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THIDIAZURON INDUCES *IN VITRO* INFLORESCENCE FORMATION IN YOUNG TRANSFORMED *DENDROBIUM* SONIA EXPLANTS ON A MODIFIED KNUDSON C MEDIUM

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ABSTRACT

In transgenic orchids, the effect of a transgene on flower color cannot be scored until the plant flowers, which may take three to five years after transformation. Inducing flowers *in vitro* would allow for much earlier screening. Therefore, we tested whether plant growth regulators (PGRs) added to a modified Knudson C (modified KC) medium could induce flowering in young-transformed *Dendrobium* Sonia explants. Five-month-old rootless explants of the *DsF3H*$_{RNAi}$ line were grown on modified KC medium supplemented with 6-benzyladenine (BA; 1.0, 2.5, and 5.0 mg L^{-1}), gibberellic acid (GA_3; 0.1, 1.0, and 5.0 mg L^{-1}), or thidiazuron (TDZ; 0.1, 0.5, and 1.0 mg L^{-1}) and observed for nine months. Inflorescences were formed only on the TDZ-supplemented medium. The 0.5 mg L^{-1} gave the highest induction rate (30%), whereas 1.0 mg L^{-1} TDZ did not induce any inflorescences and left the plants stunted. The inflorescences that formed never produced healthy floral buds and withered soon after emerging. The application of BA strongly promoted shoot formation, and GA_3 promoted root regrowth, whereas TDZ-treated plants had short leaves, long internodes, and few or no roots. A low concentration of TDZ in the KC medium is thus sufficient to switch young-transformed *Dendrobium* Sonia towards flowering, but obtaining fully developed flowers will require further work on medium composition, PGR combinations, temperature, and photoperiod.

Keywords: Cytokinin; floral transition; in vitro flowering; transgenic orchid; plant growth regulators

INTRODUCTION

Flower color is one of the most valuable traits in the ornamental orchid trade, and the demand for new colors has made it a frequent target of genetic engineering. In orchids, transgenes are usually delivered by *Agrobacterium*-mediated transformation (AMT) or particle bombardment (Baltazar-Bernal and Spinoso-Castillo, 2025), and AMT protocols for *Dendrobium* have been refined over the past few decades (Teixeira da Silva et al., 2016).

Producing transgenic plants is no longer the main difficulty; scoring the results is. Because flower color can only be judged once the plant blooms, and blooming may take three to five years depending on the variety, evaluation is slow. Therefore, a method to shorten this juvenile phase and induce flowering in transgenic lines earlier would be very useful. In vitro flowering plants are one such route. Moreover, Ding et al. (2013) and Sawettalake et al. (2017) used related approaches to study floral transition and flowering time in *Dendrobium* Chao Praya Smile. For transformed *Dendrobium Sonia*, the line studied here, *in vitro* flowering has not yet been observed.

However, before flowering can be exploited for screening, it must first be triggered reliably. This study addresses the first step: which plant growth regulator

(PGR), and at what concentration, will push young-transformed *Dendrobium* Sonia explants into flowering on a modified Knudson C medium. We compared three PGRs with different roles in orchid growth and flowering: 6-benzylaminopurine (BA), gibberellic acid (GA_3), and thidiazuron (TDZ). BA is the cytokinin most often reported to induce flowering in *Dendrobium*, including *Dendrobium* Sonia (Hee et al., 2007; Tee et al., 2008). GA_3 was included because gibberellins drive stem elongation (bolting) and modulate the floral transition, although in orchids, they tend to enhance cytokinin-induced flowering rather than induce it on their own (Wang et al., 2019). TDZ is a substituted phenylurea with strong cytokinin-like activity that remains effective at much lower concentrations than adenine-type cytokinins (Murthy et al., 1998), which is also why its tested range was set below that of BA. Each PGR was applied at three concentrations, and both floral and vegetative responses were recorded. Once established, such a protocol would allow floral traits, such as color, to be screened in transgenic lines years before field flowering.

MATERIALS AND METHODS

Plant Material and Explant Preparation

The explants were derived from a *DsF3H*$_{RNAi}$ line generated in a previous transformation study, in which successful transformation was confirmed by hygromycin selection, PCR-based T-DNA screening, and RT-qPCR expression analysis, all of which are documented in the author's dissertation (Pratama, 2023). Five-month-old-transformed *Dendrobium* Sonia plantlets, 3 cm in length, were used as explants; at this stage, the explants were fully differentiated yet still young enough to be redirected towards flowering, providing a uniform and consistent starting material for the induction test. The roots were removed in a laminar air flow cabinet because rooting and flowering are antagonistic in orchids, and root excision favors floral induction (Kostenyuk et al., 1999). Any residual medium was rinsed off with sterile distilled water before the cells were cultured.

