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Optimization of Clonal Propagation Protocol for *Arachnis annamensis via* Stem Cuttings under Controlled Nursery Conditions

Phuong Thu Pham[1], Thi Ngoc Tuyen Nguyen[1]

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ABSTRACT

Background: *Arachnis annamensis* Rolfe is a rare and endemic monopodial orchid in Vietnam, threatened by low seed germination due to obligate mycorrhizal dependence. Vegetative propagation is therefore essential for large-scale multiplication and conservation.

Methods: A stem cutting technique was evaluated under nursery conditions using 810 cuttings. Treatments included three cutting types (2, 3 and 4 nodes with aerial roots), three substrates (rice husk + sawdust + soil; rice husk + charcoal + soil; rice husk + soil) and three auxin applications (IBA 1000 ppm, NAA 1000 ppm and control).

Result: Cuttings with 3-4 nodes showed higher sprouting rates, faster shoot emergence, greater elongation and stronger rooting than 2-node cuttings. The substrate mixture of rice husk, sawdust and soil (1:1:1, v/v) provided the best aeration-moisture balance, while IBA at 1000 ppm significantly enhanced bud activation and root induction compared with NAA and control. Propagation efficiency strongly relied on the presence of aerial roots, enabling direct bud activation rather than callus-mediated regeneration. This optimized protocol represents a reliable strategy for clonal propagation and *ex situ* conservation of *A. annamensis*.

Key words: Aerial roots, *Arachnis annamensis*, *Ex situ* conservation, IBA, Monopodial orchid, Vegetative propagation.

INTRODUCTION

The Orchidaceae is one of the largest families of flowering plants, with more than 25,000 species worldwide (Gaskett and Gallagher, 2018); The Editors of Encyclopaedia (2025). Within this family, the genus *Arachnis* (Scorpion Orchids) holds both ornamental value and importance for germplasm conservation. *Arachnis annamensis* Rolfe, endemic to Vietnam and Cambodia, occurs in evergreen and semi-deciduous forests up to 1,500 m and is now threatened by habitat loss and over-collection (Averyanov and Averyanova (2013); Royal Botanic Gardens (2024). The flower morphology of this species is illustrated in Fig 1F. Studies on orchid species have shown that their morphological and reproductive characteristics play an important role in propagation and conservation strategies (Rajan *et al*., 2025).

Sexual reproduction of *A. annamensis* is limited because its dust-like, endosperm-lacking seeds require specific mycorrhizal associations for germination, resulting in extremely low regeneration under natural conditions (Li *et al.,* 2021; Ma *et al.,* 2022; Zhang *et al.,* 2022). Although in vitro culture techniques can bypass these barriers, their cost and technical demands restrict large-scale use (Shekhawat and Manokari, 2016).

Vegetative propagation *via* stem cuttings offers a practical alternative, producing uniform plants while maintaining genetic stability. Success depends on factors such as cutting type, substrate and plant growth regulators. Auxins, particularly indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA), play a central role in root and shoot induction (Le Hong *et al.,* 2024; Lubis and Harahap, 2024). However, their combined effects with cutting morphology and substrate have not been evaluated for *A. annamensis*, leaving a clear research gap.

This study therefore investigates the interactive effects of cutting node number and aerial roots, substrate composition and auxin treatments (IBA and NAA) on rooting, survival and growth of *A. annamensis* under nursery conditions. The results aim to refine propagation protocols for both ex situ conservation and sustainable cultivation of this threatened species.

[1]Faculty of Biology, Hanoi Pedagogical University 2, 32 Nguyen Van Linh Street, Xuan Hoa Ward, Phu Tho Province, Vietnam.

Corresponding Author: Phuong Thu Pham, Faculty of Biology, Hanoi Pedagogical University 2, 32 Nguyen Van Linh Street, Xuan Hoa Ward, Phu Tho Province, Vietnam.
Email: phamphuongthu@hpu2.edu.vn
ORCIDs: https://orcid.org/0000-0002-5193-1820, https://orcid.org/0009-0004-9934-538X

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MATERIALS AND METHODS

Plant materials and experimental conditions

The study was conducted from March 2023 to June 2025 at the Propagation Nursery, Hanoi Pedagogical University 2, Vietnam, using *Arachnis annamensis* Rolfe, an endemic monopodial orchid species. Mother plants were selected based on vigorous growth, healthy aerial roots and absence of pests or diseases. A representative stock plant used for cutting collection is shown in Fig 1A.

