https://doi.org/10.18406/2316-1817v18nunico20262064 e-ISSN 2316-1817

In vitro culture of forage peanut embryos under different gibberellin concentrations

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Received in: 04/08/2025
Accepted in: 14/05/2026

Abstract

Seed dormancy is a constraint in the propagation of forage peanut, limiting large-scale production. Tissue culture techniques, particularly embryo rescue, offer a promising alternative for breaking dormancy and ensuring uniform seedling establishment. This study was developed in order to evaluate the effect of gibberellic acid (GA_3) on embryo germination, with the objective of establishing an efficient protocol for *in vitro* embryo cultivation. Embryos extracted from four seed lots were cultured on Murashige and Skoog (MS) medium supplemented with five concentrations of GA_3 (0, 10, 20, 40, and 80 mg L^{-1}). Seeds were surface-sterilized with 70 % ethanol followed by 2 % sodium hypochlorite and rinsed with deionized water. Embryos were excised using a sterile scalpel in a laminar flow hood and cultured as explants for regeneration. After 21 days, germination rate, seedling growth parameters, and photosynthetic pigment contents were assessed. Statistical analyses included analysis of variance (ANOVA) and polynomial regression, with treatment effects compared using Tukey's test ($p \leq 0.05$). A quadratic response was confirmed for most parameters, underscoring the importance of dose optimization. Moderate GA_3 concentrations (40 mg L^{-1}) significantly improved embryo germination compared to the control, whereas higher concentrations (80 mg L^{-1}) negatively impacted chlorophyll content and optimal shoot and root development. GA_3 application during *in vitro* culture influenced seedling development, promoting shoot elongation and altering pigment composition. *In vitro* culture proved to be an effective approach for assessing the physiological response of *Arachis pintoi* seedlings to varying gibberellin levels, contributing to a better understanding of dormancy-breaking mechanisms in this species.

Keywords: *Arachis pintoi.* Tissue Culture. Seed Dormancy. Phytohormones.

Introduction

Forage peanut (*Arachis pintoi* Krapov. & W.C. Greg.) is a perennial, herbaceous legume species, widely recognized as a prolific and high-quality forage crop. It holds significant importance due to its application in livestock feed, attributed to its high digestibility, as well as in soil recovery and conservation on slopes and embankments. Additionally, it is used for ornamental purposes in parks and gardens (ASSIS, SILVA, AZEVEDO, 2013).

However, forage peanut seeds have high levels of post-harvest dormancy, which hinders species perpetuation and impairs crop productivity, since manual dehulling alone is insufficient to promote germination (ASSIS, KRZYZANOWSKI, DE AZEVEDO, 2018). To address this issue, plant tissue culture has been studied across various species and has demonstrated practical applicability in overcoming germination challenges (STEFANEL *et al.*, 2021).

Among the techniques within tissue culture, *in vitro* embryo culture involves the isolation and growth of zygotic embryos under sterile conditions (RAPPAPORT, 1954). This method has been successfully employed to regenerate embryos from dormant or germination-impaired seeds, as embryos possess highly totipotent tissues that serve as effective explants (LEITE *et al.*, 2021).

Nevertheless, embryo culture protocols must be customized for each species, with adjustments to optimize culture conditions, including both the composition of the nutrient medium and the precise concentration of plant growth regulators that influence explant development (LEITE *et al.*, 2021). Among these, gibberellins (GA_3) stand out for their ability to stimulate cell division, thereby promoting shoot growth and enhancing the efficiency of *in vitro* propagation (TAIZ *et al.*, 2024).

Given the lack of studies focused on the *in vitro* cultivation of *Arachis pintoi*, the present study was developed in order to establish the first protocol for *in vitro* embryo germination in this species. The effects of different concentrations of gibberellic acid (GA_3) on embryo germination and early seedling development were evaluated. This work seeks to optimize the physiological quality of regenerated plants, thereby contributing to effective propagation, conservation, and agricultural use strategies for forage peanut.

Material and methods

Four seed lots of the cultivar ‘Amarillo’ from the company BRSeeds, harvested during the 2023 growing season, were used in this study. Seeds were surface-sterilized with 70 % ethanol for one minute, followed by immersion in 2 % sodium hypochlorite solution under constant agitation for 20 minutes. After 10 minutes, the solution was replaced to ensure more efficient sterilization. Subsequently, the disinfected material was rinsed with autoclaved deionized water in a laminar flow hood for subsequent explant isolation.