Culture Medium and Culture Conditions

The basal medium was a modified Knudson C (modified KC) medium (Knudson, 1946) as described by Hee et al. (2007), supplemented with 20 g L^{-1} sucrose, 3 g L^{-1} gelrite, and 15% (v/v) coconut water, which was kept constant across all treatments as part of the basal medium. Nine treatments were prepared by adding BA (1.0, 2.5, and 5.0 mg L^{-1}), GA_3 (0.1, 1.0, and 5.0 mg L^{-1}), and TDZ (0.1, 0.5, and 1.0 mg L^{-1}). A PGR-free control was not included, as in vitro flowering in D. Sonia and related Dendrobium hybrids has only been reported on PGR-supplemented media (Tee et al., 2008; Hee et al., 2007). The tested concentrations were selected to bracket the ranges reported to be effective for orchid in vitro flowering, with the lower TDZ range reflecting its higher potency relative to the adenine-type cytokinins. The pH was adjusted to 5.3 before autoclaving at 121 °C for 20 min. Cultures were kept at 25 ± 2 °C under a 16-h photoperiod (35 µmol m^{-2} s^{-1}), subcultured monthly for four months, and then observed until inflorescence emergence and for up to 12 months. Treatments that did not flower were confirmed to be non-flowering throughout the period. The data reported here were recorded at the time of inflorescence emergence, which was approximately nine months.

Experimental Design and Statistical Analysis

A single factor experiment was conducted using a completely randomized design with nine treatments, each comprised ten replicates, with one explant per replication. Each replicate was an individual plantlet from the same *DsF3H*RNAi line, cultured separately and assigned at random, and thus represented an independent experimental unit within the clonal genotype. The inflorescence induction rate (%) was calculated as the percentage of explants forming at least one inflorescence per treatment and was compared among treatments using Fisher's exact test. Because the numbers of new shoots and new roots per explant are discrete count data containing many zero values that did not meet the assumptions of parametric analysis, they were summarized as medians with the first and third quartiles (Q1, Q3) and compared among

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treatments using the Kruskal-Wallis test, followed by Dunn's post-hoc test (Dunn, 1964) with Holm's adjustment when the Kruskal-Wallis test was significant. All analyses were performed in R version 4.2.0 (R Core Team, 2022), with significance set at $p < 0.05$.

RESULTS AND DISCUSSION

Of the three PGRs, only TDZ induced inflorescences, which did not appear until the ninth month of culture (Table 1). TDZ at 0.5 mg L^{-1} was the best treatment. Lowering the concentration to 0.1 mg L^{-1} reduced the rate to 10%, and raising it to 1.0 mg L^{-1} abolished the induction altogether. Inflorescence formation differed significantly among treatments (Fisher's exact test, $p = 0.035$). None of the inflorescences went on to form healthy floral buds; they withered shortly after appearing (Figure 1).

The vegetative responses differed sharply between the PGRs (Kruskal-Wallis test, $p < 0.001$ for both shoot and root numbers). BA promoted shoot formation, with the highest counts coming from 2.5 and 5.0 mg L^{-1} (median = 3 shoots per explant). GA_3 promoted root regrowth, peaking at 12.5 roots per explant at 1.0 mg L-1. TDZ produced neither: plants on TDZ had short leaves, long internodes, and very few or no roots (median = 0 in all three TDZ treatments), and at the two flower-inducing concentrations (0.1 and 0.5 mg L-1) they also had among the fewest shoots (medians of 1 and 0, respectively).

Table 1. Effect of different PGRs in mod KC medium on *in vitro* flowering and vegetative growth of *DsF3H*$_{RNAi}$ transformed lines of *Dendrobium* Sonia after nine months of culture

PGR	Conc. (mg L^{-1})	Inflorescence induction rate (%)	No. of shoots per explant	No. of roots per explant
BA	1.0	0	2 (2, 2) ab	8 (7.2, 8.8) ab
BA	2.5	0	3 (2, 3) a	7.5 (7, 8.8) ab
BA	5.0	0	3 (2.2, 3) a	7 (7, 7.8) abc
GA_3	0.1	0	1 (1, 1.8) abcd	5.5 (5, 7) bcd
GA_3	1.0	0	1 (1, 1) bcd	12.5 (11, 14.8) a
GA_3	5.0	0	1 (1, 1) bcd	10 (9, 11.8) ab
TDZ	0.1	10	1 (0, 1) cd	0 (0, 2) cd
TDZ	0.5	30	0 (0, 1) d	0 (0, 1.5) cd
TDZ	1.0	0	2 (1, 2) abc	0 (0, 0) d