Preliminary assessment: Role of aerial roots

Cuttings without aerial roots (2-4 nodes) were tested in standard substrate for 90 days. Survival was <5% with no shoot formation, confirming the essential role of aerial roots in maintaining viability and bud initiation. This agrees with earlier findings in monopodial orchids such as *Arachnis maingayi* (Olosunde *et al.* (2017) and studies on aerial root physiology (Hauber *et al.,* 2020). Therefore, only cuttings with aerial roots were used in the main experiment.

Cutting preparation and auxin treatment

Stem cuttings of 2, 3, or 4 nodes, each with ≥1 aerial root, were excised at a 45° angle. Cut surfaces were disinfected with lime powder or wound paste. Basal ends were soaked for 30 min in indole-3-butyric acid (IBA) or naphthaleneacetic acid (NAA) at 1000 ppm, then shade-dried for 24 h.

Experimental design

The experiment followed a completely randomized block design (RCBD) with three replications of 10 cuttings each (30 cuttings per treatment), totaling 27 treatment combinations and 810 cuttings.

Factor A- Cutting type: A1: 2 nodes + 1 aerial root; A2: 3 nodes + 1 aerial root; A3: 4 nodes + 1 aerial root.

Factor B- Substrate composition: B1: rice husk + sawdust + soil (1:1:1, v/v); B2: rice husk + charcoal + soil (1:1:1, v/v); B3: rice husk + soil (1:1, v/v).

Factor C- Rooting stimulant: C1: IBA 1000 ppm; C2: NAA 1000 ppm; C3: control (untreated).

Nursery management

Prior to planting, the substrates were sterilized with 0.1% Benomyl solution and kept under shade for 24 hours. The cuttings were then transplanted into polybags placed under polycarbonate roofing, with temperature maintained at 25-30°C and relative humidity at 75-85%. Watering was conducted twice daily (morning and afternoon), supplemented by misting to sustain humidity levels. Foliar applications of auxin treatments were repeated twice weekly, starting on the third day after planting, to enhance root initiation.

Observation criteria and measurement

After 90 days of cultivation, the following parameters were recorded.

1. $$\text{Survival rate (\%)} = \frac{\text{Number of surviving cuttings}}{\text{Total cuttings}} \times 100$$

2. Sprouting rate (%) =

$$\frac{\text{Number of cuttings with shoots}}{\text{Surviving cuttings}} \times 100$$

3. $$\text{Mean number of shoots per cutting} = \frac{\text{Total shoots}}{\text{Surviving cuttings}}$$

4. Days to first shoot emergence: Days from planting to appearance of the first shoot.

5. Shoot height (cm): Measured from shoot base to apex.

6. Number of new roots per cutting: Total new roots per cutting.

7. $$\text{Mean root length (cm)} = \frac{\text{Total root length}}{\text{Total new roots}}$$

Statistical analysis

Data were analyzed using SPSS 25.0 and Microsoft Excel 2013. One-way and two-way analyses of variance (ANOVA) were employed to assess the effects of individual and combined factors. Significant differences between means were determined using LSD test at α = 0.05.

RESULTS AND DISCUSSION

Effect of cutting type on survival and growth of *Arachnis annamensis*

Table 1 demonstrates that all cuttings bearing aerial roots maintained 100% survival after 90 days, underscoring the indispensable role of aerial roots in monopodial orchids (Fig 1B). Despite uniform survival, growth responses differed markedly with node number. Four-node cuttings exhibited the strongest performance, producing more shoots (0.92), sprouting earlier (30.56 days) and developing longer roots (4.39 cm), whereas two-node cuttings consistently showed weaker responses (0.67 shoots, 39.77 days, 3.32 cm). These contrasts are clearly illustrated in Fig 2A-C, where differences in shoot sprouting are observed among cuttings with different node numbers and auxin treatments. The results indicate that increasing node number enhances meristematic potential and internal reserves, thereby improving regeneration efficiency, while aerial roots safeguard overall survival. Similar patterns have also been reported in *Dendrobium nobile*, where cutting size strongly influenced propagation success (Colombo *et al.,* 2015). Comparable findings were observed in *Arachnis maingayi* (Olosunde *et al.* (2017); Lubis and Harahap (2024) and in *Vanilla planifolia*, another monopodial orchid with coordinated shoot-root development (Cruceru, 2024).

