In vitro medium-term conservation of vanilla (*Vanilla planifolia* Jacks. ex Andrews) through the use of ancymidol

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ABSTRACT
Objective: To evaluate the effect of ancymidol on the *in vitro* medium-term conservation of *Vanilla planifolia* by analyzing explant survival, morphological growth, and the regenerative capacity of the conserved material.
Design/Methodology/Approach: *In vitro* shoots were cultured on MS medium supplemented with 30 g L^{-1} sucrose and different concentrations of ancymidol (0, 1, 2, and 3 mg L^{-1}). After 6 and 12 months of conservation, survival percentage, shoot length, number of leaves, and number and length of roots were evaluated. After 12 months, the conserved material was transferred to MS medium containing 2 mg L^{-1} BAP to assess its regeneration capacity.
Results: Ancymidol significantly restricted shoot and root growth without affecting material survival, with all treatments showing 100% survival at both 6 and 12 months. The greatest growth inhibition was obtained with 1 mg L^{-1} ancymidol, which resulted in the shortest shoot length after 12 months compared with the control treatment. Moreover, shoots conserved under this condition retained their regenerative capacity and multiplied normally after transfer to culture medium supplemented with BAP.
Limitations/Implications: The observed response was assessed exclusively through morphological variables and regeneration capacity.
Findings/Conclusions: Ancymidol constitutes an effective alternative for the in vitro medium-term conservation of *V. planifolia*, as it reduces explant growth, maintains high viability, and allows subsequent regeneration of the conserved material.

Keywords: plant genetic resource, vanilla, natural flavoring, growth inhibitor, medium-term conservation.

Citation: Cadena-Zamudio, J. D., Ramírez-Mosqueda, M. A., Aguirre-Noyola, J. L., Caamal-Velázquez, J. H., Rodríguez-Deméneghi, M. V., & Bautista-Aguilar J. R. (2026). *In vitro* medium-term conservation of vanilla (*Vanilla planifolia* Jacks. ex Andrews) through the use of ancymidol. *Agro Productividad*. https://doi.org/10.32854/a4daqr89

Academic Editor: Jorge Cadena Iñiguez
Associate Editor: Dra. Lucero del Mar Ruiz Posadas
Guest Editor: Juan Francisco Aguirre Medina

Received: December 24, 2025.
Accepted: March 2, 2025.
Published on-line: May XX, 2026.

Agro Productividad, 19(3). March. 2026. pp: 265-274.

INTRODUCTION

Vanilla planifolia Jacks. ex Andrews is a species of high economic and phytogenetic value, recognized as the principal natural source of vanillin. Its conservation is a priority due to the genetic erosion associated with intensive clonal propagation and the limitations of conventional germplasm preservation methods (Jain *et al.*, 2023). In this context, *in vitro* conservation

represents a highly relevant alternative for preserving genetic diversity and maintaining materials of agronomic and biotechnological interest. In vanilla, both *in vitro* conservation and cryopreservation have made it possible to preserve material viability and recover regenerants with low genetic variation, thereby confirming the value of these strategies for the species (Bautista-Aguilar *et al.*, 2021; González-Arnao *et al.*, 2022). Among these alternatives, slow-growth conservation has gained particular importance, as it allows the reduction of explant growth rate without compromising either viability or regenerative capacity. This approach helps prolong intervals between subcultures and reduces risks associated with prolonged maintenance, such as contamination, loss of material, or genetic instability (Schoonraad, 2022). Its effectiveness depends on the adjustment of the culture medium, storage conditions, and the use of growth-modulating compounds (Benelli *et al.*, 2022; Lu *et al.*, 2024). In *V. planifolia*, it has been documented that modifying physical and chemical factors of the culture system, including the use of regulators such as abscisic acid, can reduce explant growth and promote temporary conservation while maintaining survival and material stability (Pasternak & Steinmacher, 2024). Among the compounds with potential for this purpose, ancymidol stands out as a growth inhibitor associated with the alteration of gibberellin biosynthesis or action, hormones involved in cell elongation and in the architecture of shoots and roots. Although gibberellin inhibitors have shown utility in minimum-growth conservation systems in different species, information regarding the use of ancymidol in *V. planifolia* remains limited (Spinoso-Castillo *et al.*, 2022; Castro-Camba *et al.*, 2022). Based on the above, the aim of the present study was to evaluate the effect of ancymidol on the *in vitro* medium-term conservation of *Vanilla planifolia* by analyzing survival, explant growth, and the regenerative capacity of the conserved material.

