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Use of bioreactors RITA® in the propagation of *Pinus patula* Schiede ex Schltdl. & Cham.

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Abstract: The objective of the present work was to evaluate the efficacy of use of the RITA® temporary immersion system in the large-scale propagation of *P. patula*. The effects of four concentrations (0.00 µM, 4.50 µM, 9.00 µM, and 13.51 µM) of 6-benzylaminopurine (BAP) on 10 hypocotyl explants were studied using a completely randomised design with three replicates per treatment. Five hypocotyl explants were grown in 250 mL RITA® containers of Woody Plant Culture Medium (WPM) supplemented with 20 g·L^{-1} sucrose and 10 mg·L^{-1} vitamins from Murashige and Skoog (MS) culture medium. The frequency of immersion of the explants into the culture medium was 2 min every 8 hours. The number of adventitious buds and calli formed, as well as shoot growth, were evaluated after 6 weeks of *in vitro* culture. The 4.50 µM concentration of BAP was the best treatment for shoot production (5 shoots per plant) and shoot length (1.32 cm). These results could help the widespread vegetative propagation of this important forest species.

Keywords: conifers; *in vitro*; micropropagation; temporary immersion system; recipient for automated temporary immersion

Pinus patula Schiede ex Schltdl. is an endemic species with significant value for Mexican forestry (Farjon, Styles 1997). It is characterised by its straight, knotless stem and its adaptation to a wide range of soil and climatic conditions (Leibing et al. 2009). Due to the high quality of its wood, this species is in great demand in the global forestry industry, so it is necessary to have enough plants of this species to contribute to the development of reforestation programs and the restoration of degraded soils (Orwa et al. 2009).

The micropropagation techniques are potentially useful for rapidly multiplying high-value forest genotypes. Currently, most of the micropropagation protocols established in *Pinus* have been developed using semisolid media (De Diego et al. 2010). Although micropropagation protocols have been successfully established for *in vitro* regeneration of *P. patula* through the two main morphogenetic routes of somatic embryogenesis and organogenesis (Sarmast 2018; Ramírez-Mosqueda et al. 2019), there is no information on the use of Temporary Immersion Systems (TIS) in *P. patula* micropropagation.

TIS systems are characteristic due to the explants being immersed in the growing medium for brief periods of time, allowing for more effective nutrient and growth regulator absorption. These systems

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Table 1. Organogenesis response of *P. patula* explants (means + SE)

BAP concentration (μM)	Response type	Shoot length (cm)	Number of shoots	Number of calli
0.00	BA	0.49 ± 0.03[b]	0.20 ± 0.07[b]	0.00 ± 0.00[b]
4.50	BA	1.32 ± 0.09[a]	5.00 ± 0.29[a]	0.00 ± 0.00[b]
9.00	BA	0.60 ± 0.04[b]	0.73 ± 0.15[b]	0.00 ± 0.00[b]
13.51	BA + CO	0.59 ± 0.05[b]	0.93 ± 0.21[b]	1.49 ± 0.27[a]

[a,b]statistically equal means with the Tukey test ($P \leq 0.05$); BAP – 6-benzylaminopurine; BA – adventitious shoot, CO – callus

have been particularly popular because they reduce crop manipulation and, as a result, production costs (Etienne, Berthouly 2002; Gomes et al. 2016; Vidal, Sánchez 2019). There are various types of bioreactors, such as the Automatic Temporary Immersion Vessel (RITA®). Propagation in RITA® bioreactors is an appealing option for the mass micropropagation of various forest species. Furthermore, it has been demonstrated that the use of these systems increases plant growth rates when compared to those based on the use of semisolids (Arencibia et al. 2017).

Three-month-old plants from *in vitro* germination of *P. patula* were used in this study, following the protocol of Ramírez-Mosqueda et al. (2019). Five hypocotyl explants (0.5 cm in length) were obtained from each plant as an initial source of explants. For propagation, a 1 000 mL RITA® temporary immersion system (150 mm × 133 mm; VITROPIC, France) was used. Explants were grown in RITA® vessels containing 250 mL of WPM (Woody Plant Medium; Sigma, USA) Culture Medium (Lloyd, McCown 1981), supplemented with 20 g·L^{-1} sucrose and 10 mg·L^{-1} vitamins from MS Culture Medium (Murashige, Skoog 1962), as well as various concentrations of 6-benzylaminopurine (BAP: 0.00 μM, 4.50 μM, 9.00 μM, and 13.51 μM).