Effect of substrate composition on survival and growth of *Arachnis annamensis*

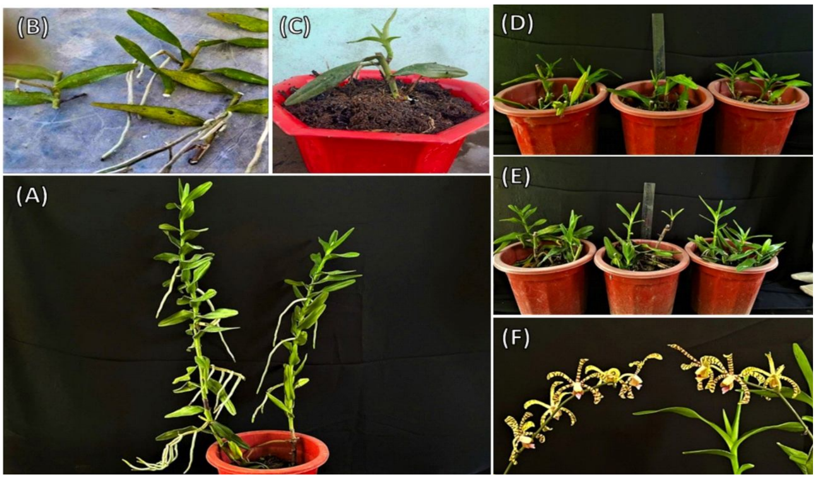
Table 2 shows that survival reached 100% across all substrates, yet growth responses varied significantly. The sawdust + rice husk + soil mixture (1:1:1, v/v) was superior, producing the highest root number (1.88), longest root length (3.96 cm) and stronger shoot growth (Fig 1C). This advantage is attributed to its balanced moisture retention and aeration around the velamen, which facilitate water uptake and root meristem activity. Similar effects have been reported in *Arachnis maingayi* and other monopodial orchids, where substrates combining water-holding capacity and porosity enhanced shoot and root development (Tan *et al.,* 2024; Olosunde *et al.,* 2017).

In contrast, rice husk + soil (1:1, v/v) gave the weakest results, with fewer roots (1.68) and shorter root length (3.61 cm), likely due to poor moisture stability and limited aeration. The charcoal-based mixture also maintained full survival but showed reduced growth, possibly because the low and unstable water-holding capacity of charcoal limited root differentiation (Warigajeshta *et al.,* 2023). Overall, these findings confirm that in *A. annamensis*, substrates are critical not only as mechanical support but also as regulators of the velamen microenvironment, which is essential for aerial root function and regeneration (Hartmann *et al.,* 2011).

Table 1: Effect of cutting type on survival and growth parameters of *Arachnis annamensis* after 90 days of propagation.

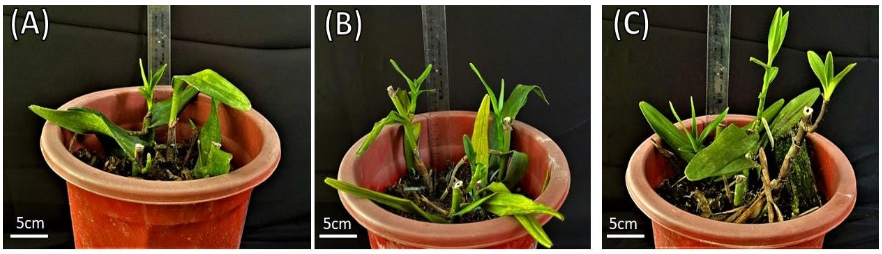
Cutting type	Survival rate (%)	Shoots per cutting (Mean±SE)	Days to first shoot emergence (Mean±SE)	Shoot height (cm) (Mean±SE)	New roots per cutting (Mean±SE)	New root length (cm) (Mean± SE)
2 nodes-1 aerial root	100	0.67±0.029b	39.77±0.19c	2.48±0.02b	1.63±0.04b	3.32±0.03c
3 nodes-1 aerial root	100	0.86±0.033a	32.83±0.81b	3.73±0.06a	1.81±0.07ab	3.63±0.06b
4 nodes-1 aerial root	100	0.92±0.030a	30.56±0.89a	3.87±0.07a	1.87±0.06a	4.39±0.07a

Note: Values are mean±SE. Different letters in a column indicate significant differences (p<0.05; one-way ANOVA, LSD test, SPSS 25.0).



