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Combined effects of cultivar, culture system, cytokinin type and concentration on *in vitro* regenerative rate of banana

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Abstract

Banana is a major fruit crop of the world. *In vitro* micropropagation allows for the mass production of propagules of high genetic and sanitary quality; however, it requires continuous protocol improvement, by testing varying conditions of *in vitro* culture system and growth regulator supplementation. This study evaluated the *in vitro* regenerative rate of banana cultivars *SCS451* Catarina (AAB) and *SCS452* Corupá (AAA) in different culture systems (permanent immersion (PIS) and intermittent immersion (IIS)) exposed to a range of concentrations (0, 5, 10 and 20 µM) of the cytokinins benzylaminopurine (BAP) and meta-topoline (mT). There were no significant differences between type of cytokinins, but the concentration showed high linear significance ($p<0.001$) and quadratic terms ($p<0.001$), as well as significant interactions with cultivar and culture system. For 'SCS451 Catarina' there were no significant differences between PIS and IIS, but for 'SCS452 Corupá' the highest regenerative rates were observed under IIS ($p<0.05$). These results indicate that BAP and mT are equally suitable for micropropagation of both tested cultivars, with satisfactory results from 5 to 15 µM. Finally, low cytokinin concentrations (~5 µM) and/or the use of liquid culture medium in IIS may allow for cost reductions in production for both cultivars.

Index terms: SCS451 Catarina; SCS452 Corupá; micropropagation; genotype dependence.

INTRODUCTION

The fruit market moves expressive amounts of financial resources around the world, and banana (*Musa* spp), along with plantains, is one of the top produced fruits worldwide, with more than 150 million tonnes in 2019 (FOOD AND AGRICULTURE ORGANIZATION – FAO, 2021). In conventional banana propagation systems, banana plants are commonly propagated by suckers or rhizome segments, which show low regeneration rate and are prone to the transmission of pests and diseases (Rodrigues; Donato, 2021). Moreover, bananas present great genetic diversity, with the main varieties used in commercial agriculture consisting of *Musa acuminata* or hybrids between *Musa acuminata* x *Musa balbisiana* (Perrier et al., 2011). One of the consequences of this genetic diversity is that many banana varieties show differential responses to environmental factors, including *in vitro* environments (Garcez; Martins; Rodrigues, 2016; Pereira et al., 2004). Plant tissue culture techniques associated to micropropagation allow for the clonal and large-scale production of pest -and disease- free propagules (Rodrigues; Donato, 2021). Biofactories were established in Brazil with the intent of producing several banana genotypes at industrial scale, by means of plant tissue culture and control of somaclonal variants and dangerous diseases (Santos-Serejo; Souza; Souza, 2016; Scherer et al., 2019). In spite of that, demand for superior-genotype banana propagules with phytosanitary certification is still high in the country, which requires efficient micropropagation protocols (Santos-Serejo; Souza; Souza, 2016; Scherer et al., 2019). In general, advancements in regeneration rates of banana micropropagation have been associated to automation and scalling-

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up, and in both cases intermittent immersion systems (IIS) were shown to be an important tool for production scale-up and cost reduction (Georgiev et al., 2014; Bello-Bello; Cruz-Cruz; Pérez-Guerra, 2019). However, variables of interest such as regenerative rate (Oliveira et al., 2011), explant oxidation (Hirimburegama; Gamage, 1997), somaclonal variation (Bairu; Aremu; Van Staden, 2011), among others, are also dependent on banana genotype.

Plant growth regulators are widely employed in micropropagation systems, especially the cytokinin N6-benzilaminopurine (BAP). However, for micropropagation in IIS, BAP was shown to be less successful in banana than other fruit crops (Escalona et al., 2003). A new compound with cytokinin-like activity, named meta-Topoline (N6-(3-hydroxy-benzyladenine)) (mT) have been employed in different *in vitro* culture systems to induce axilary bud proliferation, with satisfactory results in several plant species (Aremu et al., 2012a; Souza et al., 2019a; Souza et al., 2019b). Additionally, it was demonstrated that mT can substitute BAP for the micropropagation of certain banana genotypes (Aremu et al., 2012b; Escalona et al., 2003).

