Colegio de Postgraduados

Plant growth and early *in vitro* floral differentiation of vanilla (*Vanilla planifolia* Jacks. ex Andrews)

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ABSTRACT

Objective: To induce *in vitro* flowering of vanilla (*Vanilla planifolia*) with different plant growth regulators (PGRs) using the double-layer technique.

Design/methodology/approach: A layer of semi-solid Knudson C (KC) medium, added with 40 g L^{-1} sucrose, 15% coconut water (CW; v/v) and 7 g L^{-1} agar, was placed in 100 mL flasks. A liquid layer of the same composition without agar was placed on top. It was supplemented with different doses (mg L^{-1}) of PGRs: 6-benzyladenine (BA) (7), thidiazuron (TDZ) (6), paclobutrazol (PBZ) (0.5) and gibberellic acid (GA_3) (2). Plus two controls, C1: no PGRs + no CW; C2: no PGRs + CW. Vanilla shoots of 2 cm in length and with at least one axillary shoot were placed. They were incubated at a temperature of 26±2 °C day and 18 °C darkness, with light intensity of 55 μmol m^{-2} s^{-1} during 13 weeks. The number of shoots, leaves and roots was evaluated, as well as the length of shoots and fresh weight. Floral differentiation was evaluated at the tenth week by conventional microtechnique.

Results: The number shoots and leaves and shoot length were significantly higher in C2. The number of roots increased with PBZ 0.5 mg L^{-1}. C1 and C2 promoted higher fresh weight. Floral differentiation was observed with GA_3 2 and PBZ 0.5 mg L^{-1} treatments.

Limitations on study/implications: Further evaluation of other PGR doses and environmental conditions is required to achieve full floral differentiation of vanilla.

Findings/conclusions: CW increased vegetative growth. GA_3 and PBZ showed early floral differentiation in *Vanilla planifolia*, which is the first report of this phenomenon for the species.

Keywords: Coconut water, orchid flowering, *in vitro* double-layer culture medium, floral meristems, plant growth regulators (PGRs).

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INTRODUCTION

Vanilla planifolia is an orchid species of economic importance due to the aromatic extract that is obtained from its fruits, vanillin (Soto Arenas, 2006; Xochipa-Morante *et al.*, 2016; Bautista-Aguilar *et al.*, 2021). Vanilla plantations in Mexico begin to produce at three years after their establishment and they have an average life of 5 years; therefore, in

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many cases, there are harvests only for two years (Kelso-Bucio *et al.*, 2012, Garza-Morales, *et al.*, 2021). This long period of plant development and the short productive cycle of the crop, in addition to its susceptibility to diseases (Lee-Espinoza *et al.*, 2008; Bautista-Aguilar *et al.*, 2021), climate change, and lack of technology transference (Vázquez-Trujillo, 2020; Garza-Morales *et al.*, 2021), have caused the reduction of the cultivated surface of vanilla in Mexico. *V. planifolia* has been widely studied for its *in vitro* propagation and conservation, and until today there are several protocols about it (Gonzáles-Luna, 2003; Lee-Espinoza *et al.*, 2008; Bello-Bello *et al.*, 2015; Ramírez-Mosqueda and Iglesias-Andreu, 2015; Halim *et al.*, 2017). Although there are many protocols for plant growth of this species, no studies of *in vitro* flowering have been reported. Among all the biological phenomena, flowering is without a doubt one of the most fascinating and difficult to understand, as in the Orchidaceae family, which is why the induction of *in vitro* flowering is a valuable tool to understand it more deeply in physiological, genetic and molecular terms (Teixeira *et al.*, 2014). Flowering is influenced by environmental and endogenous factors that are integrated in perfect correspondence to trigger flowering at the adequate time (Amasino and Michaels, 2010; Tuan-Ha, 2014). *In vitro* floral induction has been reported in orchids and other ornamental species, through the use of plant growth regulators (PGRs). Among the most widely used, there are cytokinins such as BA in *Cymbidium niveo-marginatum* Mak (Kostenyuk *et al.*, 1999), in *Dendrobium* Chao Praya Smile (Hee *et al.*, 2007), and in *D. huoshanense* (Lee and Chen, 2014). Likewise, some compounds with similar action to cytokinins, such as TDZ, have shown effects that induce flowering in orchids, as in *D.* Second Love (de Melo Ferreira *et al.*, 2006), and *D. wangliangii* (Lawrie *et al.*, 2021). In addition, PBZ has also been evaluated in the genus *Dendrobium* (Te-Chato *et al.*, 2009; Zhao *et al.*, 2013), as well as GA_3 in combination with BA in *Phalaenopsis amabilis* (Semiarti *et al.*, 2015). In addition, the use of GA_3 has been reported to induce *in vitro* flowering in other species such as *Phlox paniculata* L (Anuar *et al.*, 2017). The induction of *in vitro* flowering of vanilla would open a new field for a better understanding of flowering in the species; for example, for genetic improvement referring to the quality of the aromatic compounds of the pods. Because of the aforementioned, the objective of this study was to induce *in vitro* flowering of *Vanilla planifolia* using different doses of plant growth regulators (PGRs) in a double-layer culture medium.