The pH of the medium was adjusted to 5.7, and the medium was sterilised at 1.5 kg·m^{-2} at 121 °C for 15 min. All cultures were incubated at 22 °C ± 2 °C with a photon flux density of 30–50 μE·m^{-2}·s^{-1} using fluorescent light lamps under a photoperiodic regime of 16 : 8 h (light: dark). The frequency of immersion of the explants into the culture medium was 2 min every 8 h. After 6 weeks of culture, the growth of the explants, the number of adventitious shoots, and/or calli formed were evaluated. In all experiments, a completely randomised design with 120 explants (10 explants per treatment, with 3 repetitions each) was used. Each treatment was repeated twice. After 6 weeks of culture, the effect on the number of adventitious shoots and/or calli formed was evaluated, as well as the length (cm) of the shoots. The data were statistically processed with the IBM SPSS Statistics software (Version 24, 2016). An analysis of variance (ANOVA) was performed, followed by the Tukey test ($P \leq 0.05$).

There were statistically significant differences in the morphological characteristics of shoots formed after 6 weeks of cultivation under different BAP concentrations. The 4.50 μM BAP treatment produced the largest number and size of shoots. This treatment tripled the initial shoot length (1.32 cm). The other treatments (0.00 μM, 9.00 μM, and 13.51 μM BAP) produced statistically comparable results (Table 1).

Figure 1 depicts the effect of different BAP concentrations on the size of the shoots formed. The effect of higher concentrations of cytokinin on callus formation was observed. It is noteworthy that callus formation only occurred in the WPM medium containing 13.51 μM BAP (Figure 2). These results show the effect of using higher concentrations of this cytokinin on callus formation compared to the rest of the treatments evaluated. The response of plants to various stress factors, in this case, the different concentrations of cytokinins with the capacity to stimulate a biphasic response ("hormetic effect") depending on the concentration of the stressor, has been demonstrated in numerous studies. Low doses stimulate a favourable response, while high doses of the same reagent have a negative effect on the same plant. BAP as a cytokinin has been widely used to induce adven-

Figure 1. Growth of explants exposed to different concentrations of BAP: (A) 0.00 μM; (B) 4.50 μM; (C) 9.00 μM; and (D) 13.51 μM

BAP – 6-benzylaminopurine

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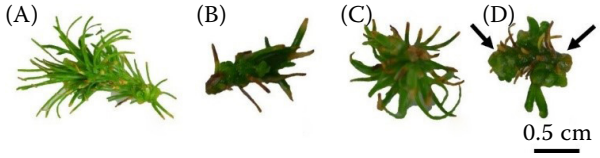

Figure 2. Organogenetic response exposed to different concentrations of BAP: (A) 0.00 µM; (B) 4.50 µM; (C) 9.00 µM; and (D) 13.51 µM; arrows indicate the formation of the cell callus

BAP – 6-benzylaminopurine

titious shoot development in the propagation of conifers (Humánez et al. 2011). It was interesting to note that relatively low doses of BAP were needed to induce shoot multiplication and elongation processes.

Several authors have found the presence of direct (Sul, Korban 2004) and indirect (Bello-Bello et al. 2012) organogenesis responses in *Pinus* species, using various concentrations of cytokinins such as BAP, Thidiazuron (TDZ), Zeatin (Z), and 2,4-dichloro-phenoxy-acetic acid (2,4-D). Although the use of BAP (4.50 µM), in combination with the use of temporary immersion systems (RITA®), allows the formation of adventitious shoots by direct organogenesis in *P. patula,* future research will evaluate the efficacy of other temporary immersion systems in the propagation of this valuable forest species. Future research will allow us to evaluate the effectiveness of other temporary immersion systems in the propagation of this valuable forest species, such as temporary immersion bioreactors (BIT®) and gravity immersion bioreactors (BIG).

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