Note: Values for shoots and roots are medians, with the first and third quartiles (Q1, Q3) of ten replicates in parentheses. Groups were compared using the Kruskal-Wallis test ($p < 0.05$) followed by Dunn's post-hoc test; within a column, medians sharing the same letter are not significantly different. Inflorescence data are presented as the induction rate (percentage of explants forming at least one inflorescence), because the median was zero in all treatments; induction was compared using Fisher's exact test. PGR = Plant Growth Regulator; BA = 6-Benzyladenine; GA_3 = Gibberellic acid; TDZ = Thidiazuron.

The developmental response of each explant was governed by both the type of PGR applied and the concentration at which it was supplied. BA is a well-known inducer of *in vitro* flowering in *Dendrobium* and is the most frequently reported regulator to induce it (Baltazar-Bernal and Spinoso-Castillo, 2025; Hee et al., 2007; Kaur, 2022; Tee et al., 2008). However, in the present study, it did not induce flowering in *Dendrobium* Sonia; instead, it promoted shoot proliferation. This is consistent with the strong dependence of BA on nutrient context: in *Dendrobium* Sonia, BA-induced inflorescences required a high-P, low-N medium (Tee et al., 2008), a regime not imposed in the present modified KC medium. In contrast, GA3 favored root regrowth, consistent with the vegetative response to GA3 reported for the same cultivar under nursery conditions by Sanghamitra et al. (2024). A comparable divergence between regulators was reported by Ríos-Barreto et al.

(2023), who found that BA, GA3, and TDZ produced different meristem responses in vanilla. TDZ was the only PGR that worked here, which is in accordance with Chang and Chang (2003), who found TDZ to be a stronger inducer than BA in *Cymbidium ensifolium*.