For each seed lot, the initial moisture content was determined using four replicates of 10 seeds. The oven-drying method at 105 °C for 24 hours was applied, in accordance with official procedures (BRASIL, 2025). Results were expressed as percentages based on the fresh weight of the seeds.

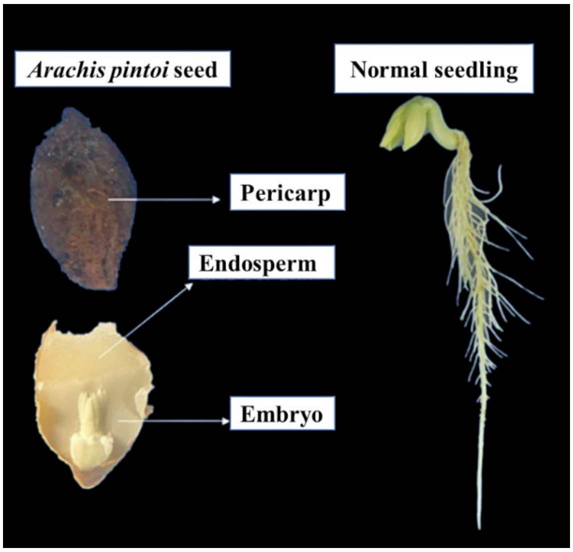
A total of 200 embryos were excised following the protocol described by Bian *et al.* (2018), using a sterile scalpel in a laminar flow cabinet, with the aim of cultivating them as explants. After removal of the pericarp, seeds were longitudinally sectioned on the side opposite the embryo to facilitate extraction.

Embryos (Figure 1) were divided into groups of 50 and aseptically transferred to test tubes containing 15 mL of Murashige and Skoog (MS) culture medium (MURASHIGE, SKOOG, 1962), supplemented with 30 g L^{-1} sucrose and solidified with 5.5 g L^{-1} agar. The plant growth regulator used was gibberellic acid (GA_3), at the following concentrations: D0 = 0 mg L^{-1} (control), D1 = 10 mg L^{-1}, D2 = 20 mg L^{-1}, D3 = 40 mg L^{-1}, and D4 = 80 mg L^{-1}. The medium pH was adjusted to 5.8 prior to agar addition and autoclaved at 120 °C and 108 kPa for 20 minutes. After inoculation, the explants were kept in a growth chamber at a controlled temperature of 25 ± 2 °C with a 16-hour photoperiod.

After 21 days (ROSSETTO, ALVES, 2008), the number of normal seedlings (Figure 1) was recorded — defined as those with well-formed and intact essential structures (shoot and root systems), demonstrating the potential to develop into healthy plants under appropriate growing conditions (BRASIL, 2025). The number of leaves per seedling was also assessed.

Shoot and root lengths were measured, with results expressed in cm per plant. Subsequently, seedlings (Figure 1) were separated into shoot and root components for evaluation of fresh biomass. These samples were then dried in a forced-air circulation oven at 50 °C until reaching constant weight to determine dry biomass.

Figure 1. Seed structures and normal seedling of Arachis pintoi. The pericarp and endosperm measure 1 cm, and the normal seedling measures 7 cm.



Source: authors (2025).

For pigment extraction, fresh forage peanut (*Arachis pintoi*) leaves (approximately 0.015 g) were collected after 21 days and transferred to Falcon tubes containing 3 mL of 80 % acetone. The tubes were wrapped in aluminum foil and stored in a refrigerator at approximately 4 °C. After 24 hours, sample absorbance was measured using a Multiskan GO spectrophotometer (Thermo Fisher Scientific) at wavelengths of 470, 645, 652, and 663 nm, according to the methodology described by Scopel, Barbosa and Vieira (2011).

Wavelength readings were performed in triplicate, using 80 % acetone as the blank. Chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid contents were calculated using the equations proposed by (LICHTENTHALER, WELLBURN, 1983).

The experiment was conducted in a completely randomized design, in a 4 × 5 factorial scheme, with five replicates consisting of two plants each, totaling 20 treatments. Four seed lots and five GA_3 concentrations were evaluated, as follows: D0 = control, D1 = 10 mg L^{-1} GA_3, D2 = 20 mg L^{-1} GA_3, D3 = 40 mg L^{-1} GA_3, and D4 = 80 mg L^{-1} GA_3.

