (Regueira et al. 2018). Micropropagation in liquid medium, providing uniform and better controlled environmental conditions, may complement or represent an alternative to the hydroponic culture systems frequently used to test the ability of willow to absorb and accumulate heavy metals and other contaminants.

The objectives of the present study were: i) to investigate the propagation of willow shoots in liquid medium by temporary and continuous immersion systems, ii) to propagate willow under photomixotrophic and photoautotrophic conditions, iii) to acclimate willow plantlets in different types of soils that could be used as alternatives to commercial peat.

Materials and methods

Willow shoots were previously established in vitro from actively growing branches collected in midsummer from a mature tree (Regueira et al. 2018).

Propagation of willow was carried out in jars (Fig. 1A) in semisolid medium (SSM) in standard conditions and in liquid medium (LM) both in standard and in photoautotrophic conditions (see below). The medium consisted of MS (Murashige and Skoog) salt and vitamin mixture (Murashige and Skoog, 1962) with half strength nitrates (MS½N) and supplemented with 0.22 µM of BA and 3% sucrose. In SSM, 0.7 % (w/v) Bacto agar was used. In some experiments with LM, sucrose was not supplemented. In standard conditions, cultures were incubated under a 16-h photoperiod provided by cool-white fluorescent lamps (photosynthetic photon flux density (PPF) of 50-60 µmol $m^{-2}s^{-1}$) at 25 ˚C light/20 ˚C dark, whereas in photoautotrophic conditions a higher PPF (150 µmol m^{-2} s^{-1}) was provided by white LEDs and CO_2-enriched air was supplied.

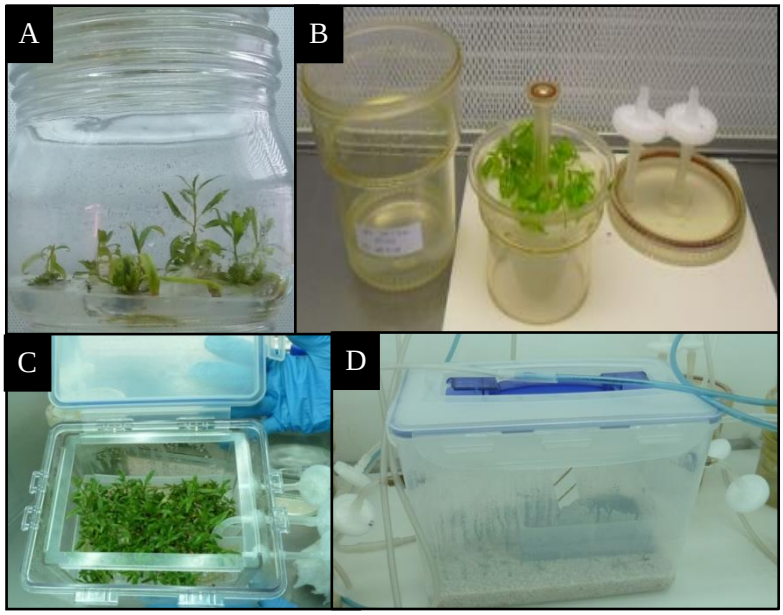

Figure 1*. Vessels used for propagation of willow. A) Glass jars. B-C) Commercial bioreactors RITA® (B) and plantform™ (C). D) In-house bioreactors made from food storage containers.*

Proliferation by temporary immersion

Shoots were cultured in liquid medium by temporary immersion (TIS) under standard conditions (low PPF and 3% sucrose). Apical and basal segments of willow were cultured in RITA® (www.vitropic.fr) and plantform™ (www.plantform.se; Welander et al. 2014) bioreactors (Fig. 1B, C) as described in Regueira et al. (2018). Three immersions of 1 min per day were applied, and in the case of plantform, additional aerations of 1 min/h were supplied.

Proliferation by continuous immersion

For continuous immersion (CIS), basal sections of willow were placed in rockwool cubes soaked in medium with sucrose (S3) or in the same medium devoid of sucrose (S0). Vessels (10 L) were made from food storage containers (Fig. 1D**)** and were equipped with 0.2 µm filters to receive forced ventilation with CO_2-enriched air (2000 ppm; 1 min/h), as described for chestnut (Vidal et al. 2017).

