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Scientific Article

Dendrobium nobile in vitro flowering induction

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Abstract

In vitro flowering is a technique used in genetic improvement that accelerates generations and favors the faster fixation of new traits of agronomic and market interest. The study aimed to establish a protocol for the *in vitro* flowering of *Dendrobium nobile*, through the combined temperature and of the growth regulator thidiazuron (TDZ) effects. Experiments was performed in a flask that was kept in a BOD incubator at 18, 21, or 24 °C or in a growth room at 26 °C. The TDZ concentrations were 0.0, 0.5, 1.0, and 2.0 mg L^{-1}. The highest percentage of flowering shoots of 13.7% occurred at a concentration of 2 mg L^{-1} of TDZ grown at a temperature of 18 °C. *Dendrobium nobile in vitro* flowering was promoted in plants cultivated in ½ MS supplemented with 30 g L^{-1} sucrose, 5.5 g L^{-1} agar, 100 mL L^{-1} coconut water, pH 6.0, and 2.0 mg L^{-1} TDZ at a controlled temperature of 18 °C and a photoperiod of 16 h. The *in vitro* flowering induction protocol of the *D. nobile* species could be used or improved for future studies.
Keywords: flowering, temperature, thidiazuron, Orchidaceae.

Resumo

Indução de florescimento *in vitro* de *Dendrobium nobile*

A floração *in vitro* é uma técnica utilizada no melhoramento genético que acelerar gerações e favorecer a fixação mais rápida de novos caracteres de interesse agronômico e do mercado. O estudo visou estabelecer um protocolo para a floração *in vitro* de *Dendrobium nobile*, através dos efeitos combinados de temperatura e do regulador de crescimento thidiazuron (TDZ). Os experimentos foram realizados em incubadora tipo BOD a 18, 21, 24 °C e, em sala de crescimento a 26 °C. As concentrações de TDZ testadas foram de 0,0, 0,5, 1,0 e 2,0 mg L^{-1}. A maior porcentagem de brotos floridos de 13,70% ocorreu na concentração de 2 mg L^{-1} de TDZ cultivados em temperatura de 18 °C. O florescimento *in vitro* de *D. nobile* foi promovido em plantas cultivadas em ½ MS suplementadas com 30 g L^{-1} de sacarose, 5,5 g L^{-1} de ágar, 100 mL L^{-1} de água de coco, pH 6,0 e 2,0 mg L^{-1} de TDZ em temperatura controlada de 18 °C e fotoperíodo de 16 h. O protocolo de indução de florescimento *in vitro* da espécie *D. nobile* poderá ser utilizado ou aprimorado para estudos futuros.
Palavras-chave: florescimento, temperatura, thidiazuron, Orchidaceae.

Introduction

Orchidaceae is one of the largest and most diverse families of the plant kingdom. Within this family, numerous species have high ornamental value such that they are classified as noble tropical flowers. Orchids of the genus *Dendrobium* are widely cultivated and commercialized due to their profusion of flowers, extensive variety of colors, shapes, sizes, year-round productivity, long shelf life, and recognized medicinal properties in some species (Sarsaiya et al., 2020; Silva and Ng, 2017; Pujari and Sankar Babu, 2022). The vast majority of species of this genus originate in Asia, and several are in danger of extinction (Sarsaiya et al., 2020). The specie *Dendrobium nobile*, popularly known in Brazil as *olho-de-boneca*, is a nonendemic species living in the South and Southeast regions of the country (REFLORA, 2020). It is characterized by erect pseudobulbs and inflorescences with flowers in the nodes of the upper halves of its purple-pink lip. It is an important medicinal species (Li et al., 2019) and is commercialized in Brazil for its ornamental aspects.

The market for ornamental plants in Brazil occupies an important position in the national agribusiness, with the potential for expansion and an important social role by generating employment and income for micro- and small producers throughout the country (Botelho et al.,

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2015). This market has extremely demanded for quality and seeks novelties. Therefore, breeding programs and studies of techniques are essentials to meet the demands and achieve the growth of this agribusiness sector (Eng and Ho, 2019; Huylenbroeck and Bhattarai, 2022; Mohammadi et al., 2021).