The data were subjected to analysis of variance (ANOVA), and gibberellic acid doses were analyzed by polynomial regression, testing both linear and quadratic models. Model selection was based on the significance of the F-test ($p \leq 0.05$) and a coefficient of determination (R^2) greater than 0.60. For the quadratic models, the optimal GA concentration (maximum point) was determined by setting the first derivative of the fitted equation equal to zero and solving for x, i.e., $x = \frac{-b}{2a}$, where a and b are the coefficients of the quadratic and linear terms, respectively. Mean comparisons for individual seed lot effects were performed using Tukey's test ($p \leq 0.05$). Statistical analyses were performed using R software (R Core Team, 2024), with the 'emmeans' (LENTH, 2017) and 'stat' (KARTIKEYA BOLAR, 2019) packages.

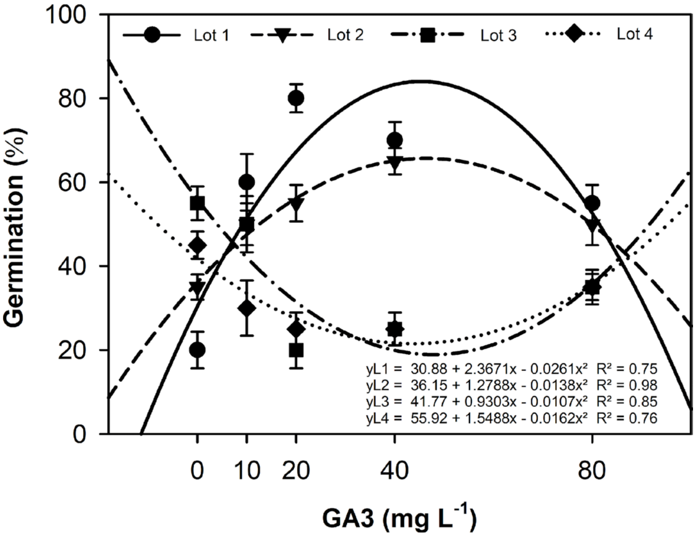
Results

Upon evaluating the seed moisture content across the lots, no variation greater than 3 % was observed (Lot 1: 6.36 %, Lot 2: 8.64 %, Lot 3: 8.95 %, Lot 4: 9.36 %). These values fall within the recommended range for conducting physiological tests, as uniform seed moisture among different lots is essential for standardized evaluations and for obtaining reliable and consistent results (KRZYZANOWSKI *et al.*, 2020).

The effect of different GA_3 concentrations on the germination of forage peanut seeds from the four seed lots is illustrated in Figure 2. All lots exhibited a quadratic response to gibberellin application, indicating that the tested GA_3 concentrations influenced germination rates following dormancy breaking.

The optimal GA_3 concentrations for each seed lot, based on the regression equations presented in Figure 2, are shown in Table 1, along with the maximum germination percentage observed for each lot.

Figure 2. Percentage of germinated embryos as a function of different GA_3 concentrations.



Source: authors (2025).

Although the optimal GA_3 concentration was similar across all four seed lots, Lot 4 reached the highest germination percentage at 93 %, followed by Lot 1 with approximately 85 % germinated embryos when treated with 40 mg L^{-1} GA_3. However, higher concentrations led to a decrease in germination rates for both lots.

In contrast, Lots 2 and 3 had reduced physiological responses to the applied GA_3doses, with germination percentages below 65 %. A 31 % difference was observed between Lots 4 and 3, representing the highest and lowest maximum germination values, respectively.

Lot 4 already reached high germination rates even without the addition of the hormone, likely due to its initially high seed vigor. On the other hand, the poor responsiveness of Lot 3 may be attributed to physiological deficiencies or the presence of damage within that particular seed batch.

A significant effect of the seed lot factor was revealed by the analysis of variance for most of the evaluated variables, except for dry weight. This indicates that the physiological and genetic characteristics of the seed lots had a substantial influence on seedling development parameters (Table 2).

The GA_3 dose factor had a significant effect only on shoot length and leaf number, suggesting that the application of the growth regulator predominantly influenced aerial vegetative growth, without significantly affecting root development, fresh weight, or dry weight.

Table 1. Optimal GA_3 concentrations and maximum germination percentages for the four forage peanut seed lots.

Seed Lot	Optimal GA3 dose (mg L^{-1})	Maximum Germination (%)
1	45,34 a	85,0 a
2	46,33 a	63,0 b
3	43,49 a	62,0 b
4	47,79 a	93,0 a

Means followed by the same letter do not vary from each other according to Tukey's test ($p \leq 0.05$).
Source: authors (2025).

Table 2. Summary of ANOVA for SHL (shoot length), RL (root length), FW (fresh weight), DW (dry weight), LN (leaf number).