MATERIALS AND METHODS

Plant material

In vitro vanilla plants (*Vanilla planifolia* Jacks. ex Andrews), previously established from axillary buds at the National Center for Genetic Resources of INIFAP, were used as the source of explants. The plants were established on MS medium (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} sucrose and 2 mg L^{-1} BAP (6-benzylaminopurine), and the pH was adjusted to 5.8. Phytagel® (2.5 g L^{-1}) was used as the gelling agent. The culture media were sterilized in an autoclave at 1.5 kg cm^{-2} and 121 °C for 15 min. Cultures were maintained at 24±2 °C under a 16-h light/8-h dark photoperiod, with a light intensity of 50 μmol m^{-2} s^{-1}.

Effect of ancymidol on the *in vitro* medium-term conservation of vanilla

Individual vanilla shoots (*V. planifolia*) 2 cm in length, bearing one leaf and one root, were transferred to MS medium supplemented with 30 g L^{-1} sucrose and different concentrations of ancymidol (PhytoTechnology Laboratories®) (ANC: 0, 1, 2, and 3 mg L^{-1}). The pH was adjusted to 5.8±0.1, and 2.5 g L^{-1} Phytagel® was added. A volume of 20 mL of culture medium was dispensed into 25×150 mm test tubes in order to avoid subculturing for 12 months. Subsequently, the culture media were sterilized in an autoclave at 1.5 kg cm^{-2} and 121 °C for 15 min. Under a laminar flow hood, one

individual shoot was inoculated per test tube. Cultures were maintained at 24±2 °C under a 16-h light/8-h dark photoperiod, with a light intensity of 50 μmol m^{-2} s^{-1}. Two evaluation intervals were established, at 6 and 12 months, during which the following variables were assessed.

Morphological variables evaluated

After 6 and 12 months of culture, the following morphological variables were evaluated: survival percentage, calculated by dividing the number of living plants multiplied by one hundred by the number of plants established per treatment. Shoot length was recorded in centimeters (cm) using a Chicago Brand® digital caliper. The number of leaves was determined visually, considering only leaves longer than 5 mm. The number of roots was also counted visually, considering only roots longer than 5 mm. Root length was recorded in centimeters (cm) using a Chicago Brand® digital caliper.

Regeneration

After 12 months of *in vitro* conservation, only the shoots derived from the 1 mg L^{-1} ANC treatment were transferred to MS medium supplemented with 30 g L^{-1} sucrose and 2 mg L^{-1} BAP, and the pH was adjusted to 5.8. Phytagel® (2.5 g L^{-1}) was used as a gelling agent. 35 mL of culture medium were dispensed into 150 mL glass jars. Sterilization and incubation procedures were the same as those described above. After 1 month, the number of shoots per explant, shoot length, number of leaves, and the number and length of roots were recorded.

Statistical analysis

A completely randomized design was used in all experiments. Thirty shoots were used per treatment, and the experiments were conducted twice. The data obtained were statistically processed using IBM SPSS Statistics software (version 21). Analysis of variance (ANOVA) was performed, followed by Tukey's test ($p \leq 0.05$), in order to determine whether significant differences existed among treatments. Normality and homogeneity of variance were assessed using the Kolmogorov-Smirnov and Levene tests, respectively. When variables did not meet these assumptions, they were transformed using the natural logarithm (ln).