Stimulating *in vitro* flowering is a technique that can be combined with genetic improvement programs, especially for late-flowering plants (Kaur, 2022). According to Silva et al. (2014), orchids generally take 3 to 13 years to produce a flowering plant from seeds and have well-defined flowering seasons and juvenile periods that vary depending on environmental conditions and the genetic composition of the genus, species, or hybrid, so the success of breeding programs in orchids has come slowly. Several studies in the literature that research the factors that influence the genetic expression for the flowering of orchids (Nohales and Kay, 2019; Zheng et al., 2019).

In vitro flowering is a technique used to accelerate generation times, favor the faster fixation of new traits, especially in plants with difficult regeneration or rooting (Bridgen et al., 2018), and clarify the factors or mechanisms involved in plant flowering (Wang et al, 2021). This technique enables us to manipulate conditions to induce the transition from the vegetative to the reproductive phase. *In vitro* flower morphogenesis depends on several physical and chemical factors, in addition to intrinsic and extrinsic stimuli (Bridgen et al., 2018).

Dendrobium nobile and its group of hybrids, some authors report the dependence of their flowering on environmental conditions, particularly the need for low temperatures, the conditioning of the adult plant, variations in the levels of endogenous hormones, and gene regulation (Campos and Kerbauy 2004; Silva et al., 2014). Silva et al. (2014) suggested that lower temperatures may improve the flower phenotype *in vitro*. Synthetic cytokinins such as 6-benzylaminopurine (BAP), thidiazuron (TDZ), and 2-isopentenyl adenine (2iP) are among the growth regulators studied in the *in vitro* flowering induction process (Silva et al., 2014; Wang et al., 2019).

Although experiments with different species and hybrids of the genus *Dendrobium* and the use of culture media with different compositions are found in the literature, there is no specific protocol for the control of *Dendrobium nobile* flowering in an *in vitro* environment. The most recent study by Wen et al. (2017) demonstrated that flowering genes are expressed at low temperature but can also be induced by TDZ. A protocol with a simple and well-defined culture medium could promote studies of the physiological and molecular mechanisms that control the flowering phase of the species (Silva et al., 2014), in addition to contributing to the production of flower stems and the development of new cultivars in less time. This study aims to establish a protocol for the *in vitro* flowering of *Dendrobium nobile*, through the combined thidiazuron (TDZ) and effects of temperature.

Materials and Methods

The plants used in the experiment were derived from *Dendrobium nobile* seeds grown in the nursery of the Federal University of Lavras. The seeds were germinated in KC medium (Knudson, 1946) supplemented with 20 g L^{-1} sucrose, 100 mL L^{-1} coconut water, 5.5 g L^{-1} agar, and 2 g L^{-1} activated charcoal. The pH was adjusted to 6.0. A total of 40 mL of culture medium was added to glass flasks, which were kept in a growth room at 26 °C with a photoperiod of 16 h (40-56 µmol m^{-2} s^{-1}) for 60 days. After seed germination, the seedlings were individualized and transferred to new culture medium, remaining in a growth room for another 60 days until the start date of the experiment. Correct ½ MS medium (Murashige and Skoog, 1962) with 30 g L^{-1} sucrose, 5.5 g L^{-1} agar, 100 mL L^{-1} coconut water, and pH 6.0 was used. The TDZ concentrations tested were 0.0, 0.5, 1.0, and 2.0 mg L^{-1}. Culturing was performed in a flask contained 40 mL of culture medium that was kept in a biochemical oxygen demand (BOD) incubator at a controlled temperature of 18, 21, or 24 °C or in a growth room at 26 °C. The photoperiod in both environments (BOD and growth room) was 16 h photoperiod, with irradiance of 40-56 µmol m^{-2} s^{-1}.

The plants used did not have roots, and they were all standardized to have 4 cm (± 1.0 cm) shoots for the experiment. A completely randomized design was used with eight treatments and six replicates, each replicate consisting of a flask with three plants.

After four months, the number of shoots, number of leaves, shoot length (cm), length of the largest root (cm), dry weight (g) of shoot and root, and percentage of flowering plants were calculated. The correlations between the studied variables were calculated as Spearman's rho coefficient, yielding a Spearman correlation matrix.