Acclimation and effect of the type of soil

Rooted shoots obtained by CIS under photomixotrophic and photoautotrophic conditions were selected for evaluating different soils during the acclimation step. Both groups of plants were transferred to a phytotron and planted in commercial peat for a first acclimation step. One month later, the plantlets were measured and their roots were carefully washed to eliminate the rests of peat. Then, the plantlets were transferred to new pots in the greenhouse.

Three types of soil were used:
1) commercial peat,
2) soil from an oak forest, with high organic matter content,
3) crop soil with low organic matter content.

Six weeks later, the plants were removed from the soil, and parameters related to the growth response of the 6 groups of plants (2 micropropagation systems and 3 soils) were recorded. In addition, the hydrolytic enzymatic activities of the soils were determined as described by Trasar-Cepeda et al. (2008) and dehydrogenase activity by the method of Camiña et al. (1998).

Results

Proliferation by temporary immersion
The appearance of willow shoots cultured in standard conditions with 3% sucrose both in SSM and in LM by temporary immersion is presented in **Fig. 2**, and the proliferation obtained in each type of container is presented in **Fig.3.**

Figure 2*. Willow shoots cultured with 3% in standard conditions. (A) Glass jars. (B) RITA®. (C) plantform™.*

As can be observed in Fig. 3, TIS favored shoot proliferation. Similar results were observed in other woody plants such as calabash tree, apple, teak and pistachio (Murch et al. 2004, Zhu et al. 2005, Quiala et al. 2012, Akdemir et al. 2014). The highest multiplication coefficient was observed in plantform™ bioreactors, confirming the results obtained in a previous study (Regueira et al. 2018). Similar results were reported for chestnut (Vidal et al. 2015). Basal shoots produced more segments than apical explants, and consequently were used for experiments in CIS.

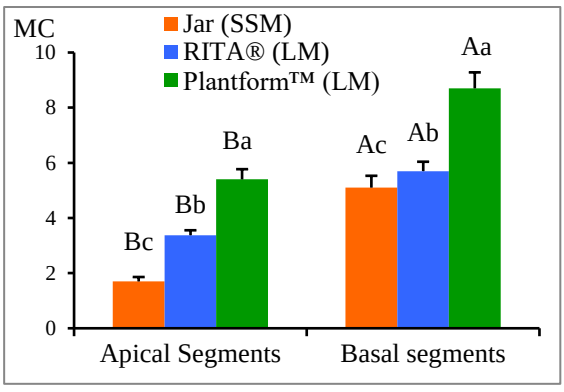

Figure 3*. Effect of type of explant and the culture system on the multiplication coefficient (MC) of willow shoots. SSM: semisolid medium. LM: liquid medium.*

Proliferation by continuous immersion
Basal shoots were successfully propagated in continuous immersion photoautotrophic conditions (high PPF and CO_2 supply), both with supplementation of 3% sucrose (photomixotrophic growth) and without

sucrose (photoautotrophic growth). Shoots cultured without sucrose presented a vigorous aspect (Fig. 4A). All shoots formed roots spontaneously (Fig. 4B).

***Figure 4**. Proliferation of willow under photoautotrophic conditions. (A) Willow shoots cultured in CIS without sucrose. (B) Shoots with roots formed spontaneously in the proliferation medium.*

The multiplication coefficient of shoots proliferated in CIS is shown in Fig. 5. The differences between shoots cultured with or without sucrose were not significant for any of the parameters tested, although the explants cultured without sugar showed a slightly higher multiplication coefficient. The beneficial effects of culturing woody plants in photoautotrophic conditions using large vessels with forced ventilation has been reported by other authors as Zobayed (2005) and Xiao et al. (2011).

