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RESEARCH ARTICLE

Response Surface Methodology (RSM) for the growth optimization of *Clonostachys rosea* TNAU CR04 under varying temperatures, pH and water activity

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Abstract

This study examined the saprophytic fungus *Clonostachys rosea* TNAU CR04, noted for its strong antagonistic capabilities against several plant pathogens, as a prospective biological control agent in sustainable agriculture. Moreover, this study aimed to optimize the growth conditions of *C. rosea* by examining the interactive effects of temperature, pH and water activity (aw) on its mycelial development. To accomplish this objective, we employed response surface methodology (RSM), specifically using a Box Behnken design, which allowed for a systematic exploration of these three critical variables across 17 experimental trials. The analysis revealed that temperature and pH positively affected growth, whereas relatively high-water activity negatively affected growth. The ideal conditions identified were 30 °C, pH 6.5 and aw of 0.88, resulting in a maximum radial growth of 44.80 mm. Model validation showed a strong correlation between the predicted and actual outcomes, with an R^2 value of 0.9901. This research underscores the necessity of optimizing environmental parameters to improve the efficacy of *C. rosea* in agricultural applications. Future studies should focus on validating these findings under field conditions and examining the influence of additional environmental variables on various *C. rosea* strains to enhance the formulation of biofungicides and promote sustainable pest and disease management.

Keywords

biological control; experimental design; mycelial growth; plant pathogens

Introduction

The sustainable management of plant diseases has increasingly focused on biological control methods, which ensure environmental safety, reduce chemical residue levels, prevent pest resistance and support long-term, cost-efficient and environmentally responsible pest and disease management practices. Biological control primarily relies on the use of beneficial organisms and their products to manage harmful pests and plant diseases, offering an effective alternative to the extensive use of chemical fertilizers and pesticides (1). Among various biocontrol agents, *Clonostachys rosea* (formerly known as *Gliocladium roseum*) (2) is a promising saprophytic filamentous fungus classified under the phylum Ascomycota. It is widely distributed globally and thrives in various habitats, with its highest occurrence in soil. As a highly effective mycoparasite, *C. rosea* has significant biological control potential against

numerous fungal plant pathogens, nematodes and insects. By acting as a mycoparasite, *C. rosea* targets fungal pathogens and degrades them through the release of lytic enzymes, such as chitinases and glucanases. The fungus also generates antimicrobial secondary metabolites and volatile organic compounds that further inhibit pathogen proliferation. It competes aggressively for nutrients and space, depriving pathogens for critical resources. Moreover, *C. rosea* establishes itself as an endophyte in plant tissues, serving as a protective barrier against pathogen invasion (3, 4). With broad-spectrum antagonistic activity against a range of plant diseases, such as *Fusarium* spp. (5), *Botrytis cinerea* (6), *Sclerotinia sclerotiorum* (7) and *Rhizoctonia* spp. (8), *C. rosea* is an effective mycoparasitic fungus.

Research has shown that the strain *Clonostachys rosea* TNAU CR04, isolated and characterized at Tamil Nadu Agricultural University (TNAU), has high antagonistic activity against *Alternaria* spp. and *Fusarium* spp. (9, 10). However, maximizing the growth and effectiveness of biocontrol agents such as *C. rosea* requires an understanding of how environmental factors such as temperature, pH and water activity (aw) influence its growth and efficacy. It was reported that water activity (aw), pH and temperature are critical abiotic factors governing the germination and growth potential of the organism (11). For instance, pH regulates fungal cell membrane integrity and nutrient availability, while temperature directly influences enzyme kinetics and metabolic processes. *C. rosea* has an optimal temperature range for growth and enzyme production, generally between 20 °C and 30 °C. Within this range, metabolic activity is optimized, facilitating accelerated fungal growth, sporulation and the synthesis of antimicrobial metabolites and volatile organic molecules (12, 13). Spore germination, mycelial development and the synthesis of bioactive compounds are all influenced by water activity, which measures the free water available for microbial metabolism (14). Optimizing the developmental conditions of antagonistic fungi like *C. rosea* in laboratory environments is essential for augmenting their efficacy in field applications. This requires a comprehensive understanding of these parameters. To date, no research has addressed the collective influence of these environmental factors on the growth of *C. rosea*.

Response surface methodology (RSM) is a frequently adopted experimental approach for evaluating the effects of different variables and optimizing processes such as enzyme production, microbial growth and culture conditions in biological systems (15-18). This methodology facilitates the concurrent examination of interactions among many factors and the influence of multiple process variables on the resultant outcome (19). Multiple experimental combinations are carried out to minimize the impact of irrelevant factors that cause unexplained variance in actual microbial growth (20). This design also reduces the number of experimental runs in a variety of industrial applications, as it is a less time-consuming and more efficient method (21).

Through the application of RSM, this study aims to evaluate the interactive influence of temperature, pH and water activity on the culture characteristics of *Clonostachys rosea* TNAU CR04. The outcomes will contribute to optimizing the mass production of *C. rosea* this biocontrol agent and formulating strategies for its field application to prevent various plant pathogens from establishing infection.

Materials and Methods

Organism

The *Clonostachys rosea* TNAU CR04 strain (GenBank Accession No: ON926975) used in this study was previously characterized for its notable antagonistic properties, such as mycoparasitism, enzyme production, antimicrobial compound synthesis and competitive resource acquisition (10). The strain was sourced from the Culture Collection Centre, Department of Plant Pathology, TNAU, Coimbatore, Tamil Nadu, India. Fungal cultures were purified on PDA media and kept at 4°C for use in further optimization studies.