Results and Discussion

The balance of auxins and cytokinins, provided adequately through the culture medium, is essential for the development of explants *in vitro*, as is the temperature. These factors influence not only growth but also plant development processes, such as flowering, and when combined they can accelerate physiological processes (Taiz et al., 2017).

In addition, the effects of plant hormones were studied under different experimental conditions and in orchid species from the *Phalaenopsis*, *Dendrobium*, *Oncidium*, *Doritaenopsis*, *Spathoglottis* and *Cymbidium* families. The results report varied effects in relation to the application of these hormones (Silva et al., 2014; Wang et al., 2019). In monopodial and sympathetic orchids such as Phalaenopsis and Dendrobium, synthetic cytokinins such as BAP, TDZ, 2iP stimulate flowering, while synthetic auxins such as NAA suppress the effect (Silva et al., 2014; Wang et al., 2019).

TDZ concentration was positively correlated with the number of shoots, total number of leaves, shoot fresh and dry weights, and total number of flowers. The total number of roots, mean number of roots, root fresh and dry weights, and shoot length were negatively correlated with TDZ concentration (Table 1).

Table 1. Spearman correlation matrix for *Dendrobium nobile* grown *in vitro*.

	C	T	NS	TNL	MNL	MSL	SFW	SDW	TNR	MNR	LLR	RFW	RDW	TNFL
C	1	$1{,}00\times10^{0}$	$6{,}28\times10^{-4}$	$5{,}01\times10^{-4}$	$3{,}37\times10^{-1}$	2.27×10^{-3}	4.22×10^{-5}	$4{,}46\times10^{-4}$	2.17×10^{-5}	3.61×10^{-4}	6.09×10^{-4}	1.62×10^{-4}	3.18×10^{-4}	5.53×10^{-6}
T	0.0000	1	5.02×10^{-1}	1.40×10^{-1}	6.91×10^{-3}	4.86×10^{-2}	1.46×10^{-1}	2.14×10^{-2}	8.63×10^{-1}	8.71×10^{-1}	6.02×10^{-1}	5.16×10^{-1}	4.54×10^{-1}	4.19×10^{-2}
NS	0.3742*	0.0760	1	5.36×10^{-28}	4.99×10^{-1}	1.64×10^{-6}	1.01×10^{-12}	2.08×10^{-8}	4.41×10^{-5}	1.82×10^{-12}	1.79×10^{-12}	2.85×10^{-6}	9.12×10^{-6}	3.88×10^{-3}
TNL	0.3804*	0.1666	0.8877*	1	3.86×10^{-6}	1.34×10^{-3}	8.70×10^{-16}	7.63×10^{-11}	5.79×10^{-5}	3.44×10^{-10}	1.45×10^{-10}	1.12×10^{-5}	5.19×10^{-6}	3.53×10^{-3}
MNL	0.1087	0.2997*	0.0767	0.4905*	1	5.37×10^{-3}	1.46×10^{-3}	1.18×10^{-3}	1.62×10^{-1}	2.52×10^{-1}	1.14×10^{-1}	1.98×10^{-1}	5.28×10^{-2}	4.58×10^{-1}
MSL	-0.3365*	0.2213*	-0.5064*	-0.3526*	0.3085*	1	2.59×10^{-1}	7.66×10^{-1}	6.43×10^{-4}	2.40×10^{-5}	4.78×10^{-5}	1.67×10^{-4}	1.06×10^{-3}	2.89×10^{-2}
SFW	0.4411*	0.1639	0.6934*	0.7523*	0.3501*	-0.1278	1	1.06×10^{-29}	3.50×10^{-5}	2.98×10^{-8}	1.68×10^{-7}	7.05×10^{-4}	9.82×10^{-5}	2.12×10^{-2}
SDW	0.3835*	0.2568*	0.5772*	0.6488*	0.3563*	-0.0338	0.8990*	1	2.61×10^{-3}	7.90×10^{-5}	2.54×10^{-4}	2.98×10^{-2}	3.58×10^{-2}	4.55×10^{-2}
TNR	-0.4556*	0.0196	-0.4401*	-0.4339*	-0.1577	0.3735*	-0.4452*	-0.3321*	1	2.24×10^{-33}	6.47×10^{-24}	4.55×10^{-15}	4.55×10^{-16}	2.02×10^{-2}
MNR	-0.3891*	0.0184	-0.6878*	-0.6312*	-0.1295	0.4534*	-0.5720*	-0.4267*	0.9196*	1	8.51×10^{-37}	5.22×10^{-16}	5.21×10^{-16}	1.84×10^{-2}
LLR	-0.3750*	0.0591	-0.6880*	-0.6415*	-0.1780	0.4383*	-0.5454*	-0.3982*	0.8547*	0.9348*	1	1.70×10^{-16}	1.54×10^{-18}	1.98×10^{-2}
RFW	-0.4095*	0.0737	-0.4962*	-0.4695*	-0.1454	0.4088*	-0.3710*	-0.2431*	0.7398*	0.7560*	0.7639*	1	1.30×10^{-35}	1.68×10^{-1}
RDW	-0.4473*	0.0848	-0.4736*	-0.4848*	-0.2173	0.3595*	-0.4216*	-0.2351*	0.7570*	0.7560*	0.7941*	0.9299*	1	9.60×10^{-2}
TNFL	0.4835*	-0.2280*	0.3194*	0.3225*	0.0842	-0.2444*	0.2573*	0.2243*	-0.2592*	-0.2630*	-0.2600*	-0.1558	-0.1874	1