The cultivars SCS451 Catarina (AAB) and *SCS452 Corupá* (AAA) are two superior genotypes of Prata and Cavendish subgroups, respectively. 'SCS451 Catarina', widely known as Prata Catarina, is an essentially derived variety of 'Branca de Santa Catarina', being shorter, more resistant to Panama disease (*Fusarium oxysporum* f. sp. *cubense* - *Foc* race 1) and more productive (Lichtemberg et al., 2011a). 'SCS452 Corupá', widely known as Nanicão Corupá, is an essentially derived variety of 'Nanicão', being equally productive but shorter (Lichtemberg et al., 2011b).

Within this context, the present study aimed at evaluating the combined effects of banana cultivar (SCS451 Catarina and *SCS452 Corupá*), culture system (permanent and intermittent), type of cytokinin supplemented to the culture medium (BAP and mT), and cytokinin concentration in the medium (0, 5, 10 and 20 µM), on the regenerative rate of adventitious banana shoots *in vitro*.

MATERIAL AND METHODS

The explants of 'SCS451 Catarina' and *'SCS452 Corupá'* were collected from Basic Plants Orchars (Breeding Plants Orchards) at Company of Agricultural Research and Rural Extension of Santa Catarina (Epagri). The experiment was carried out at the Plant Developmental Physiology and Genetics Laboratory of the Federal University of Santa Catarina (LFDGV/UFSC), Brazil. The basal culture medium (MSB) was constituted of MS salts (Murashige; Skoog, 1962) supplemented with Morel vitamins (Morel; Wetmore, 1951) and sucrose (30 g L^{-1}). Medium pH was adjusted to 5.8±0.05 with NaOH (1.0 N) and/or HCl (0.5 N) prior to autoclave sterilization for 20 min at 1.1 atm and 121 ºC. *In vitro* cultures were kept in a growth chamber at 25±2 °C, with 16:8 h (light:dark) photoperiod, and light intensity of 70-80 µmol m^{-2} s^{-1}, provided by cool white fluorescent lamps.

Explants were obtained from adult healthy mother-plants of cultivars SCS451 Catarina and SCS452 Corupá. Suckers were collected from the mother-plants and immediately subjected to disinfestation procedures according to Scherer et al., 2019. Explants were subsequently reduced in size to 1.0x1.0x2.0 cm (length, width, height), and inoculated for establishment *in vitro* in glass flasks (69 x 121.7 mm / 350 mL) containing 50 mL of MSB gelled with 2.5 g L^{-1} Phytagel®, and containing a range of BAP or mT concentrations: 0.1, 1, 10 or 20 µM, plus the control (MSB free of growth regulators). These cultures were transferred every 4 weeks to fresh medium, and maintained in the same treatments for 5 subcultures, in order to obtain a sufficient number of shoots for a large factorial experiment. During each subculture of this initial multiplication phase, old leaves and roots were excised, shoots were cut transversally (~1cm) above the apical meristem and longitudinally at the center of each shoot, taking care not to split the shoot completely, with the intention of breaking shoot apical dominance and promoting the formation of adventitious shoots.

The experiment was set up with four predictive variables: i) culture system (permanent immersion system (PIS), with 50 mL of gelled medium in 350 ml glass culture flasks (Figure 1A, 1B and 1C); or intermittent immersion system (IIS), with 600 ml of liquid medium in double-flask apparatuses using 5L PET bottles (Figure 1D and 1E); ii) cytokinin supplemented to the culture medium (BAP or mT); iii) cytokinin concentration (0, 5, 10 or 20 µM); and iv) banana cultivar (SCS451 Catarina or SCS452 Corupá). Shooting

units used in the 5 µM treatments (BAP or mT) derived from cultures previously established in 0.1 µM and 1 µM BAP or mT, respectively, which showed very low regenerative rates during the initial multiplication phase - similar to those of the control treatment (0 µM) - while shooting units used in 0, 10 and 20 µM treatments of either BAP or mT were transferred to medium containing the same cytokinin type and concentration used in the multiplication phase.