SV	df	Mean Square				
		SHL	RL	FW	DW	LN
Lot (L)	3	23.571 *	1.825 **	0.066 **	0.0032 ns	11.090 **
GA_3 Dose (D)	4	19.200 *	0.048 ns	0.013 ns	0.0003 ns	7.109 *
Interaction (L x D)	12	6.726 ns	0.492 ns	0.009 ns	0.0021 ns	2.930 ns
Residual	80	6.631	0.444	0.012	0.0014	2.382
C.V. (%)		60.7	25.76	7.9	3.36	40.05

* *Significant at 5 % ($p < 0.05$); ** Highly significant at 1 % ($p < 0.01$); ns = not significant.

Source: authors (2025).

No significant interaction was observed between seed lot and GA_3 dose (L × D) for any of the analyzed variables (Table 2), indicating a consistent response pattern to the growth regulator across all lots. This suggests that, despite differences in initial physiological quality among the seed lots, evidenced by germination percentage, shoot and root length, and photosynthetic pigment content, the dose-response pattern to GA_3 application was uniform across lots.

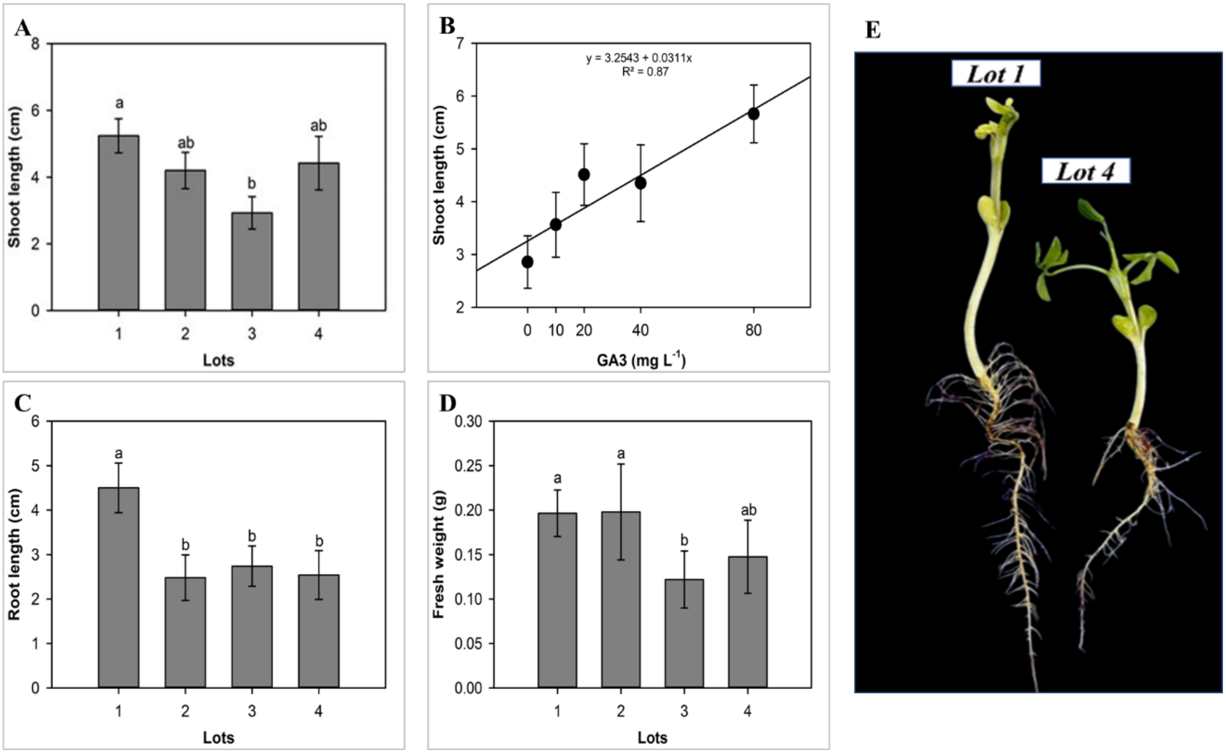
Analysis of Figure 3 reveals that the morphological traits differed significantly among the seed lots. Lot 1 had superior performance in all evaluated variables, with greater shoot length (Figure 3A), longer root length (Figure 3C), and higher fresh weight (Figure 3D), being statistically different from the other lots in all cases ($p < 0.05$).