MATERIALS AND METHODS

Establishment of induction to flowering in a double-layer culture medium

To establish the experiment in double-layer mediums for flowering induction, the methodology proposed by Sim *et al.* (2007) was adapted.

Plant material

Shoots with length of approximately 2 cm were used, with at least one axillar shoot of *Vanilla planifolia*, from material from Papantla, Veracruz, Mexico, and they were multiplied through several *in vitro* sub-cultures, in a RITA® type temporary immersion system (TIS), supplemented with 2 mg L^{-1} of BA, plus 0.5 mg L^{-1} of naphthaleneacetic acid (NAA), plus 30 g L^{-1} of sucrose.

Culture medium

The culture medium that induced flowering consisted of a double-layer medium, which included: Knudson's semi-solid medium (C KC; Phytotechlab®), supplemented with 40 g L^{-1} of sucrose (Phytotechlab®), 150 ml L^{-1} of sterilized coconut water (v/v), from fresh immature coconuts from the local market, and 7 g of agar (Sigma®). The liquid phase consisted of the same composition as the semi-solid phase, except for the agar. The semi-solid phase was placed in containers in the amount of 20 mL, and once gellified, 10 mL of liquid medium were added.

Treatments

The treatments consisted in doses of PGRs: BA, TDZ, PBZ and GA_3 (Sigma®), with two controls, C1: without PGRs and without coconut water; C2 without PGRs supplemented with coconut water (Table 1). Both phases of the medium were supplemented with those doses. Glass containers of 240 ml capacity were used, where four explants were placed per container, considered as the experimental unit with four repetitions per treatment.

The pH was adjusted to 5.7 before sterilization at 121 °C for 20 minutes in an autoclave (STIK® model Mj-504-A) at 1.5 atmospheres of pressure. They were incubated at a temperature of 26±2 °C daylight and 18 °C darkness, with a luminous intensity of 55 μmol m^{-2} s^{-1}, during 13 weeks.

Morphological analysis

Ten weeks after culture in a double-layer medium for flowering induction, part of the growing tissue was extracted from each explant by treatment, which contained mainly structures different from the commonly observed growth were observed *in vitro*, with the aim of performing anatomical cuts and observing a possible morphological change with floral structures.

The samples were placed in a fixing solution for herbaceous plant tissues, because they are very soft tissues (FAA) at 50%. Then, the samples were dehydrated (Sandoval, 2005) and processed through the conventional technique of inclusion in paraffin, dyed with safranine 0 at 0.1%, complemented with fast green at 0.1%, mounted with synthetic resin and observed with the photonic microscope (Johansen, 1940).

Table 1. Flowering inductive treatments in double-layer medium supplemented with different doses of plant growth regulators (mg L^{-1}).

Treatments	CW	BA	TDZ	PBZ	GA_3
C1	0%	0	0	0	0
C2	15%	0	0	0	0
BA	15%	7	0	0	0
TDZ	15%	0	6	0	0
PBZ	15%	0	0	0.5	0
GA_3	15%	0	0	0	2

CW: coconut water; BA: 6-benzyladenine, TDZ: thidiazuron, PBZ: paclobutrazol, GA_3: gibberellic acid.