RESULTS AND DISCUSSION

Effect of ancymidol on the *in vitro* medium-term conservation of vanilla

First evaluation, 6 months: Significant differences were observed among the different ancymidol concentrations evaluated (Table 1). A survival rate of 100% was recorded in all treatments. In general terms, ANC reduced shoot length in comparison with the treatment lacking ANC. However, a hormetic effect was observed, in which low concentrations negatively affected shoot length (Figure 1). The shortest shoots, measuring 2.62 cm, were obtained with the addition of 1 mg L^{-1} ANC to the culture medium, followed by shoots of 3.88 and 3.89 cm obtained with 2 and 3 mg L^{-1} ANC, respectively. The highest shoot length in vanilla (4.94 cm) was observed in the control treatment (without ANC).

Table 1. Effect of ancimidol on the *in vitro* preservation of vanilla (*Vanilla planifolia* Jacks. ex Andrews) after 6 months of culture.

ANC (mg L^{-1})	Survival (%)	Shoot length (cm)	Number of leaves	Number of roots	Root length (cm)
0	100.00±0.00	4.94±0.38a	3.80±0.20a	2.70±0.36a	3.37±0.32a
1	100.00±0.00	2.63±0.15c	2.40±0.30b	3.00±0.25a	1.32±0.08b
2	100.00±0.00	3.88±0.17b	3.20±0.32a	3.50±0.22a	1.00±0.06b
3	100.00±0.00	3.89±0.19b	3.50±0.37a	3.40±0.26a	1.36±0.11b

The values represent the mean±standard error. Values followed by a different letter denote statistically significant differences according to (Tukey, P≤0.05).

Figure 1. Effect of ancymidol on the *in vitro* conservation of *Vanilla planifolia* after 6 months of culture. A) MS+0 mg L^{-1} ANC, B) MS+1 mg L^{-1} ANC, C) MS+2 mg L^{-1} ANC, and D) MS+3 mg L^{-1} ANC.

The treatment with 1 mg L^{-1} ANC produced the lowest number of leaves (2.40), whereas the control treatment produced shoots with 3.80 leaves. Regarding the number of roots, no significant differences were observed among treatments. With respect to root length, the addition of ANC reduced root development per shoot. Nevertheless, a visible increase in root thickness was noted in treatments containing ANC compared with the control treatment. Shoots with roots measuring 3.37 cm in length were obtained in the control treatment, whereas treatments containing ancymidol showed root lengths ranging only from 1.00 to 1.36 cm.

Second evaluation, 12 months: Significant differences were observed among the different ancymidol concentrations evaluated (Table 2). A survival rate of 100% was recorded in all treatments. Overall, ANC reduced shoot length in comparison with the treatment without ANC. However, a hormetic effect was observed, in which low

concentrations negatively affected shoot length (Figure 2). The shortest shoots, measuring 4.25 cm, were obtained with the addition of 1 mg L^{-1} ANC to the culture medium, followed by shoots of 4.87 and 5.13 cm obtained with 2 and 3 mg L^{-1} ANC, respectively. The greatest shoot length in vanilla (10.63 cm) was observed in the control treatment (without ANC).

Unlike the previous evaluation, the control treatment (0 mg L^{-1} ANC) produced the lowest number of leaves (5.10), whereas the treatments containing ancymidol promoted the formation of a greater number of leaves. Regarding root number, the ANC treatments stimulated root formation and visibly increased root thickness (Figure 2). In contrast to the previous evaluation, vanilla plants exposed to ANC required the formation of a greater number of roots and, in particular, showed an increase in root thickness, a response that appears to be specific to this type of plant growth inhibitor. As for root length, the addition of ANC reduced the length of roots produced by vanilla shoots. Nevertheless, a clear visible

Table 2. Effect of ancimidol on the *in vitro* preservation of vanilla (*Vanilla planifolia* Jacks. ex Andrews) after 12 months of culture.