Upper triangular matrix has p values; lower triangular matrix has the Spearman's ρ coefficients; * significant correlation at 5% probability; C - TDZ concentration; T - temperature; NS - number of shoots; TNL - total number of leaves; MNL - mean number of leaves per shoot; MSL - mean shoot length; SFW - shoot fresh weight; SDW - shoot dry weight; TNR - total number of roots per shoot; MNR - mean number of roots; LLR - length of the longest root; RFW - root fresh weight; RDW - root dry weight; TNFL - total number of flowers.

These correlations are explained by the fact that certain concentrations of cytokinins added alone to the culture medium induce shoot cell division, proliferation, and morphogenesis. In addition, TDZ is a synthetic cytokinin with stronger activity than other cytokinins, and its reasons for *in vitro* application are quite varied. It can be used to prevent leaf yellowing, improve photosynthetic activity, induce dormancy breaking, induce callus production, and induce somatic embryogenesis, as well as induce the formation of shoots (Dinani et al., 2018).

In some species, temperature and vernalization are possible inducers of the flowering process (Wang et al., 2019). However, these factors do not always have similar effects between plants grown *in vitro* and *ex vitro* (Silva et al., 2014). In this study, the mean number of leaves, shoot length, and shoot dry weight showed positive correlations with temperature, but the total number of flowers showed a negative correlation with temperature.

In addition, there were positive correlations between the number of shoots, average and total numbers of leaves, fresh weight, and shoot dry weight and negative correlations between the average shoot length, total and average numbers of roots, root fresh and dry weights, and the total number of flowers (Table 1). Possibly, this plant regulator activated genetic information translators for floral development, but it cannot be stated that in vitro flower development resulted from the balance between meristem size and coordination of organogenesis. This balance is expected in an ex vitro situation, in nature, as reported by Chandler (2011).

In *Dendrobium*, flowering is generally promoted by a low-temperature environment. Wang et al. (2009) reported that a constant temperature of 25 °C promoted the flowering of nonperfect flowers in *D. nobile*. When the temperature regime was lower (23 °C day/18 °C night), perfect flowers developed, albeit at low percentages. In hybrids such as *Dendrobium Chao Praya Smile* and *Dendrobium Madame Thong-In*, flowering occurs in environments with high temperature (Campos and Kerbauy, 2004; Silva et al., 2014).