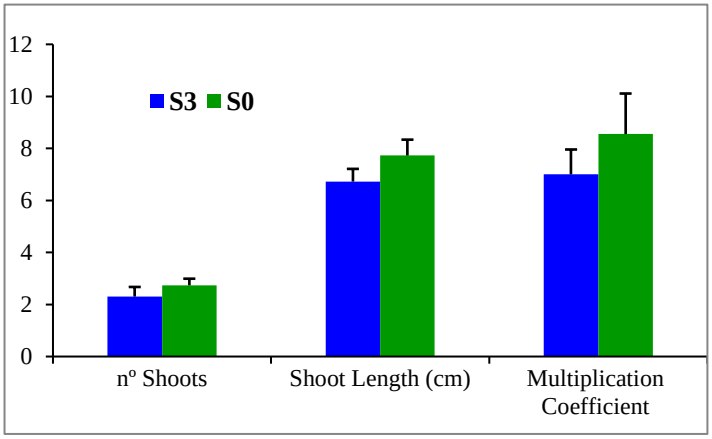

***Figure 5**. Proliferation of willow under photomixotrophic and photoautotrophic conditions.*

Acclimation and effect of the type of soil

Willow plantlets obtained in CIS in S0 and S3 were transferred to commercial peat in a phytotron for a first acclimation step. After 4 weeks, plantlets were measured (Fig. 6A) and distributed evenly between the soil treatments described in the Materials and methods section. After 6 weeks in the greenhouse, both plants and soils were characterized (Fig. 6B, Fig. 7, Fig. 8). All plants survived acclimation, although differences in the height and vigor of plants growing in the three types of soil were observed. However, the supplementation of sucrose during micropropagation did not affect the posterior growth of the plantlets (Fig. 6B, Fig. 7).

The height of the aerial and root zone of the plants after six weeks in the greenhouse is shown in Fig. 6B. Peat produced higher shoots than the other soils, whereas the highest development of the roots was obtained in crop soil (Fig. 6B). Acclimation in a soil that allows a relatively good growth of the aerial part and a good development of the root system could be advantageous for the subsequent transplanting of willows to field conditions. Usually the soils of plantations are not as rich in nutrients as the other soils used in this study (peat and forest soil), and plants cultured in the crop soil seemed to be well adapted to these restrictive conditions.

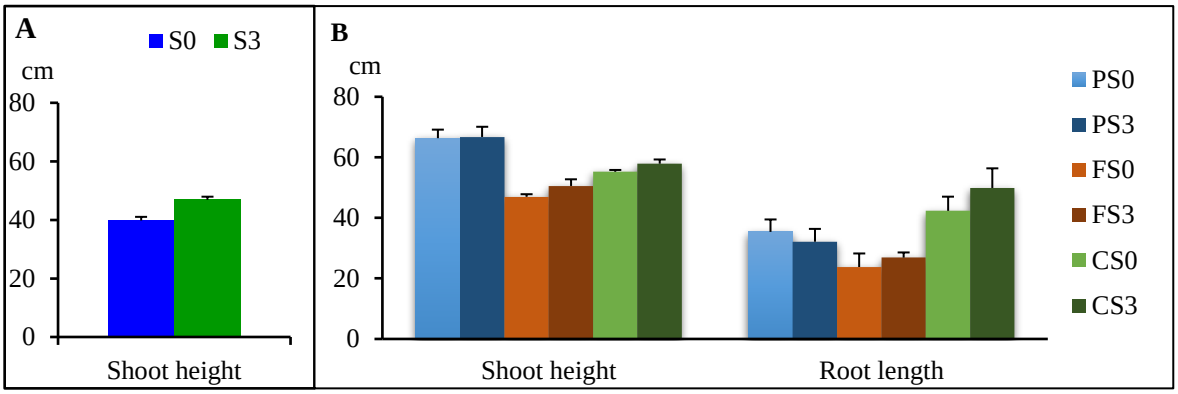

***Figure 6**. A) Shoot height of willow plantlets grown in CIS with 3% sucrose (S3) and without sucrose (S0) after one month in the phytotron. B) Shoot and root length of willow plantlets cultured for 6 weeks in the greenhouse in three types of soil. PS0, PS3: plants grown in peat and micropropagated without sucrose (PS0) or with 3% sucrose (PS3). FS0, FS3: plants grown in a forest soil and micropropagated without sucrose (FS0) or with 3% sucrose (FS3). CS0, CS3: plants grown in a crop soil and micropropagated without sucrose (CS0) or with 3% sucrose (CS3)*