The experiment had a completely randomized design, and was repeated four times. In order to ensure a proportion of 50 ml culture medium per inoculated shooting unit in each treatment, experimental units (EUs) were constituted by one shooting unit in PIS (one shooting unit per culture flask, with six EUs per repetition), and by three shooting units in IIS (with two EUs per repetition and two repetitions per double-flask apparatuses). In both cases all shooting unit were divided in the middle as previously described (Figure 1A and 1B). After 4 weeks the mean number of shoots per EU and per repetition was recorded and the regenerative rates calculated as [(final shoot number - initial shoot number)/initial shoot number]. For each treatment, a four data-points were obtained and used in the statistical analysis, each consisting of the average regenerative rate in four repeated experiments.

Experimental results were analyzed with multiple regression, using R language (R Core Team, 2017). An initial Gaussian (normal) regression model was built for regenerative rate, including the all predictive variables and additionally including a quadratic term for cytokinin concentration (to account for a possible curvilinear dose-response indicated by previous exploratory analysis), and all possible interactions. After building the initial model, stepwise selection of effects was carried out based on the Corrected Akaike Information Criterion

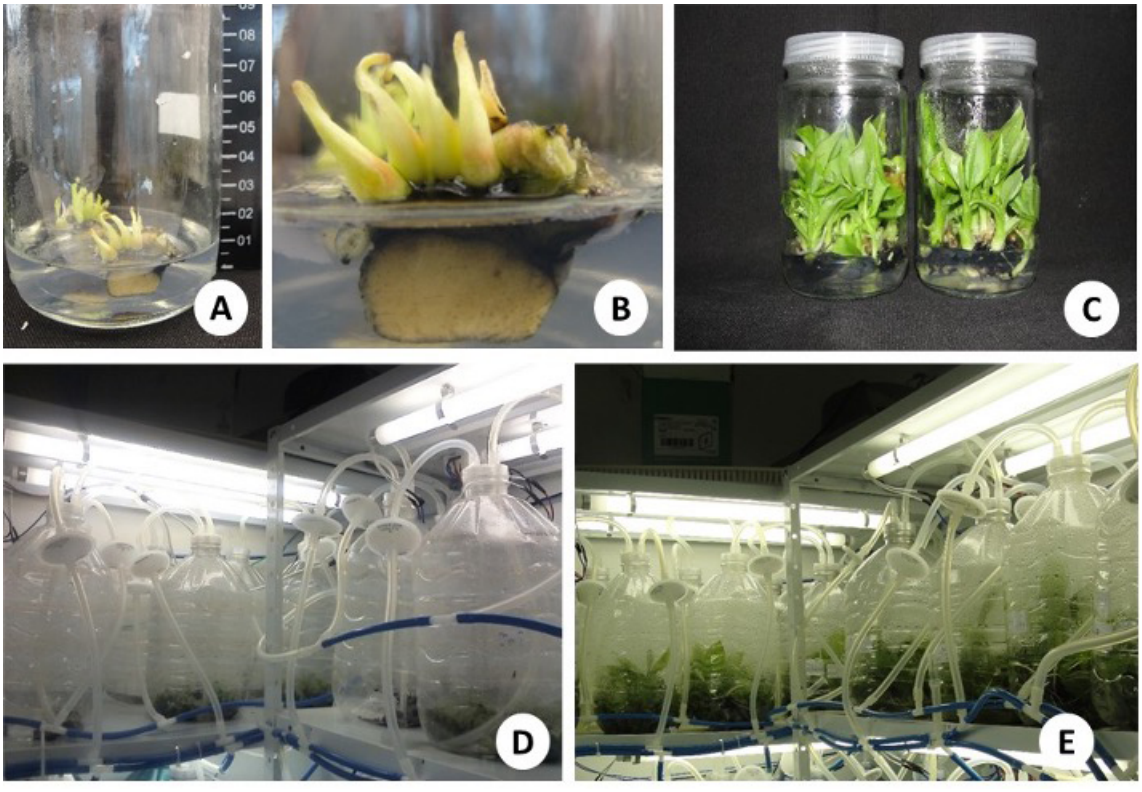

Figure 1: Culture systems evaluated on the regenerative rate of banana shoots (cultivars SCS451 Catarina (AAB) and SCS452 Corupá (AAA)). A and B) Shooting units newly inoculated in permanent immersion system with gelled medium (PIS); C) Shoots developed in PIS; D) Shooting units newly inoculated in intermittent immersion system with liquid medium (IIS); E) Shoots developed in IIS.

(cAIC) (Hurvich; Tsai, 1989; Burnham; Anderson, 2004). Model assumptions of residual normality and equal variance were graphically inspected using diagnostic plots of residuals. After detecting evidence for unequal variances across regenerative rate, we employed heteroskedasticity-consistent covariance matrix estimation (Long; Ervin, 2000) to estimate coefficients and standard errors. Variables and/or interactions were considered significant when $p<0.05$, and means and tendencies were compared by 95% confidence intervals (Cumming; Fidler; Vaux, 2007).

RESULTS AND DISCUSSION

The regenerative rate of the two genotypes was effectively modulated by the tested experimental conditions, with all combinations of cultivar, culture system and cytokinin type presenting significant curvilinear trends along the cytokinin concentration gradient (Figure 2). Culturing shooting units on medium free of cytokinins resulted only in growth and no proliferation of new shoots, regardless of culture system and cultivar. In