(A) Stock plant ready for harvesting cuttings.
(B) Stem segments with aerial roots prepared for cutting.
(C) Cutting planted in rice husk + sawdust + soil (1:1:1, v/v).
(D) Shoot sprouting of 3-node cutting treated with 1000 ppm NAA.
(E) Shoot sprouting of 4-node cutting treated with 1000 ppm IBA.
(F) Flower morphology of *Arachnis annamensis*.

Fig 1: Clonal propagation of *Arachnis annamensis via* stem cuttings under nursery conditions.



(A) 2-node cutting treated with 1000 ppm IBA.
(B) 3-node cutting treated with 1000 ppm NAA.
(C) 3-node cutting treated with 1000 ppm IBA.
Scale bar = 5 cm.

Fig 2: Shoot sprouting in cuttings under different node numbers and auxin treatments.

Effect of rooting stimulant treatments on survival and growth of *Arachnis annamensis*

Table 3 demonstrates the strong effect of exogenous auxins on the propagation of *A. annamensis*. IBA at 1000 ppm gave the best performance, with more shoots (1.11), faster sprouting (29.71 days), taller shoots (3.71 cm) and stronger rooting (2.22 roots, 4.19 cm). NAA at the same concentration also improved growth but was consistently less effective, while untreated controls showed the weakest response, with delayed sprouting (58.40 days), shorter shoots (1.83 cm) and poor rooting (0.58 roots, 2.18 cm). Representative shoot sprouting responses under NAA and IBA treatments are illustrated in Fig 1D and 1E, respectively.

These findings confirm the superior efficiency of IBA, consistent with previous reports across horticultural crops (Hartmann *et al.,* 2011; Babu *et al.,* 2018). Comparable improvements in root induction following IBA application have also been reported in vegetative propagation studies using stem cuttings (Kumari *et al*., 201); Maninderdeep and Singh, 2022). Its advantage is attributed to its greater chemical stability and lower susceptibility to enzymatic degradation, which allows sustained physiological activity within the cutting tissue. By contrast, the weaker effect of NAA may result from its higher conjugation rate, which reduces the pool of active auxin and potential inhibitory effects under prolonged exposure (Grigoriadou *et al.,* 2021; Somasundaram *et al.,* 2021).

In monopodial orchids, auxins are central to both the release of axillary buds from apical dominance and the initiation of root meristems, explaining the coordinated shoot and root development observed in treated cuttings. This aligns with established auxin-cytokinin interactions in regeneration signaling pathways (Shekhawat and Manokari, 2016; Lubis and Harahap, 2024). Comparable responses have been reported in *Dendrobium nobile* and *Cattleya sp.*, where IBA enhanced propagation success through direct organogenesis, *i.e.*, organ formation without an intervening callus stage (Venturieri and Pickscius, 2013; Lubis and Harahap, 2024). The very weak regeneration in untreated controls underscores that endogenous auxin alone is insufficient for reliable vegetative propagation in *A. annamensis*.

Interaction effects of cutting type and substrate composition on survival, sprouting rate and shoot production of *Arachnis annamensis*

Table 4 shows that all cutting-substrate combinations maintained 100% survival after 90 days, confirming the essential role of aerial roots in viability. However, sprouting and shoot production varied significantly with the interaction between node number and substrate. Four-node cuttings in the rice husk + sawdust + soil mixture (1:1:1, v/v) achieved the highest sprouting rate (84.44%) and shoot number (0.94), while two-node cuttings consistently produced the lowest values (66.67%, 0.67), regardless of substrate.