The regulation of flowering in *Dendrobium* cultivation is very important due to the limited flowering season of the species of this genus. Controlled induction would improve the commercialization of flowers in other seasons. The concentrations of cytokinins vary greatly between species; for most species of the genus *Dendrobium*, high concentrations reduce the formation of flowers (Silva et al., 2014). However, Zhang et al. (2019) found, for *Dendrobium 'Sunya Sunshine'*, that 30 mg L^{-1} TDZ effectively promoted the formation of flower buds (87.1%). In the present study, a higher percentage of flowering shoots occurred under 2 mg L^{-1} TDZ. At a temperature of 18 °C with a concentration of 2 mg L^{-1} TDZ, 13.70% of the shoots flowered. Thus, the TDZ concentration influenced the floral induction of plants *in vitro*, since in the absence of the regulator, the temperature variation was not sufficient to induce flowering (Figure 1).

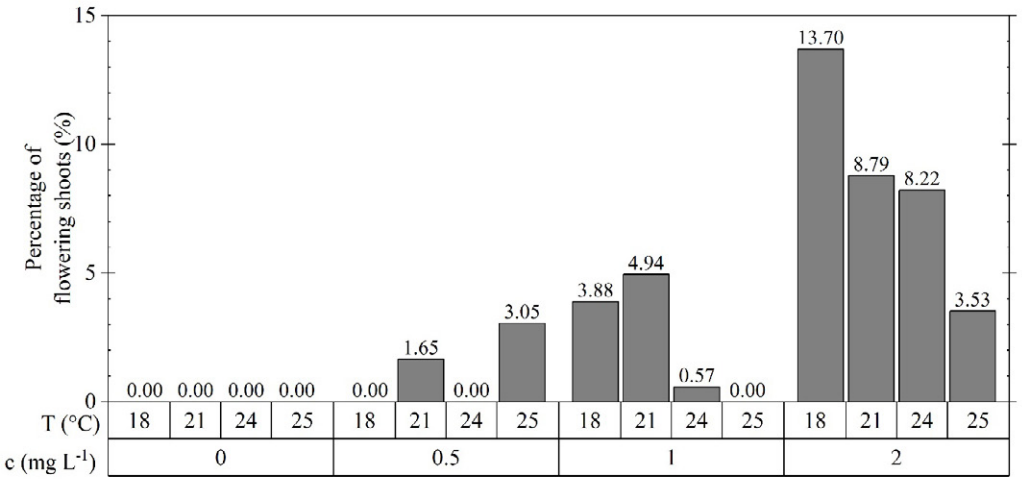

Figure 1. Percentage of flowering shoots as a function of temperature (T) and TDZ concentration (c) for *in vitro* induction of *Dendrobium nobile* flowering.

Most flowering shoots had four or five leaves (23% and 21%, respectively), 88% had no roots, and 8% had one or two roots. The roots of the flowered shoots ranged from 3 to 8 cm. The average shoot length of most shoots ranged from 15 to 25 cm (23% of plants). The higher the mean number of leaves and shoot length were, the lower the flowering rate was. Regarding the number of flowers per bud, 67% of the flowered buds had one flower, while 23% had two flowers, 6% had three flowers, and 4% had four flowers (Figure 2).