The superior performance of Lots 1 and 4 in embryo germination is confirmed in Table 1, where both lots reached the highest maximum germination percentages (85 % and 93 %, respectively), with statistically equivalent optimal GA concentrations. However, despite this similarity in germination response, Lot 1 outperformed Lot 4 in seedling vigor, as indicated by greater shoot and root length (Figures 3A and 3C), a difference also visually evident in the representative seedlings shown in Figure 3E. Such performance may be attributed to greater integrity of the embryonic tissues, enhanced mobilization of seed reserves, and higher metabolic activity during germination. In vigorous seedlings, such as those from Lot 1 (Figure 3E), it is common to observe greater efficiency in the utilization of carbohydrate and protein reserves, which promotes cell elongation.

The linear response observed between GA_3 concentrations and shoot length (Figure 3B; $R^2 = 0.87$) indicates that, even after dormancy was overcome, shoot elongation continued to increase with the application of the growth regulator, particularly in seedlings from Lot 1. In contrast, lower mean values for shoot length, root length, and fresh weight were recorded for Lots 2, 3, and 4, with Lot 3 presenting the lowest averages among all evaluated lots — further suggesting reduced physiological quality.

According to Table 3, the interaction between seed lots and GA_3 doses was highly significant ($p < 0.01$) for all photosynthetic pigment variables: chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids. The regression response curves for the analyzed variables across the four seed lots under different gibberellin concentrations are presented in Figure 4. The variation in CHL *a* accumulation (Figure 4A) observed among seed lots in the regression models corroborates previous findings regarding physiological differences primarily associated with seed quality. Greater CHL *a* accumulation

Figure 3. A) Shoot length (cm) of Arachis pintoi seedlings from different seed lots; B) Fitted curve for GA_3 doses as a function of shoot growth; C) Root length (cm) of seedlings from different seed lots; D) Fresh weight (g); and E) Representative normal forage peanut seedlings from Lots 1 and 4.



Source: authors (2025).

was recorded in lots of higher physiological vigor, such as Lot 1, indicating greater responsiveness to the applied GA_3 doses. In contrast, reduced or non-significant increases in CHL *a* were detected in lots of lower vigor, such as Lot 3, suggesting metabolic limitations, reduced chloroplast functionality, and potentially compromised photosynthetic activity.

Higher expression of chlorophyll *b* was found in Lots 1 and 3 at the 80 mg L^{-1} dose, while lower expression levels were recorded in Lots 2 and 4 expression with increasing GA_3 concentrations (Figure 4B). Total chlorophyll followed a trend similar to CHL *a* and CHL *b*, with no substantial increase under higher GA_3 doses (Figure 4C). Carotenoid levels decreased with increasing GA_3 concentrations