general, for all tested combinations of culture system and cultivar, the regenerative rate showed an increase from 0 to 10 µM cytokinin, reaching estimated peak rates between 10 and 15 µM, from where a decrease ensues towards concentrations above 20 µM (Figure 2). Cytokinin type (BAP or mT) did not present a significant simple effect ($p = 0.13$), nor interactions with other variables. Cytokinin concentration, on the other hand, showed highly significant linear ($p<0.001$) and quadratic terms ($p<0.001$), as well as significant interactions with other variables.

Specifically, the most complex significant effect was a three-way interaction ($p = 0.021$) between cultivar, culture system, and the quadratic term for cytokinin concentration, indicating that the regenerative rate curves induced by BAP and mT gradients are dependent on the combination of cultivar (SCS451 Catarina or SCS452 Corupá) and culture system (PIS or IIS) used (Figure 3). Inspection of 95% regression confidence envelopes shows that the quadratic trends of regenerative rate across cytokinin concentration (5 to 20 µM) do not differ significantly between cultivars in PIS (Figure 3A and 3B), however, when cultured in IIS, 'SCS452 Corupá' presented the highest estimated values comparatively to 'SCS451 Catarina' (Figure 3C and 3D). It should be noted that 'SCS452 Corupá' in IIS (Figure 3D) presented higher variability and less accuracy in estimations than all other combinations of cultivar and culture system, as denoted by a wider 95% regression confidence envelope. This greater variability was derived from more "extreme" observations (repetitions with growth rates above 5) at midrange cytokinin concentrations (5 and 10 µM).

According to the observed results, the use of either BAP or mT has a pronounced effect on banana regenerative rate *in vitro*, with even low cytokinin concentrations (5 µM) producing significant improvements over culture conditions without supplementation (0 µM), although the estimated trends indicate further improvements could be obtained in media with 10-15 µM of either tested cytokinin. The use of low growth regulator concentrations is beneficial in reducing culture medium cost, and also, reducing the rate of somaclonal variation, since higher growth regulator concentrations tend to correlate with greater probability of somaclonal variation (Bairu; Aremu; Van Staden, 2011). The cytokinin BAP is widely used in plant tissue culture, but limitations regarding regenerative rate and high-concentration effects, such as somaclonal variation (Santos; Rodrigues, 2004; Bairu; Aremu; Van Staden, 2011), should be considered. In this sense, other studies show mT as a promising substitute to BAP, inducing high regenerative rates and a lower inhibition of root formation in the acclimation stage (Aremu et al. 2012c; Werbrouck et al., 1996; Escalona et al., 2003; Roels et al., 2005).