ANC (mg L^{-1})	Survival (%)	Shoot length (cm)	Number of leaves	Number of roots	Root length (cm)
0	100.00±0.00	10.63±0.16a	5.10±0.50b	4.70±0.57b	5.55±0.49a
1	100.00±0.00	4.25±0.19c	7.50±0.60a	8.90±0.69a	1.21±0.13b
2	100.00±0.00	4.87±0.21b	7.30±0.55a	7.70±0.94a	1.05±0.08b
3	100.00±0.00	5.13±0.22b	7.90±0.60a	8.30±0.71a	1.16±0.08b

The values represent the mean±standard error. Values followed by a different letter denote statistically significant differences according to (Tukey, $P \leq 0.05$).

Figure 2. Effect of ancymidol on the *in vitro* conservation of *Vanilla planifolia* after 12 months of culture. A) MS+0 mg L^{-1} ANC, B) MS+1 mg L^{-1} ANC, C) MS+2 mg L^{-1} ANC, and D) MS+3 mg L^{-1} ANC.

increase in root thickness was observed in the ANC treatments compared with the control. Shoots with roots measuring 5.55 cm in length were obtained in the control treatment, whereas the treatments containing ancymidol showed root lengths ranging only from 1.05 to 1.21 cm.

In vitro regeneration of the conserved material

The success of the *in vitro* conservation protocol was confirmed by the micropropagation of vanilla shoots from material conserved for 12 months in ancymidol. An average of 8 shoots per explant was obtained, with each shoot reaching an average length of 2.35 cm and producing one root with an average length of 1.24 cm (Figure 3).

The results demonstrate that ancymidol is an effective alternative for the *in vitro* medium-term conservation of *Vanilla planifolia*, as it maintained 100% survival over 6 and 12 months while significantly restricting shoot and root growth (Table 1 and Table 2). This response is particularly relevant in slow-growth conservation systems, whose objective is to modulate explant development without compromising either viability or regenerative capacity. In this regard, the growth deceleration induced by ancymidol represents a substantial operational advantage, as it allows longer intervals between subcultures and reduces germplasm handling, with the consequent decrease in the risks of contamination, physiological deterioration, and unwanted variation. This behavior is consistent with that described in vanilla and in other species maintained under minimum-growth conditions, where the controlled reduction of development is a central component for the efficient conservation of plant material (Bautista-Aguilar *et al.*, 2021; Coelho *et al.*, 2020; Lu *et al.*, 2024). At 6 months, the inhibitory effect of ancymidol was evident both in the morphological variables and in the phenotypic response of the shoots (Table 1 and Figure 1). The concentration of 1 mg L^{-1} produced the shortest shoot length, whereas the control treatment showed the greatest growth, thus confirming the regulator's capacity to decelerate development without affecting survival. This response is consistent with the mode of action