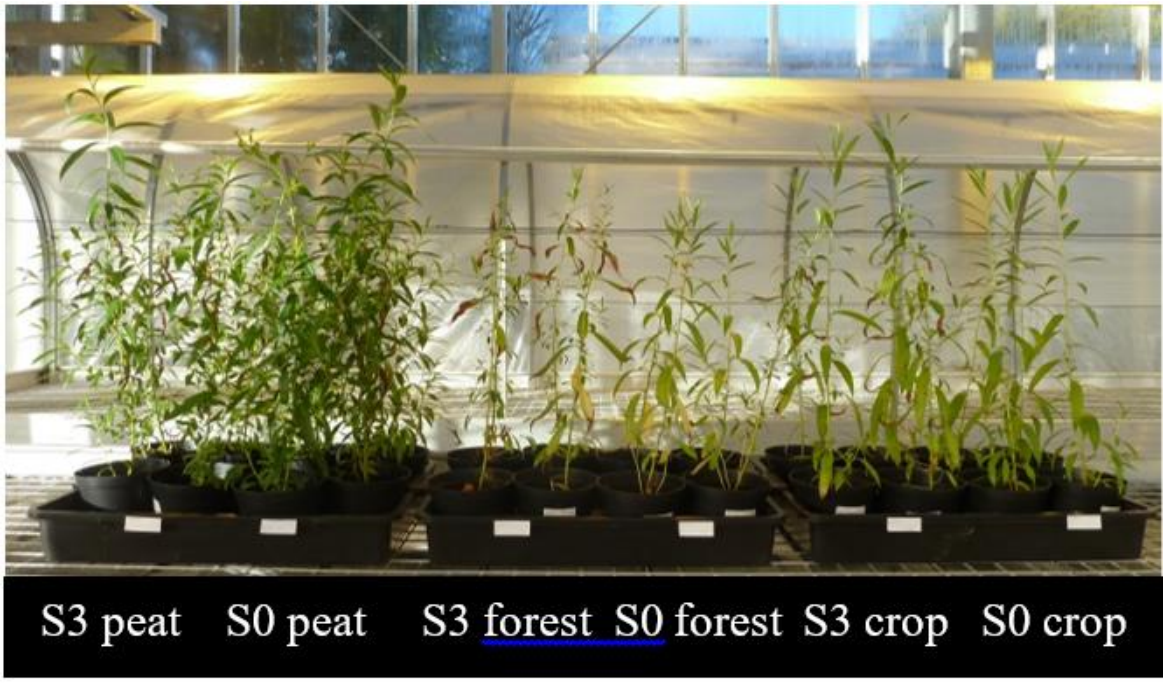

***Figure 7**. Willow shoots micropropagated with (S3) or without sucrose (S0) growing in different types of soil (peat, forest soil and crop soil).*

The preliminary enzymatic assays indicated that the values of the enzymatic activities were not affected by the micropropagation conditions of the plantlets. For this reason, and to simplify the graphic representation, the enzymatic activities corresponding to plantlets growing in the same soil were pooled together (Fig. 8), irrespective that the shoots were cultured with or without sucrose during the micropropagation stage.

The results indicate that the enzymatic activities were more influenced by the type of soil than by the presence of plants (Fig. 8). Enzyme activities were analyzed using the bulk soil. The lack of significant differences between the enzymatic activity of the soil with and without plants suggests both that the

main influence of plants and plant roots occurred on the rhizhosphere soil, and also more than six weeks are probably required to detect this influence in the bulk soil.