Statistical analysis

The treatments were distributed in a completely random design. The normality of the data was determined through the Shapiro-Wilk test, and the homoscedasticity test through the Levene test. The range of a transformation was carried out, and the data were analyzed through ANOVA and means comparison with Tukey's test (0.05%). The data were analyzed through R software, version 4.1.0 (R Core Team, 2021).

RESULTS AND DISCUSSION

The treatments affected the plant growth of *V. planifolia*. It was observed that the number of shoots presented significant differences between treatments (Figure 1). The S/PGRs+CW15% treatment was statistically higher since it presented the highest number of shoots (6.5) (Figure 1A) and the highest number of leaves (17.2) (Figure 1B). Likewise, it was statistically higher in the length of the shoots, with them being 35% higher than the shoots in BA, TDZ and GA_3 (Figure 1D).

The results indicate that coconut water can have a positive effect on plant growth. According to some authors this effect can be due to the cytokinins present in this compound (Gupta, 2016). Coconut water has been used for the germination and the proliferation of different orchid species. In *Dendrobium* 'Gradita 31', adding coconut water at 15% to the culture medium increased both the number of protocorms generated (37%) and the fresh weight of the protocorms from 0.28g to 0.46 g (Winarto and Teixeira, 2015).

In this experiment, the CW showed a higher number of vanilla shoots, with an average of 6.5 shoots per explant, compared to BA, which yielded 4 shoots per explant, even when BA is one of the cytokinins most frequently used in *in vitro* culture for the proliferation

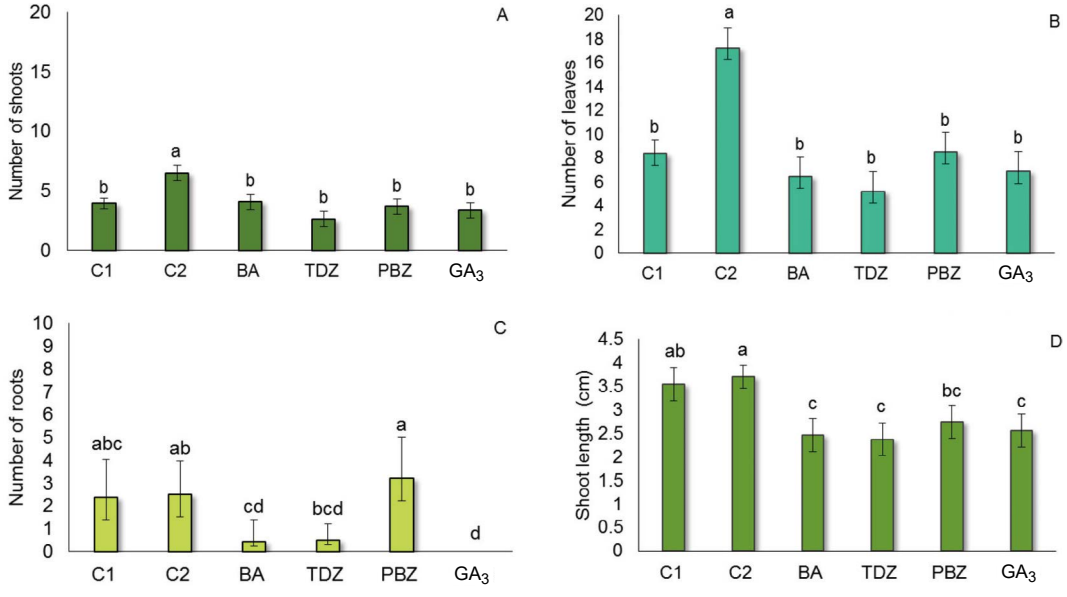

Figure 1. Number of shoots (A), leaves (B), roots (C), length of shoots (D) derived from nodal segments of *Vanilla planifolia*, in flowering inductive treatments based on different doses of plant growth regulators (mg L^{-1}): BA, 7; TDZ, 6; PBZ, 0.5; GA_3, 2. All the doses supplemented with coconut water. Bars with different letters + SD indicate significant differences (Tukey, $p \leq 0.05$).