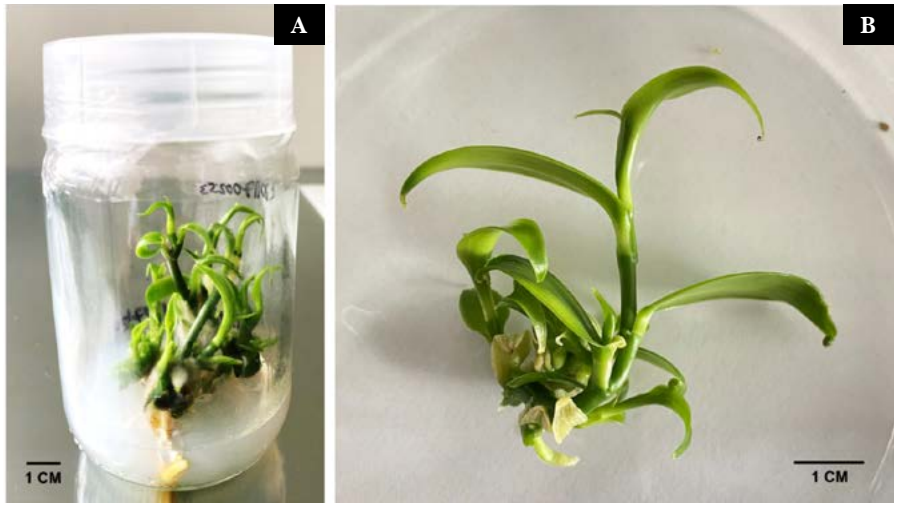

Figure 3. *In vitro* regeneration of *Vanilla planifolia* from explants preserved for 12 months in medium supplemented with ancymidol. A) *In vitro* multiplication, and B) Regenerated plant material.

of gibberellin inhibitors, whose interference limits cell elongation and promotes compact phenotypes. From a conservation standpoint, this effect is desirable, as it allows viable explants to be maintained under a restricted growth dynamic compatible with prolonged periods of in vitro storage (Spinoso-Castillo *et al.*, 2022; Aljaser *et al.*, 2021). This pattern was maintained and even became more pronounced at 12 months, when the difference between the control treatment and the ancymidol treatments was more marked (Table 2 and Figure 2). In the absence of the regulator, shoots exhibited considerable elongation, whereas 1 mg L^{-1} maintained the most restricted growth, with 4.25 cm compared to 10.63 cm in the control. These results indicate that this concentration provides the best balance between growth restriction and viability maintenance, a key condition in *in vitro* conservation protocols. Likewise, they suggest that ancymidol exerts a stable regulatory effect over time, capable of prolonging the conservation period without compromising the biological integrity of the material. This response is in agreement with reports in other species, where gibberellin inhibitors have reduced shoot elongation while maintaining high survival and subsequent recovery, thereby supporting their usefulness in minimum-growth conservation strategies (Spinoso-Castillo *et al.*, 2022; Cruz-Cruz *et al.*, 2022). With regard to the possible hormetic effect, the data suggest that *V. planifolia* exhibits a sensitivity window to ancymidol in which an intermediate dose is more efficient in restricting growth without compromising survival. Rather than strict hormesis, the results appear to reflect a concentration-dependent nonlinear response, a phenomenon frequently observed in in vitro systems exposed to compounds that alter hormonal homeostasis. Since gibberellins participate in key processes of plant growth, such as cell elongation, axial expansion, and the morphological organization of organs, changes in their balance may translate into differential developmental responses depending on the dose applied (Bagale *et al.*, 2022; Castro-Camba *et al.*, 2022). In this context, the fact that 1 mg L^{-1} was more effective than the higher concentrations in restricting shoot elongation suggests a specific sensitivity of the system to moderate levels of the inhibitor. This finding is consistent with what has been described in other plant models, where the optimal concentration of a growth regulator does not necessarily correspond to the highest dose, but rather to the one capable of modulating development while preserving explant viability and regenerative competence (van Gelderen *et al.*, 2023; Sánchez-Mendoza *et al.*, 2025). A particularly interesting aspect of the present study was the response of the root system in the presence of ancymidol. At 6 months, although no significant differences were detected in root number, a reduction in root length was recorded relative to the control. Subsequently, at 12 months, the ancymidol treatments promoted a greater number of roots, although these were shorter than those observed in the absence of the regulator. In addition, a qualitative tendency toward thicker roots was observed in the treatments supplemented with ancymidol. Taken together, this pattern suggests that gibberellin restriction not only affects aerial elongation, but also modifies root architecture, probably through adjustments in meristematic activity, cell proliferation, and the organization of root growth. Several studies have shown that gibberellin regulation exerts a substantial influence on root system dynamics; therefore, alterations in this hormonal axis may generate shorter roots, though not necessarily a homogeneous suppression of all components of root growth (Qin *et al.*, 2022; Barker *et al.*,