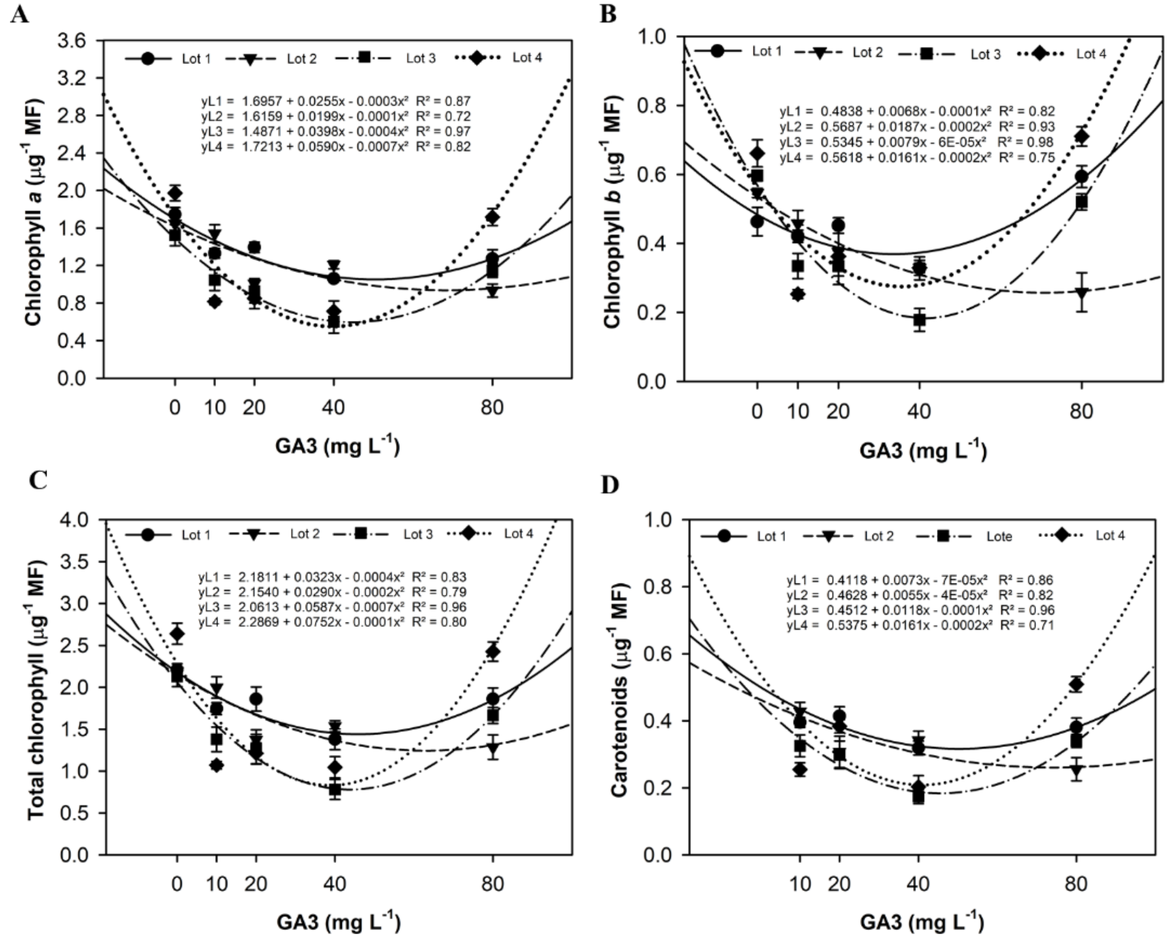
Table 3. Summary of ANOVA for CHL *a* (chlorophyll *a*), CHL *b* (chlorophyll *b*), TC (total chlorophyll), and CAR (carotenoids).

SV	df	Mean Square			
		CHL a	**Chl b**	**TC**	**CAR**
Lot (L)	3	0.426 *	0.136 **	0.301 *	0.036 **
GA_3 Dose (D)	4	1.957 **	0.036 **	0.057 **	0.163 **
Interaction (L x D)	12	0.376 **	0.049 **	0.154 **	0.029 **
Residual	80	0.107	0.006	0.018	0.008*
C.V. (%)		26.8	4.8	5.9	24.9

*Significant at 5 % ($p < 0.05$); ** Highly significant at 1 % ($p < 0.01$).

Source: authors (2025).

Figure 4. Fitted curves of photosynthetic pigments as a function of different GA_3 doses. A) Fit for chlorophyll *a*; B) Fit for chlorophyll *b*; C) Fit for total chlorophyll; D) Fit for carotenoids.



Source: authors (2025).

in all lots, except for Lot 4, which exhibited the highest concentration at 80 mg L^{-1} (Figure 4D).

Variation in the average number of leaves among the different forage peanut seed lots was also identified (Figure 5A). Lot 1 reached the highest average number of leaves per seedling and was statistically superior to Lot 3 ($p < 0.05$), which had the lowest observed value. Lots 2 and 4 presented intermediate values, with no significant difference compared to Lots 1 and 3.