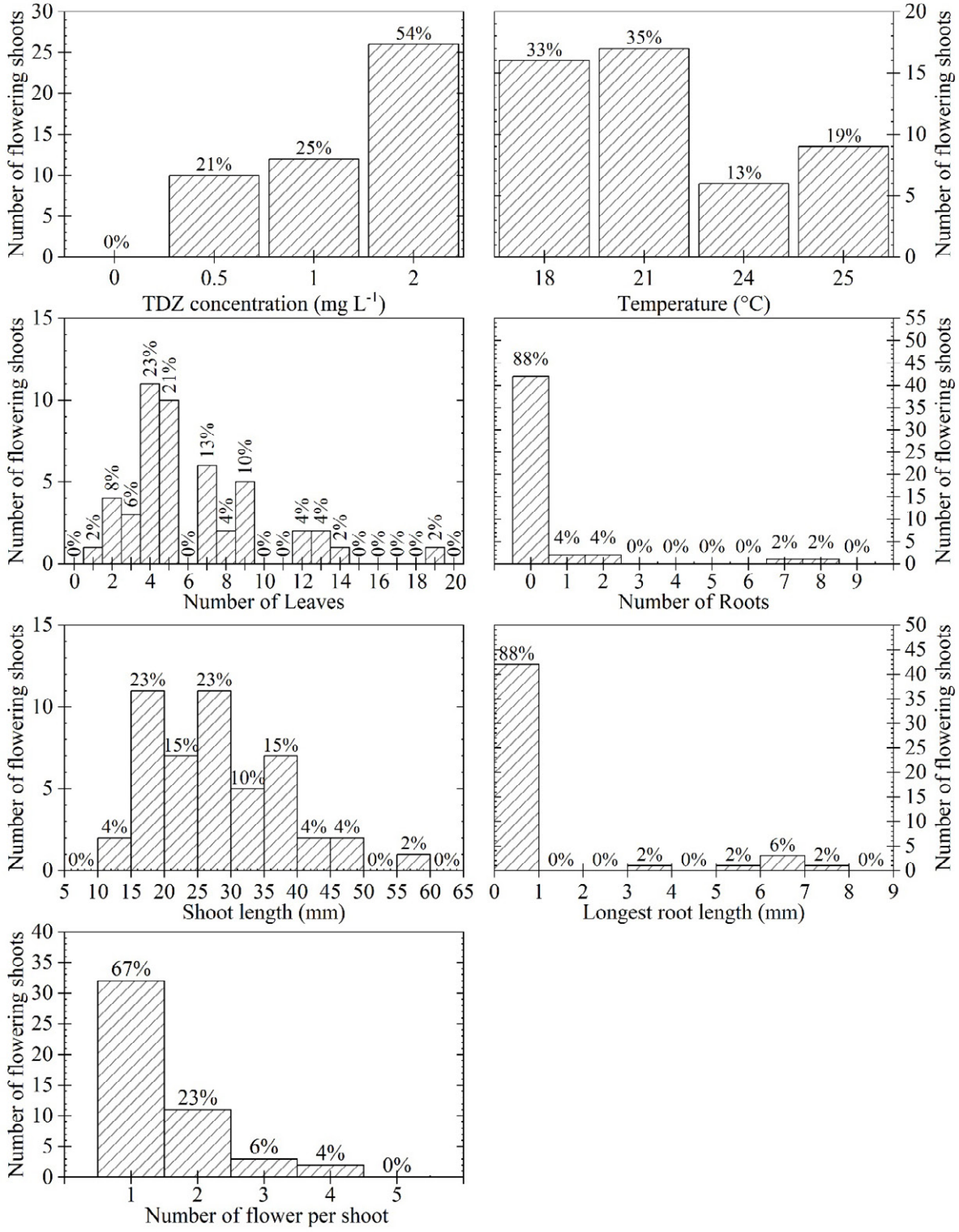

Figure 2. Percentage of flowering shoots as a function of TDZ concentration, temperature, number of leaves, number of roots, shoot length, longest root length and number of flower per shoot, for *in vitro* induction of *Dendrobium nobile* flowering.

In a study with *D. wangliangii*, Zhao et al. (2013) observed that lower TDZ concentrations provided better multiplication percentages of this species, with a lower flowering rate. The TDZ 2 mg L^{-1} concentration was more efficient for the formation of inflorescences in *D. wangliangii*, but it was not more efficient for the formation of shoots.

In view of the many different results described in the literature for species of the same genus, possibly TDZ activates genetic information translators for floral development in *Dendrobium*. However, it cannot be said that the development of flowers in vitro is a result of the balance between meristem size and coordination of organogenesis, which is expected for plants in general. Despite the advances in studies on the *in vitro* flowering of plants, many phenomena of this process are incipient (Pujari and Sankar Babu, 2022) requiring investigations, especially molecular ones, so that we can say with greater certainty how the regulators act in the process in vitro flowering of orchids. Furthermore, we believe that the *ex vitro* use of regulators can help accelerate the process of flowering in a greenhouse, which can be tested in order to generate new technologies for the ornamental plant market.

The inflorescence of the species used in this study increased from the upper nodes of the oldest stem and usually without leaves of the plant (Figure 3a). This behavior is common in several species of the genus *Dendrobium*.