Concerning culture system, '*SCS451* Catarina' did not present significant differences between PIS and IIS, while '*SCS452 Corupá*' showed a significantly different trend, and higher regenerative rates in IIS. Escalona et al. (2003) and Roels et al. (2005) studies comparing banana multiplication rates using different BAP and mT concentrations in PIS and IIS, observed significantly higher regenerative rates in medium containing 4.44 µM mT under IIS. Intermittent immersion systems increase the interface between plant/culture and culture medium, and also prevent the formation of nutrient gradients within the medium, thus possibly maximizing nutrient absorption and positively influencing the growth

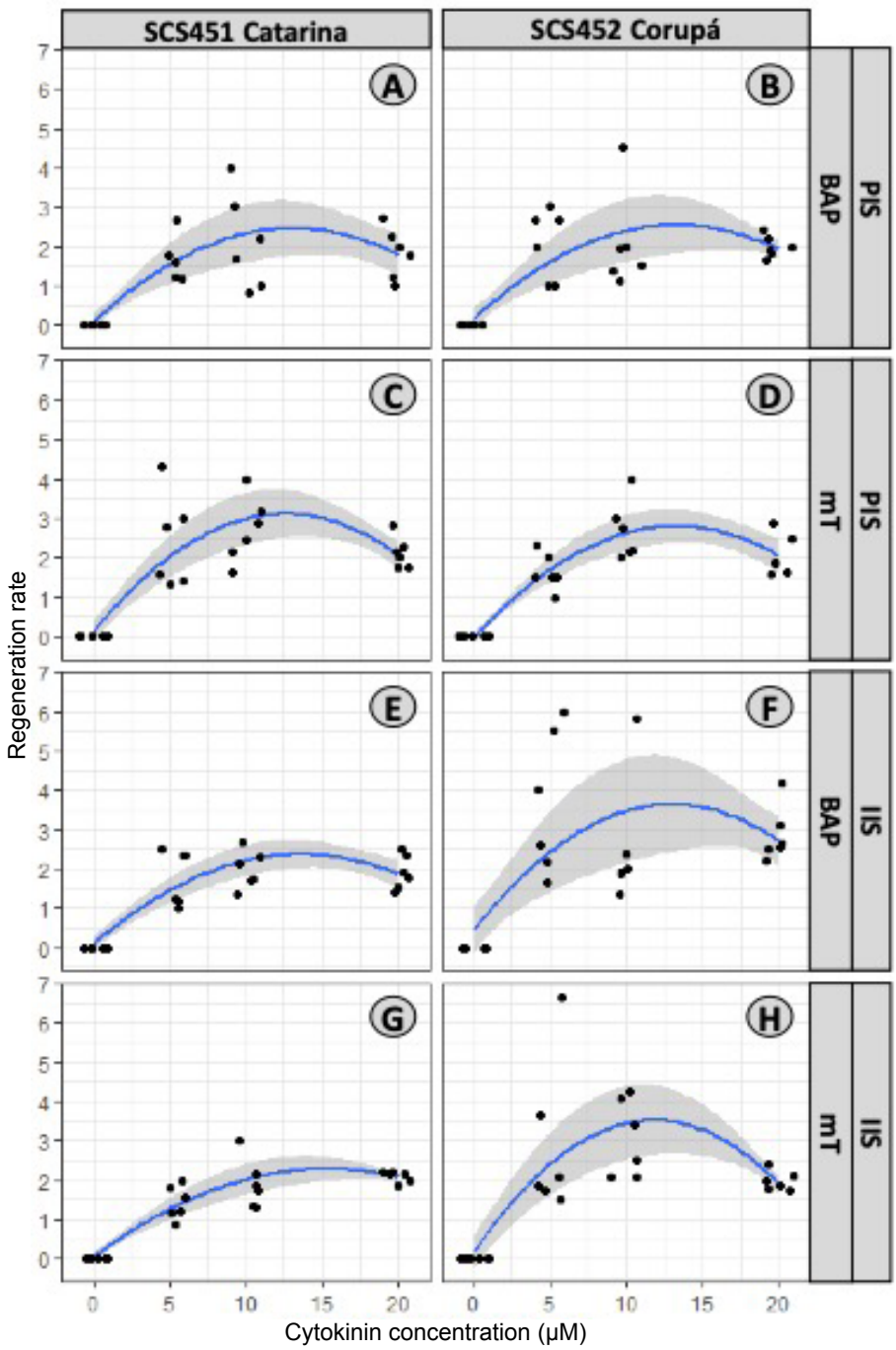

Figure 2: *In vitro* regenerative rates of banana cultivars SCS451 Catarina (AAB) and SCS452 Corupá (AAA) cultured in permanent immersion (PIS) or intermittent immersion (IIS) systems, using media supplemented with a range of concentrations of either Benzylaminopurine (BAP) or meta-Topoline (mT) (0, 5, 10 and 20 μM). A) Regenerative rates of 'SCS451 Catarina' cultured with BAP in PIS; B) Regenerative rates of 'SCS452 Corupá' cultured with BAP in PIS; C) Regenerative rates of 'SCS451 Catarina' cultured with mT in PIS; D) Regenerative rates of 'SCS452 Corupá' cultured with mT in PIS; E) Regenerative rates of 'SCS451 Catarina' cultured with BAP in IIS; F) Regenerative rates of 'SCS452 Corupá' cultured with BAP in IIS; G) Regenerative rates of 'SCS451 Catarina' cultured with mT in IIS; H) Regenerative rates of 'SCS452 Corupá' cultured with mT in IIS. Lines indicate estimated trends, shaded bands depict 95% confidence envelopes, and dots depict observed values (horizontally dislocated around the true x-axis value to improve visualization).

rate (Bello-Bello; Cruz-Cruz; Pérez-Guerra, 2019). Furthermore, according to Steinmacher et al. (2011), IIS also allow for the periodic renewal of the *in vitro* atmosphere, thus promoting not only uniform nutrient distribution, but also facilitated cellular respiration and lower gas accumulation inside the IIS vessel; lastly, IIS uses liquid medium, which contributes to cost reduction when compared to permanent immersion systems with gelled medium (Bello-Bello; Cruz-Cruz; Pérez-Guerra, 2019; Steinmacher et al., 2011; Cejas et al., 2011). However, the results herein presented indicate that not all banana cultivars respond in the same way to intermittent immersion culture systems.

The results obtained in this study agree with previous reports on *Musa* spp. micropropagation (Lima; Moraes, 2006; Roels et al., 2005), in the sense that regenerative rates were largely affected by cytokinin concentration, and were also genotype-dependent. Beyond that, this study contributes additional information regarding the importance of the combined effects of banana cultivar, culture system, and cytokinin concentration in the regenerative rates of banana adventitious shoots *in vitro*. This suggests that each cultivar or genotype may require specific ideal conditions of culture system, type and concentration of cytokinins, in order to maximize a commercial scale of true-to-type plantlet production.