(Halim *et al.*, 2017; Inderiati *et al.*, 2019). Better results were obtained in this study by adding coconut water, compared to other studies. Carranza-Álvarez *et al.* (2021) indicate that it generated 4.6 shoots per explant with the addition of 3% (v/v) of coconut water, although they do not report significant differences in comparison to their control. It is important to consider that in this study the BA was used in higher doses to attempt to induce flowering in vanilla, compared to the doses normally reported for vegetative growth, which could decrease the number of shoots. Some authors report thresholds of BA doses for plant growth. Lee-Espinosa *et al.* (2008) indicate that dose of 2.1 mg L^{-1} generated an average of 18 shoots per vanilla explant, although the use of the dose under or over this dose generated a lower number of shoots per explant. Similarly to the data in this study, Abebe *et al.* (2009) reported that the dose of 3 mg L^{-1} of BA generated up to 4.2 shoots per explant in vanilla, after 45 days of growth. Meanwhile, Inderiati *et al.* (2019) showed that dose of 1.5 mg L^{-1} of BA in vanilla promoted the highest number of shoots per explant (3.27). Thus, the doses most frequently reported for vanilla proliferation are from 1 to 3 mg L^{-1}. In previous studies (data not shown), different doses of BA were tested to try to understand its potential effect on the induction of flowering in vanilla, and in addition to the reports for other orchid species, a high dose was used in this study, although a decrease was found in the plant growth (Figure 1A, B and C).

The treatment with 0.5 mgL^{-1} of PBZ significantly increased the average number of roots per explant (3.2) (Figure 1C), compared to the doses of BA (0.43), TDZ (0.5), and GA_3 which inhibited it completely. These results are similar to what was reported by Bello-Bello *et al.* (2015) who indicate that the dose of 1 mg L^{-1} generated on average 3.8 roots per explant. And this result is similar to those reported in other species. In *Dendrobium* Friederick's, the dose of 0.5 mg L^{-1} of PBZ promoted the highest average number of roots per explant (6; Te-Chato *et al.*, 2009). Although PBZ increased the number of roots in vanilla, these roots were, in addition, quite thickened, inhibiting the aerial growth, which is why exploring it for rooting during its phase of *ex vitro* acclimation is proposed. These results are similar to what Gimenes *et al.* (2018) report, who mentioned that the PBZ in *Zygopetalum crinitum* (Orchidaceae) seedlings decreased the length of the aerial part and roots, and in addition, promoted their thickening, although the authors mention that in the acclimation phase, the application of PBZ did not imply a higher survival of the orchid *Z. crinitum*. Meanwhile, Wen *et al.* (2013) report that in *Dendrobium nobile*, the application of 0.8 mg L^{-1} of PBZ increased by 41.6% the survival rate in the acclimation of this species, in comparison to a control. Therefore, it would be pertinent to explore this effect in vanilla. In addition to this, the treatment with GA_3 inhibited root emission, which contrasts with what was reported by Coello *et al.* (2010), who indicate that GA_3 is an important factor for the promotion of roots in *Guarianthe skinneri*. Other authors report similar results from those in this study, such as Rodrigues *et al.* (2015), who indicate that the dose of 5 mg L^{-1} GA_3 decreased to 1.87 the number of roots in *Cyrtopodium saintlegerianum*, compared to the control without GA_3 (4.53 roots per explant). This shows that each species can have differential responses in the presence of the same growth regulator.

The length of shoots was statistically higher in C2 (Figure 1D), compared to GA_3, BA and TDZ. Similar results were reported by Carranza-Álvarez *et al.* (2021), since the

addition of coconut water at 3% (v/v) increased the lengths of vanilla shoots, from 2.0 cm to 3.5 cm, compared to the control. On the other hand, Roy *et al.* (2012) mention that higher doses of TDZ in *Cymbidium giganteum* decrease the length of explants. For example, 0.2 mg L^{-1} of TDZ generated shoots of 1.85 cm, while the dose of 2.0 mg L^{-1} reduced their length to 0.67 cm. In contrast to our results, it has been reported that GA_3 increases the elongation of shoots in other species, for example, doses of 5 and 10 mg L^{-1} promoted the highest average length, from 7.74 and 8.06 cm, respectively, in comparison to the dose without GA_3 (4.53 cm), in the *in vitro* culture of *Cyrtopodium saintlegerianum* (Rodrigues *et al.*, 2015). However, these reports differ from our results, since this study found that dose of 2 mg L^{-1} of GA_3 generated shoots of 2.5 cm, while the control promoted shoots of 3.5 cm of length.