These results demonstrate that longer cuttings with more nodes provide greater reserves of carbohydrates, proteins and hormones, together with more active axillary meristems. When combined with the favorable aeration and moisture balance of the sawdust-based medium, these factors created optimal conditions for sprouting, as also illustrated in Fig 1C. Similar findings in *Arachnis maingayi* and woody ornamentals confirm that node number and substrate quality jointly determine regeneration efficiency (Hartmann *et al.,* 2011; Olosunde *et al.,* 2017).

The consistently weaker performance of two-node cuttings highlights the limitations of reduced meristem availability and internal reserves, even under favorable substrates. Overall, the interaction effects underline that

Table 2: Effect of substrate composition on survival and growth of *Arachnis annamensis* after 90 days of propagation.

Substrate composition	Survival rate (%)	Shoots per cutting (Mean±SE)	Days to first shoot emergence (Mean ± SE)	Shoot height (cm) (Mean±SE)	New roots per cutting (Mean±SE)	New root length (cm) (Mean±SE)
Rice husk + sawdust + soil (1:1:1, v/v)	100	0.85±0.033a	33.08±0.84a	3.51±0.071a	1.88±0.065a	3.96±0.066a
Rice husk + charcoal + soil (1:1:1, v/v)	100	0.82±0.032a	34.80±0.79a	3.40±0.069a	1.81±0.063a	3.86±0.064ab
Rice husk + soil (1:1, v/v)	100	0.78±0.030a	34.24±0.74a	3.32±0.072a	1.68±0.059b	3.61±0.066b

Note: Total sample: 270 cuttings per substrate (90 control, 180 treated). Values are mean±SE. Different letters in a column indicate significant differences (p<0.05; one-way ANOVA, LSD test).

Table 3: Effect of auxin treatments on survival and growth of *Arachnis annamensis* after 90 days of propagation.

Rooting treatment	Survival rate (%)	Shoots per cutting (Mean±SE)	Days to first shoot emergence (Mean±SE)	Shoot height (cm) (Mean±SE)	New roots per cutting (Mean±SE)	New root length(cm) (Mean±SE)
IBA 1000 ppm	100	1.11±0.019a	29.71±0.48a	3.71±0.05a	2.22±0.04a	4.19±0.045a
NAA 1000 ppm	100	1.06±0.015b	31.86±0.39b	3.53±0.05b	2.17±0.04a	3.97±0.044b
Control (untreated)	100	0.27±0.027c	58.40±0.51c	1.83±0.03c	0.58±0.05b	2.18±0.038c

Note: Values are mean±SE. Different letters in a column denote significant differences (p<0.05; One-way ANOVA, LSD test, SPSS 25.0).

Table 4: Interactive effects of cutting type and substrate composition on survival, sprouting rate and shoot production of *Arachnis annamensis* after 90 days of propagation.

Cutting type	Substrate composition	N	Survival rate (%)	Sprouting rate (Mean±SE)	Shoots per cutting (Mean±SE)
2 nodes-1 aerial root	Rice husk + sawdust + soil (1:1:1, v/v)	90	100	66.67±5.0bc	0.67±0.05c
2 nodes-1 aerial root	Rice husk + charcoal + soil (1:1:1, v/v)	90	100	66.67±5.0bc	0.67±0.05c
2 nodes-1 aerial root	Rice husk + soil (1:1, v/v)	90	100	66.67±5.0bc	0.67±0.05c
3 nodes-1 aerial root	Rice husk + sawdust + soil (1:1:1, v/v)	90	100	78.89±4.33ab	0.93±0.06a
3 nodes-1 aerial root	Rice husk + charcoal + soil (1:1:1, v/v)	90	100	76.67±4.48ab	0.86±0.06ab
3 nodes-1 aerial root	Rice husk + soil (1:1, v/v)	90	100	74.44±4.62b	0.78±0.05b
4 nodes-1 aerial root	Rice husk + sawdust + soil (1:1:1, v/v)	90	100	84.44±3.84a	0.94±0.05a
4 nodes-1 aerial root	Rice husk + charcoal + soil (1:1:1, v/v)	90	100	83.33±3.95a	0.93±0.05a
4 nodes-1 aerial root	Rice husk + soil (1:1, v/v)	90	100	82.22±4.05a	0.89±0.05ab

Note: Different letters within a column indicate significant differences ($p<0.05$) based on two-way ANOVA followed by LSD post-hoc test using SPSS 25.0. Values are presented as mean±standard error (SE).