2021). Nevertheless, since root thickness was not quantified, this observation should remain a qualitative morphological appraisal, useful for interpreting the overall explant response, but insufficient on its own to support a formal anatomical conclusion (Jing *et al.*, 2024; Velandia *et al.*, 2024). More broadly, the results obtained here are consistent with the rationale of slow-growth *in vitro* conservation and with previous findings in vanilla, where growth deceleration has been achieved without compromising the subsequent recovery of the conserved material. In *V. planifolia*, it has been documented those materials regenerated after in vitro conservation procedures can maintain adequate vegetative growth and low levels of genetic variation, which reinforces the importance of identifying regulators capable of restricting development without affecting the biological stability of germplasm. Complementarily, studies in other species indicate that the effectiveness of minimum-growth conservation systems depends largely on the fine adjustment of variables such as genotype, explant type, and culture medium composition, which explains why the response to growth inhibitors is usually highly specific to the experimental system (González-Arnao *et al.*, 2022; Nurtaza *et al.*, 2024). In this sense, the response observed in the present work provides evidence that ancymidol can be incorporated as a functional tool within vanilla conservation protocols, particularly when the aim is to combine growth restriction, sustained viability, and subsequent recovery of the material (González-Arnao *et al.*, 2022; Nurtaza *et al.*, 2024; Benelli *et al.*, 2022; Benke *et al.*, 2025; Muñoz *et al.*, 2019). The recovery of the conserved material after 12 months undoubtedly constitutes one of the most relevant findings of the study, as it demonstrates that the growth restriction induced by ancymidol did not compromise the morphogenic capacity of the explants. In particular, shoots derived from the 1 mg L^{-1} treatment resumed growth after transfer to MS medium supplemented with BAP, reaching an average multiplication of eight shoots per explant. This result confirms that ancymidol acted as an effective regulator for temporarily limiting development during the conservation phase, yet without irreversibly affecting the regenerative competence of the plant material. From the perspective of *in vitro* conservation, this attribute is fundamental, since the effectiveness of a protocol should not be assessed exclusively by survival during storage, but also by the actual possibility of reactivating, multiplying, and subsequently reusing the conserved germplasm. In this regard, the response obtained in *V. planifolia* agrees with previous studies on vanilla, orchids, and other species maintained under slow-growth conditions, in which sustained viability and subsequent recovery represent essential criteria for validating the biological and operational usefulness of *in vitro* conservation systems (Bautista-Aguilar *et al.*, 2021; Cruz-Cruz *et al.*, 2022; Lu *et al.*, 2024). Altogether, the evidence generated indicates that ancymidol, particularly at 1 mg L^{-1}, constitutes an efficient alternative for the *in vitro* medium-term conservation of *Vanilla planifolia*, as it significantly reduces shoot growth, maintains 100% survival for 12 months, and allows the subsequent regeneration of the conserved material. Therefore, this regulator may be considered a promising tool to strengthen *ex situ* vanilla conservation strategies under *in vitro* culture conditions, especially in contexts where restricted growth, sustained viability, and subsequent morphogenic recovery must be integrated within a single germplasm management scheme.

CONCLUSIONS

Ancymidol proved to be an effective tool for the *in vitro* medium-term conservation of *Vanilla planifolia*, maintaining 100% survival for 12 months while significantly restricting shoot and root growth. Among the concentrations evaluated, 1 mg L^{-1} was the most effective, as it achieved the greatest reduction in shoot elongation without compromising material viability or its subsequent regenerative capacity. Furthermore, explants conserved under this condition recovered their morphogenic competence after transfer to multiplication medium, thereby confirming the functional stability of the protocol. Taken together, these results indicate that the use of ancymidol represents a promising alternative for strengthening *ex situ* vanilla conservation strategies through slow-growth systems under *in vitro* conditions.

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