The treatment with BA and TDZ promoted oxidation of the explants 6.25 and 25%, respectively (data not shown). This is similar to other growth variables (number of leaves, length of explant), the treatments with PGRs also affected negatively the fresh weight of the shoots generated in *V. planifolia (*Figure 2) since the control treatments were statistically higher.

Floral differentiation of *Vanilla planifolia*

The treatments with GA_3 and PBZ (Figure 3) showed formation of tissues different from the vegetative ones reported in vanilla. In the cuts carried out, it was observed that treatments of GA_3 and PBZ presented flattening of the meristem dome (Figure 3 B-D), sign of the floral initiation. Meanwhile, the rest of the treatments presented a convex meristem dome, characteristic of vegetative growth (Figure 3A). The use of various PGRs has been reported to attain *in vitro* flowering. Te-chato *et al.* (2009) achieved 29% of *in vitro* floral induction in *Dendrobium* Friederick's, with the addition of 0.05 mg L^{-1} of PBZ. The addition of PBZ and TDZ, in concentrations of 1 mg L^{-1} in each PGR have promoted up to 62.2% of *in vitro* flowering in *Dendrobium nobile* (Wang *et al.*, 2009). Likewise, GA_3 has been used

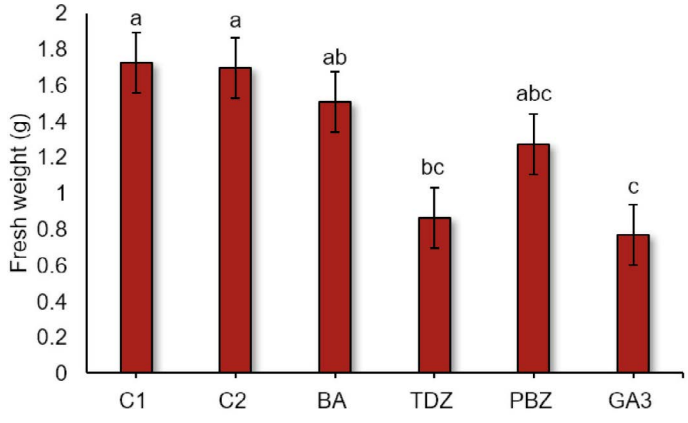

Figure 2. Fresh weight (g), of shoots derived from nodal segments of *Vanilla planifolia*, in flowering inductive treatments based on different doses of plant growth regulators (mg L^{-1}): BA, 7; TDZ, 6; PBZ, 0.5; GA_3, 2. All the doses were supplemented with coconut water. Bars with different letters + SD indicate significant differences (Tukey, $p \leq 0.05$).