Table 5: Interactive effects of cutting type and rooting stimulant treatments on shoot emergence time, shoot height and new root formation of *Arachnis annamensis* after 90 days of propagation.

Cutting type	Rooting treatment	N	Days to shoot emergence (Mean±SE)	Shoot height (cm) (Mean±SE)	New roots per cutting (Mean±SE)
2 nodes-1 aerial root	IBA 1000 ppm	90	39.88±0.25c	2.56±0.036b	1.58±0.052d
2 nodes-1 aerial root	NAA 1000 ppm	90	39.66±0.27c	2.4±0.03b	1.69±0.049d
2 nodes-1 aerial root	Control (untreated)	90	-	-	-
3 nodes-1 aerial root	IBA 1000 ppm	90	27.13±0.32d	4.15±0.045a	2.51±0.076b
3 nodes-1 aerial root	NAA 1000 ppm	90	29.97±0.38d	3.93±0.043a	2.37±0.062b
3 nodes-1 aerial root	Control (untreated)	27	61.33±1.01a	1.66±0.05e	0.57±0.069f
4 nodes-1 aerial root	IBA 1000 ppm	90	22.11±0.21e	4.43±0.009a	2.57±0.053a
4 nodes-1 aerial root	NAA 1000 ppm	90	24.72±0.25e	4.27±0.009a	2.44±0.053a
4 nodes-1 aerial root	Control (untreated)	45	56.64±0.35b	1.93±0.032d	0.59±0.069f

Note: Different letters within a column indicate significant differences ($p<0.05$) based on two-way ANOVA followed by LSD post-hoc test using SPSS 25.0. Values are presented as mean±standard error (SE).

both cutting morphology and substrate composition must align to maximize propagation success in *A. annamensis*.

Interaction effects of cutting type and rooting stimulant on shoot emergence time, shoot height and new root formation of *Arachnis annamensis*

Table 5 shows that cutting type combined with auxin strongly influenced shoot emergence, shoot height and root formation. The best results came from three-node cuttings treated with IBA 1000 ppm, which sprouted earliest (27.13 days), produced the tallest shoots (4.15 cm) and formed the most roots (2.51 per cutting). Two-node cuttings performed poorly regardless of auxin and untreated controls failed to sprout or root.

The superior outcome of the IBA + three-node treatment reflects the greater reserves present in cuttings with more nodes, while IBA consistently outperformed other treatments. Fig 2C illustrates the vigorous shoot development in this combination. Similar findings have been reported in tropical orchids and woody plants, confirming IBA as the most effective auxin for rooting and shoot promotion (Hartmann *et al.,* 2011; Grigoriadou *et al.* 2021).

In monopodial orchids, propagation relies on axillary meristems and aerial roots. Healthy aerial roots secure water uptake and hormone transport, while IBA stimulates both rooting and the release of axillary buds from apical dominance. The weak performance of two-node cuttings, even with auxin, shows the limitations of reduced reserves and meristematic potential. Thus, successful propagation of *A. annamensis* requires combining appropriate cutting morphology with effective auxin treatment.

CONCLUSION

This study developed an efficient clonal propagation protocol for the endangered monopodial orchid *Arachnis annamensis* using stem cuttings under controlled nursery conditions. Cutting length, substrate composition and auxin treatment significantly influenced survival, sprouting and rooting performance. Cuttings with three to four nodes showed better regeneration due to higher carbohydrate reserves and a greater number of axillary nodes available for bud activation, with four-node cuttings providing the most reliable growth and rooting performance. The presence of at least one healthy aerial root on each cutting proved essential for maintaining viability and ensuring successful regeneration. The substrate composed of rice husk, sawdust and soil (1:1:1, v/v) offered optimal moisture retention and aeration, supporting aerial root function. Among the auxin treatments, indole-3-butyric acid (IBA) at 1000 ppm consistently outperformed naphthaleneacetic acid (NAA) because of its greater chemical stability and longer physiological activity. Overall, the combination of four-node cuttings, the sawdust-based substrate and IBA treatment provides an effective strategy for large-scale propagation and supports the *ex situ* conservation of this valuable endemic orchid.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

This study did not involve human participants or animal experiments and therefore informed consent/ethical approval was not required.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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