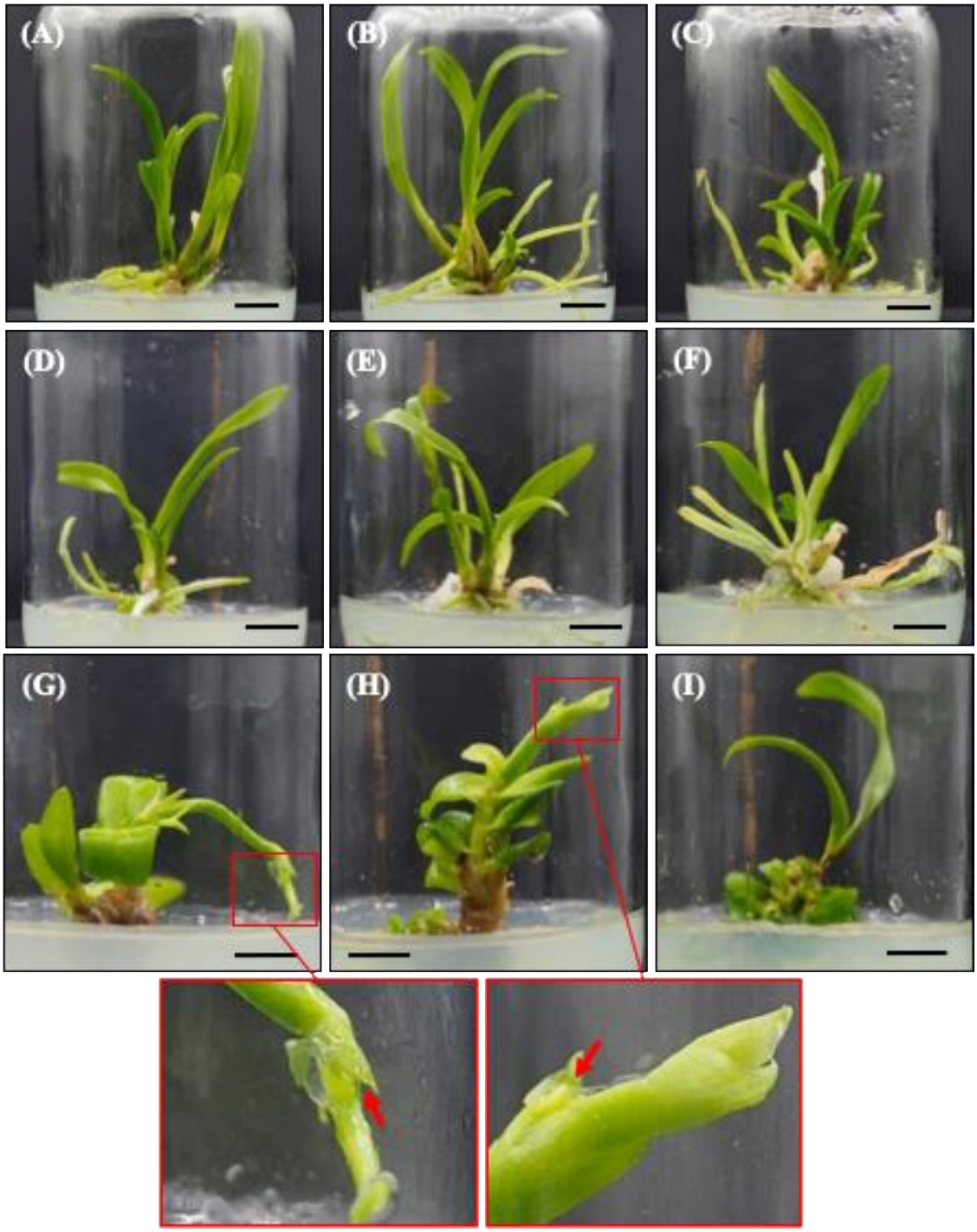

Figure 1. *In vitro* flowering of *DsF3H*$_{RNAi}$ transformed lines after nine months of culture on modified KC medium with different PGRs and concentrations. Explants were cultured on modified KC supplemented with BA (A, B, C; 1.0, 2.5, and 5.0 mg L^{-1}), GA3 (D, E, F; 0.1, 1.0, and 5.0 mg L-1), and TDZ (G, H, I; 0.1, 0.5, and 1.0 mg L^{-1}). Expected flower buds are indicated by the red arrows. Scale bar = 1 cm.

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The mechanism by which cytokinins, such as TDZ, induce these changes has been studied for some time. Scorza (1979) proposed that cytokinins act in floral evocation by controlling early mitotic activity, triggering the precocious initiation of axillary meristems, and accelerating appendage production. Later work put this on a molecular footing: D'Aloia et al. (2011) showed that cytokinin promotes floral transition in *Arabidopsis* by activating *TSF*, a paralog of *FT*, and a recent genetic analysis by Bartrina et al. (2025) confirmed cytokinin as a positive regulator of flowering acting through the floral integrator *SOC1*, the FD-FT/TSF module, and components of the age pathway. Therefore, cytokinin is only one input among several that converge on floral integrators (Peer et al., 2021; Rehman et al., 2023).

However, inducing an inflorescence was not the same as obtaining a flower. The buds never developed and soon withered. This is not surprising: recent syntheses of orchid *in vitro* florigenesis note that inflorescence growth, bud development, and anthesis usually call for a different medium, temperature, and photoperiod than induction does (Baltazar-Bernal and Spinoso-Castillo, 2025; Kaur, 2022), and Zhao et al. (2013) found that leaving plants on TDZ medium for too long damaged flower development and caused morphological variation and early withering. Part of the answer may lie in the hormonal regime itself. Single hormone treatments can induce inflorescences, but sequential or combined hormone treatments tend to increase the induction frequency and produce normal flowers in several orchids (Chang and Chang, 2003; Nadal et al., 2023; Wang et al., 2009; Wu et al., 2024). Temperature is also important; Wang et al. (2009) observed deformed flowers in *Dendrobium nobile* at 25 °C but normal ones once the seedlings were moved to a cooler 23 °C/18 °C (light/dark) regime for 45 days. The practical implication is that induction, as reported here, needs to be followed by a separate development stage with its own medium, hormone combination, temperature, and photoperiod. In its present form, therefore, the protocol only reaches the induction stage and does not yet yield the developed flowers needed to evaluate flower color, which is the ultimate aim of screening transgenic lines.

CONCLUSION

TDZ in a modified Knudson C medium induced inflorescences in young-transformed *Dendrobium* Sonia explants within nine months, and 0.5 mg L^{-1} was the best concentration (30% induction rate). Although the induction rate was modest, TDZ was the only regulator that could trigger floral induction in transformed *D.* Sonia, whereas BA and GA3 did not induce flowering and instead promoted shoots and roots. The inflorescences never became healthy flowers, which shows that floral induction and flower development are two separate problems requiring two different sets of conditions. Nevertheless, a low concentration of TDZ is a viable entry point for shortening the juvenile phase of transgenic *Dendrobium* Sonia, provided that the induction rate can be improved. The next steps are to test multi-stage PGR regimes and to tune the temperature and photoperiod after induction, so that the transgene's effect on flower color can eventually be read off *in vitro*.

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