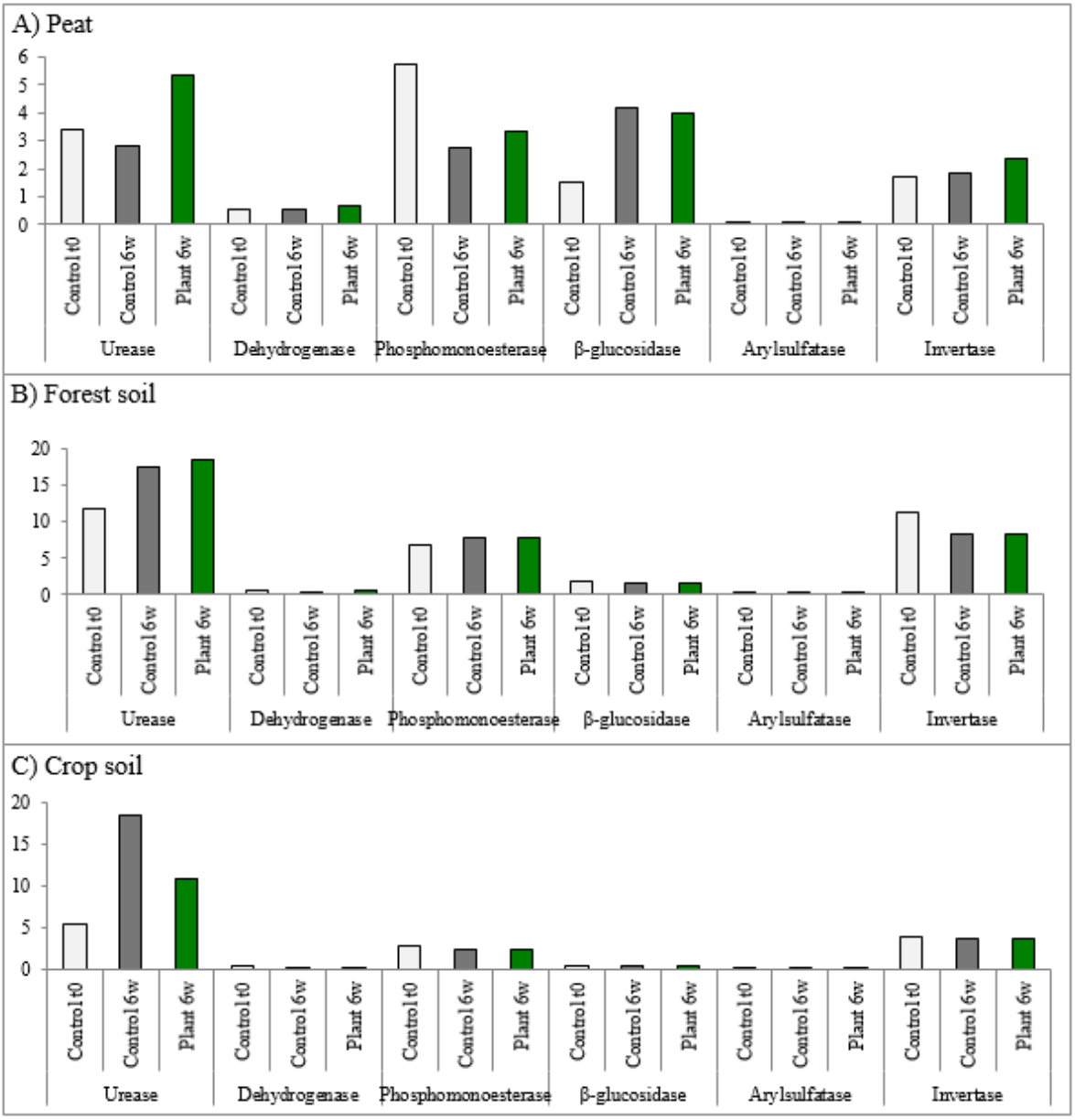

***Figure 8**. Effect of the type of soil, the time of sampling and the presence of plant on enzymatic activities. Control t0: control without plant at time 0; Control 6w: control without plant after 6 weeks; Plant 6w: samples with plants after 6 weeks. Enzymatic activities in µmoles of product g^{-1} h^{-1}.*

In conclusion, willow shoots were successfully propagated in liquid medium by temporary immersion with sucrose supplementation and by continuous immersion without sucrose. Shoots propagated under photomixotrophic and photoautotrophic conditions were satisfactorily acclimated. Commercial peat, forest soil, and crop soil were valid for willow acclimation. Plants grown in peat showed higher aerial growth, but those grown in crop soil showed more roots.

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