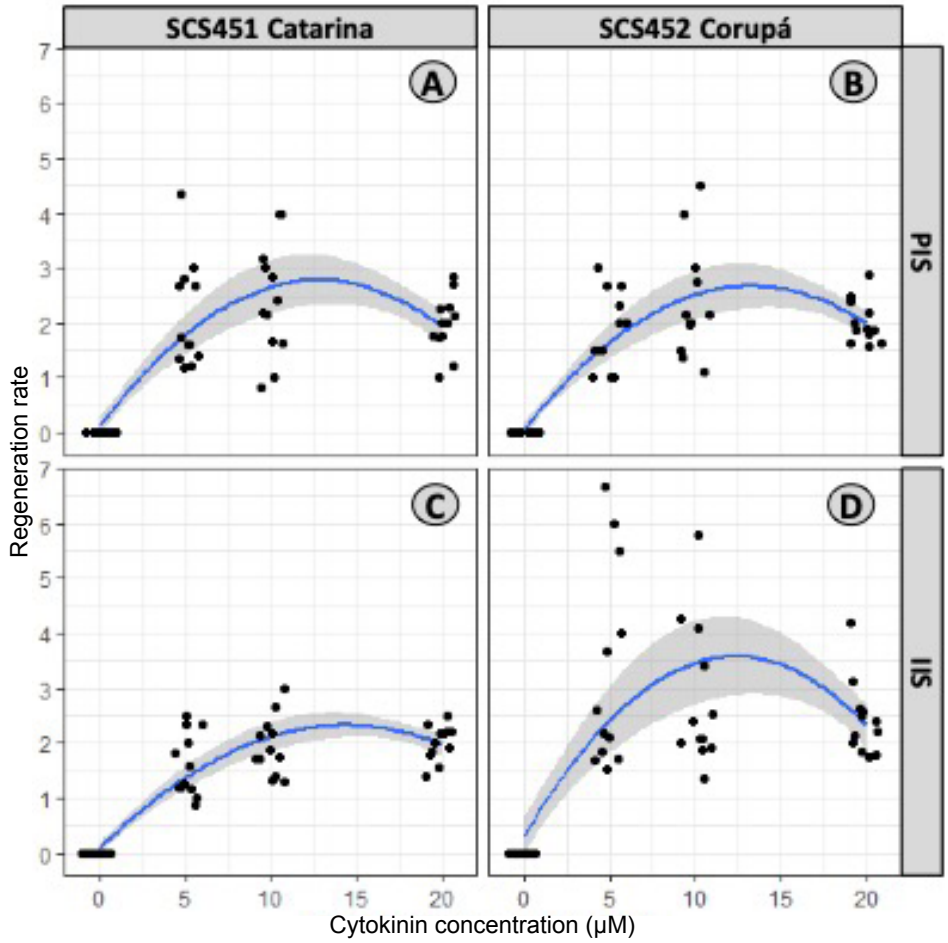

Figure 3: A) Regenerative rates of 'SCS451 Catarina' cultured with cytokinins (BAP and mT) in PIS; B) Regenerative rates of 'SCS452 Corupá' cultured with cytokinins (BAP and mT) in PIS; C) Regenerative rates of 'SCS451 Catarina' cultured with cytokinins (BAP and mT) in IIS; D) Regenerative rates of 'SCS452 Corupá' cultured with cytokinins (BAP and mT) in IIS. Lines indicate estimated trends, shaded bands depict 95% confidence envelopes, and dots depict observed values (horizontally dislocated around the true x-axis value to improve visualization).

CONCLUSIONS

Cytokinin supplementation to the culture medium, in the form of either BAP or mT, is essential for adventitious shoot proliferation in banana cultivars *SCS451* Catarina and *SCS452 Corupá*, regardless of culture system (PIS or IIS). Low cytokinin concentrations of ca. 5 µM are enough to elicit satisfactory results, although peak proliferation is estimated to occur between 10 and 15 µM of either BAP or mT. It is noteworthy that liquid culture medium in IIS allowed for the successful multiplication of *Musa in vitro*, at commercially viable rates, for both tested cultivars. '*SCS452* Corupá' had its multiplication rate significantly improved in IIS, while '*SCS451* Catarina' showed no difference between PIS and IIS. This suggests that, although the *in vitro* response varies with banana genotype, intermittent immersion systems have the potential to provide commercially viable regeneration rates of *Musa sp.* 'SCS451 Catarina' and 'SCS452 Corupá'.

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