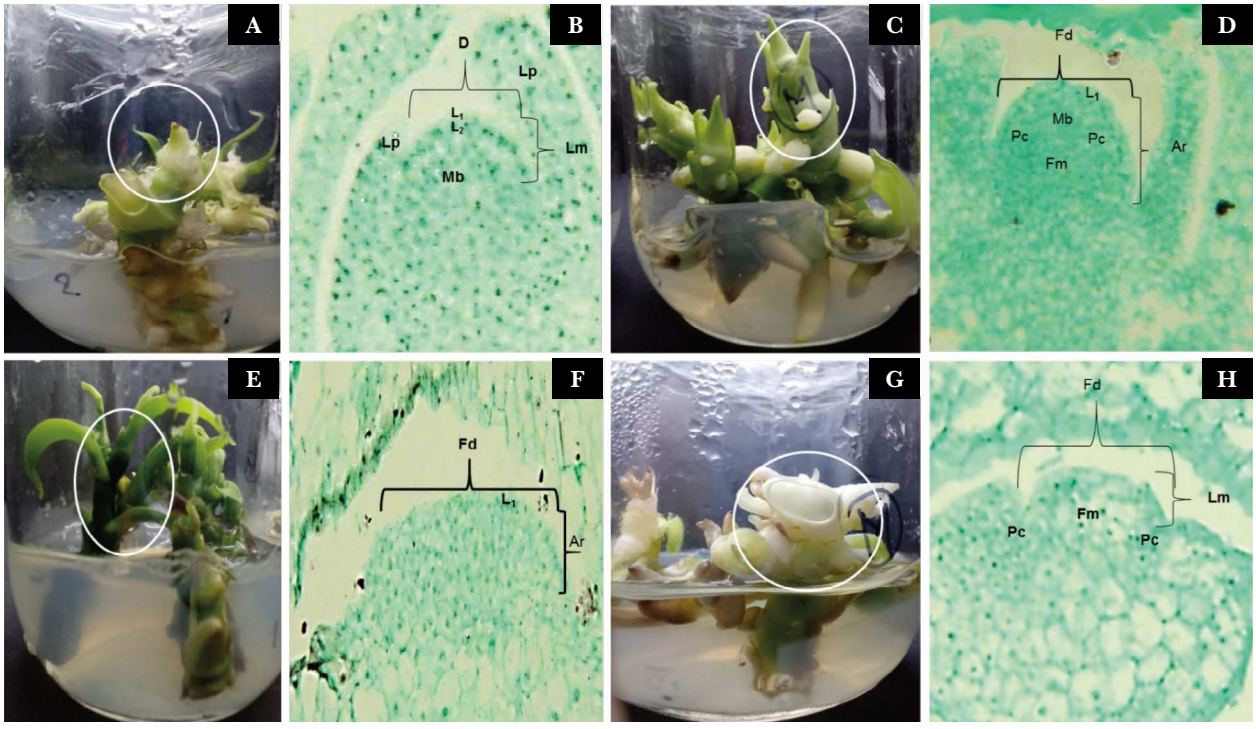

Figure 3. *In vitro* growth of shoots (A, C, E, G) with fragments for anatomical cuts (white circles) and longitudinal sections of apical shoots (B, D, F, H) of *Vanilla planifolia* subjected to treatments that induce flowering with PGRs (mg L^{-1}): A-B: BA, 7; C-D: PBZ, 0.5; E, F, G, H: AG_3, 2. B) Vegetative apical meristem. D and F) Apical meristem at the beginning of floral differentiation, which consists in the lengthening of the meristem body (Ra) and beginning of dome flattening (Da). H) Apical meristem at the beginning of floral differentiation where dome flattening is seen. Cm: meristem body; D: curve dome; Da: flat dome; L: meristem length; Mf: fundamental meristem; Pc: procambium Pf: leaf primordium; Ra: region of apical meristem lengthening; T1: tunic 1 or protodermis; T2: tunic 2.

to attain flowering induction. Semiarti *et al.* (2013) indicate that the dose of 15 mg L^{-1} of GA_3 plus 9 mg L^{-1} of BA can induce flowering initiation in *Phalaenopsis amabilis* (L.) Blume. Britto *et al.* (2003) indicate that *in vitro* flowering was observed in *Ceropegia bulbosa* Roxb. var. *Bulbosa* cultivated in Gamborg B5 medium with addition of 1 mg L^{-1} GA_3 + 0.5 mg L^{-1} BA. Chaari-Rkhis *et al.* (2006) indicate that they obtained floral induction in some olive cultivars such as "Marsaline" and "Picholine" with dose of 10 mg L^{-1} of GA_3. One of the most frequently reported PGRs for *in vitro* floral induction in orchids, mainly *Dendrobium*, is BA (Kostenyuk *et al.*, 1999; Hee *et al.*, 2007; Sim *et al.*, 2007, Zhao *et al.*, 2013), although in this study the treatment with BA did not show characteristics of floral initiation in vanilla (Figure 3A).

CONCLUSIONS

A high dose of BA affects negatively the vegetative growth of vanilla. The use of PBZ increases the number and thickening of roots. Coconut water in the culture medium promotes a greater growth of vanilla. The GA_3 and PBZ generate changes in the meristems, which indicate early floral differentiation. This study is a first report that shows the inductive potential of *in vitro* flowering of *Vanilla planifolia*, although it is necessary to

conduct more research to attain complete meristem differentiation and floral development of the species.

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