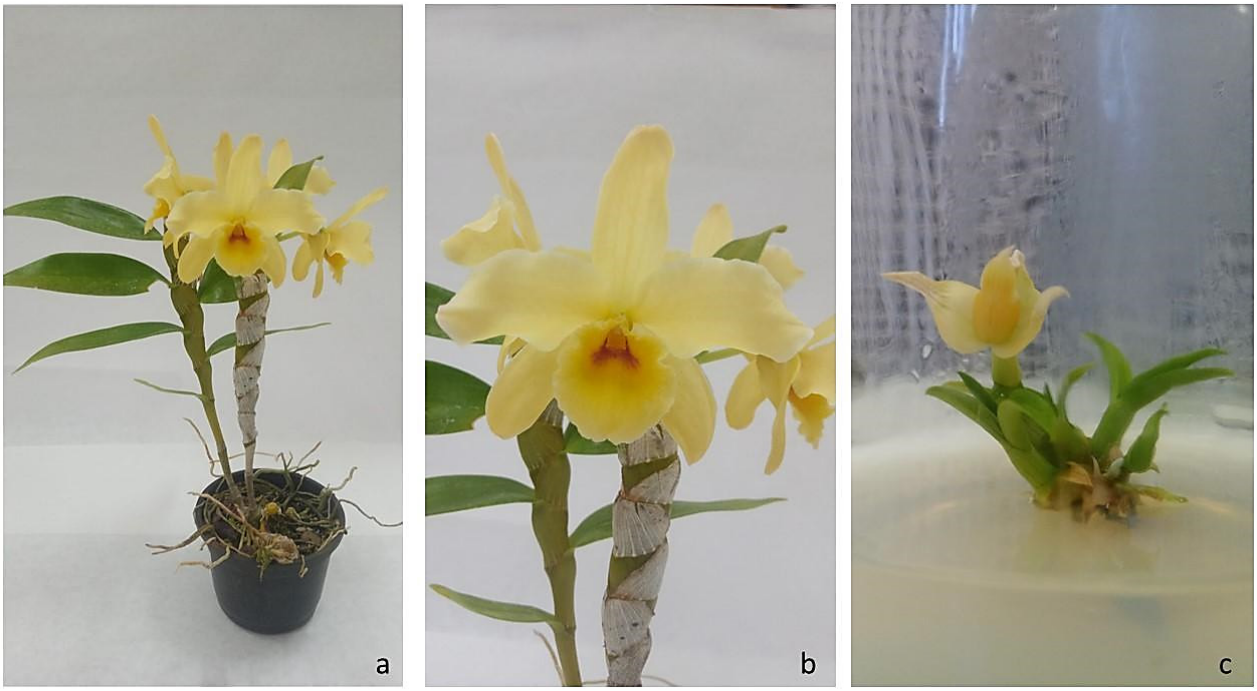

Figure 3. Growth of *Dendrobium nobile* inflorescence (a). Flower color *ex vitro* (b). Flower color *in vitro* (c).

The flowers usually have a yellow color with a red pigmented lip (Figure 3b). However, the *in vitro* inflorescences did not accurately reflect the full color of the flowers, but the presence of yellow petals was noted (Figure 3c).

Conclusions

This study found that culture in ½ MS medium with 30 g L^{-1} sucrose, 5.5 g L^{-1} agar, 100 mL L^{-1} coconut water, pH 6.0, and 2.0 mg L^{-1} TDZ at a controlled temperature of 18 °C and a photoperiod of 16 h induced *in vitro* flowering in *Dendrobium nobile*. The *in vitro* flowering induction protocol of the *D. nobile* species could be used or improved for future studies.

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Author contribution

MCN, VAR: Conceptualization. MCN, GVSA, JHNF: Data curation. MCN, JHNF: Formal analysis. MP: Funding acquisition. MCN, VAR: Investigation. MCN, VAR: Methodology. MCN: Project administration. MP: Resources. JHNF: Software. MCN, JHNF: Validation. MCN, JHNF: Visualization. MCN: Writing – original draft. MCN, JHNF, MVR: Writing – review & editing.

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