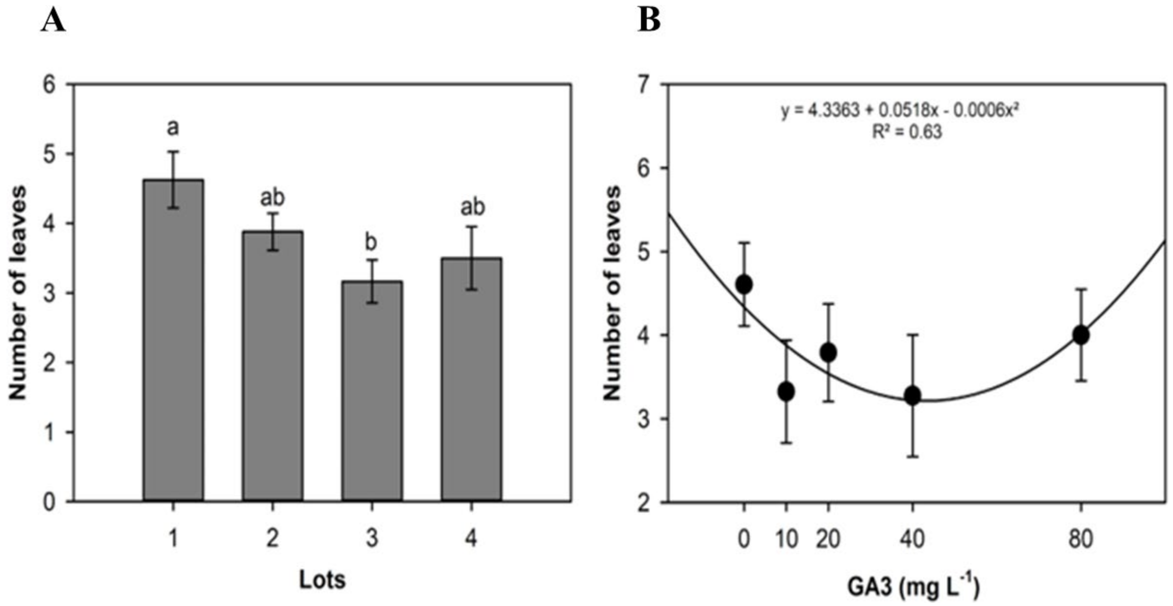
In the fitted regression curve (Figure 5B), the highest average number of leaves was observed in the control treatment (0 mg L^{-1}), followed by a decreasing trend as the GA_3 dose increased. The highest concentrations (40 and 80 mg L^{-1}) resulted in reduced leaf emergence.

Discussion

The use of gibberellic acid (GA_3), a phytohormone widely employed to stimulate plant growth and development, has proven effective in overcoming seed dormancy (TAIZ *et al.*, 2024). In the study developed by Silva, Landgraf, and Machado (2013), the application of GA_3 significantly increased the germination rate of *Brachiaria brizantha* seeds ('MG 5' and 'Marandu' cultivars).

The present study confirmed the significant influence of gibberellin (GA_3) on seedling growth, with variables related to physiological vigor and photosynthetic pigment content showing different responses depending on the seed lot and the applied dose. Higher embryo

Figure 5. A) Number of leaves as a function of different seed lots; B) Fitted curve for number of leaves in response to GA_3 dose.



Source: authors (2025).

germination percentages were recorded for Lots 1 and 4 as GA_3 concentrations increased up to 40 mg L^{-1}, followed by significant reductions at the highest dose of 80 mg L^{-1}. Such reductions may be explained by hormonal imbalance or potential toxicity associated with excessive GA_3 concentration. In contrast, studies using intact seeds reported limited GA_3 effectiveness, likely due to restricted hormone uptake through the impermeable seed coat (BAUTE *et al.*, 2026), reinforcing the advantage of embryo culture as an approach to circumvent physical dormancy barriers.

The low germination rates observed in Lots 2 and 3 (Table 1) may be related to reduced physiological viability of the seeds in these lots. In both cases, none of the tested GA_3 doses were sufficient to significantly enhance embryo germination, which remained below 65 %, as confirmed by the regression analyses. This behavior suggests that the initial poor seed quality compromised the response to the growth regulator. Seeds with low physiological quality exhibit limited responses to GA_3 treatment, indicating that the initial seed quality can compromise the effectiveness of growth regulators. Domingues Neto *et al.* (2024) valued not only for the quality of their fruit but also for their medicinal properties. Conventional propagation through seeds faces challenges due to irregular and slow germination, affecting the time required for seedling formation and the viability and uniformity of plantations. The use of plant growth regulators has been explored as a strategy to overcome these barriers, improving both the rate and uniformity of seed germination. This study aimed to evaluate the influence of seed imbibition with plant growth regulators on the germination and subsequent growth of yellow and sweet passion fruit seedlings. Gibberellic acid (GA3 also observed that the effects of growth regulators such as GA_3 on the germination and seedling development of sweet passion fruit (*Passiflora alata*) and yellow passion fruit (*Passiflora edulis*) varied depending on seed physiological quality.

The analysis of variance (ANOVA) revealed significant differences between seed lots and GA_3 doses for various variables, but the interaction between lot and dose was significant only for chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents. This suggests that while some variables, such as shoot length and leaf number, are more strongly influenced by the intrinsic characteristics of each lot, chlorophyll content responds more specifically to the combination of lot and GA_3 dose. This occurs because the expression of photosynthetic pigments may be modulated by both the genetic composition of the lot and the presence of growth regulators (TAIZ *et al.*, 2024).

The effect of GA_3 on shoot length (Figure 3B) demonstrates that the application of the phytohormone promotes cell elongation, thereby increasing seedling length. This effect is expected, as gibberellin stimulates the expression of genes associated with cell expansion (MARCOS FILHO, 2015).

However, seed lots exhibited variable responses, possibly due to differences in initial seed vigor. This result reinforces the role of gibberellin as a growth promoter while also highlighting the influence of seed lot vigor on the observed outcomes.

Marinho *et al.* (2021)such as pre-soaking. Thus, this study aimed to evaluate the effects of pre-soaking with gibberellin on the physiological potential of two sweet corn seed lots with contrasting vigor levels under ideal and water-deficit conditions. Initially, the seed lots were characterized for germination and physiological potential through first count, germination, accelerated aging, cold test, normal seedling length, normal seedling dry mass, field emergence, emergence speed index, and tetrazolium test. Subsequently, the seeds were pre-soaked with water and 0.4 and 0.8 g L^{-1} gibberellic acid, in addition to the control (without pre-soaking analyzed the impact of GA_3 pre-soaking treatments on sweet corn seed lots with different vigor levels. It was demonstrated that water soaking improved performance in high-vigor seeds, whereas application of 0.4 g L^{-1} GA_3 favored low-vigor seeds. These findings indicate that initial seed quality plays a more decisive role in seedling development than the concentration of gibberellin applied.

Regarding fresh weight, no significant differences were found among Lots 1, 2, and 3; however, Lot 4 had a lower mean value (Figure 3D). This result may be attributed to the lower initial vigor of Lot 4, which did not benefit from GA_3 application. Similar outcomes were observed for leaf number, where Lot 3 had the lowest number of leaves, while the other lots did not differ significantly from each other (Figure 5A). Moreover, GA_3 doses did not significantly influence leaf number, and higher concentrations resulted in values lower than the control (Figure 5B). This suggests that although gibberellin promotes elongation, it may discourage or even inhibit leaf development at elevated concentrations. This trend aligns with findings by Stefanel *et al.* (2021), who reported a positive effect of GA_3 on the *in vitro* elongation of *Eugenia involucrata*; however, concentrations above 2 μM GA_3 in ½ MS medium led to a reduction in the number of shoots per explant.

The significant interaction between seed lots and GA_3 doses on chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents indicates that the response of photosynthetic pigments depends on both the initial physiological quality of the lot and the GA_3 concentration applied. A decrease in chlorophyll *a* content was observed in Lots 1 and 2 with increasing GA_3 concentrations, while low levels were maintained across all treatments in Lots 3 and 4 (Figure 4A). This pattern may suggest that higher doses of gibberellin interfere with chlorophyll biosynthesis, a phenomenon previously reported in studies on the sensitivity of photosynthetic pigments to plant growth regulators (TAIZ *et al.*, 2024). Higher chlorophyll *b* expression was detected in Lots 1 and 3 at 80 mg L^{-1} GA_3, whereas lower expression levels were recorded in Lots 2 and 4 with increasing hormone concentrations (Figure 4B). This variability in pigment responses among lots is likely associated with differences in physiological vigor and genetic background.

Carotenoid levels decreased with increasing GA_3 doses in all lots, except for Lot 4, which exhibited higher carotenoid content at 80 mg L^{-1} (Figure 4D). Carotenoids play a critical role in light absorption and photoprotection against oxidative stress (UENOJO, MARÓSTICA JUNIOR, PASTORE, 2007). The observed decrease in carotenoid content at higher GA_3 concentrations may indicate a limitation in the seedlings' antioxidant capacity, while the atypical response of Lot 4 may suggest greater adaptability of this specific lot to stress induced by elevated GA_3 levels.

Therefore, moderate GA_3 concentrations (~40 mg L^{-1}) significantly enhanced embryo germination compared to the control, whereas higher concentrations (80 mg L^{-1}) negatively affected chlorophyll content and optimal shoot and root development.

In vitro culture proved to be an effective approach for assessing the physiological response of forage peanut seedlings to different gibberellin concentrations, providing deeper insight into the mechanisms involved in overcoming physiological dormancy in this species.

Conclusions

A quadratic response was confirmed for most parameters, underscoring the importance of dose optimization. Moderate GA_3 concentrations (40 mg L^{-1}) significantly improved embryo germination compared to the control, whereas higher concentrations (80 mg L^{-1}) negatively impacted chlorophyll content and optimal shoot and root development. GA_3 application during *in vitro* culture influenced seedling development, promoting shoot elongation and altering pigment composition.

Acknowledgements

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG)

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