Culture medium

The medium employed for culturing the fungi was potato dextrose agar (PDA) (water activity, aw = 0.88). Glycerol was added in varying concentrations to adjust the aw to 0.93 and 0.98 at three different pH (5.5, 6.5 and 7.5) before autoclaving. The aw of all the media was measured using an AquaLab series 3 instrument located at the Centre for Post-Harvest Technology, TNAU, Coimbatore. A 5 mm culture disc from a 10-day-old *C. rosea* culture plate was subsequently removed with a cork borer and inoculated onto a PDA plate according to the different treatments.

Experimental design for the optimization of culture conditions via RSM

A Box-Behnken design with three variables (temperature, pH, and water activity) was used to study their combined effects on the mycelial growth of *C. rosea* across three levels. Unlike full factorial designs, the Box-Behnken design minimizes the number of experimental trials while still capturing key variable interactions. Table 1 delineates the levels and ranges of the factors. Furthermore, it bypasses the severe experimental conditions characteristic of central composite designs, hence ensuring more dependable optimization for *C. rosea*. This design is particularly useful for exploring quadratic response surfaces, producing a second-order polynomial model while minimizing the number of experimental runs. The total number of experimental runs required is calculated via the formula

$$N = k^2 + k + Cp \qquad \text{Eqn.01}$$

where *k* indicates the number of factors (3) and Cp is the number of central point replications (3).

This design, implemented via Design Expert 13, led to 17 experimental runs. The second-degree polynomial model is expressed as:

Table 1. Actual factor levels for Box-Behnken Design

Independent Variables	Symbols	Actual levels		
		Low (-1)	Medium (0)	High (+1)
Temperature	A	20	25	30
Water Activity	B	0.88	0.93	0.98
pH	C	5.5	6.5	7.5

$Y= b0 + b1A + b2B + b3C + b12AB + b13AC+ b23BC + b11A^2 + b22B^2 + b33C^2$ (Eqn. 02)

where Y is the predicted response; A, B and C are independent variables; b0 is the offset term; b1, b2, b3 are linear effects; and b11, b22 and b33 are interaction effects. The model was evaluated via regression analysis and ANOVA as per equation 2. The model's adequacy was assessed via R^2 analysis and model validation, while the significance of the equation was determined through F-value testing at the 5 % significance level. To visually interpret the relationships between the factors and the response and to optimize the conditions, the model equations were transformed into contour plots by Design Expert 13 software.

Data recording

Mycelial growth was analyzed under the interactive effects of temperature, pH and water activity by measuring the fungal colony diameter (mm) in two perpendicular directions at a 90° angle. The values were averaged from three replicates and the colony radius was calculated by dividing the mean diameter by two (22).

RSM model validation

The RSM model was validated by implementing the predicted conditions to achieve optimal growth of *C. rosea*. The predicted outcomes were then compared with the actual experimental results for validation.

Results and Discussion

Experimental results for the optimization of culture conditions via RSM

The Box-Behnken design (BBD) of response surface methodology (RSM) was employed to optimize three important variables for enhancing the growth of *C. rosea* TNAU CR04 in PDA media. The results from 17 experimental trials are summarized in Table 2. The experimental data were subjected to regression analysis to develop a mathematical model, which facilitated the identification of optimal growth conditions for maximizing mycelial growth. ANOVA was used to test the statistical significance of the polynomial model equation, which was performed via Design-Expert 13 software. The resulting response was fitted to a second-order polynomial equation, as shown below.

Radial Mycelial Growth (mm)

$Y = 40.63 + 0.8625A - 1.71B + 4.00C - 0.4750AB - 1.10AC - 2.17BC + 0.3388A^2 + 1.01B^2 - 6.69C^2$ (Eqn. 03)

where Y represents the radial mycelial growth (mm), A represents the temperature, B represents the water activity and C represents the pH of the medium.

The impact of the variables on mycelial growth was analyzed through equation (2). Temperature (A) had a positive coefficient (0.8625), indicating that an increase in temperature within the experimental range promotes fungal radial growth. Elevated temperatures promote the growth of *C. rosea* by increasing enzyme activity, enhancing metabolic processes such as ATP synthesis and facilitating food absorption, however solely within its ideal temperature range. Beyond this optimal range, excessively high temperatures can lead to enzyme denaturation, stress responses and impaired growth. Conversely, water activity (B) exhibited a negative effect (-1.71), suggesting that higher water activity suppresses growth. pH (C) demonstrated the most significant enhancement of fungal growth, as indicated by its positive coefficient of 4.00. The analysis confirmed the significance of the linear effects of A, B and C and their respective interactions. Additionally, the interaction effect between water activity (B) and pH (C) was significant (Table 3).

In the Box-Behnken design, the actual maximum radial growth observed was 44.80 mm at 30 °C, pH 6.5 and 0.88 aw of the medium, which suggests that these conditions enhance enzyme activity and metabolic efficiency, crucial for maximizing its biocontrol efficacy. The predicted and actual responses, along with the design matrix, are also presented in Fig. 1 and Table 2.

Table 2. Predicted and experimental responses of the dependent variable in a three factor Box–Behnken Design

Run	Factor 1	Factor 2	Factor 3	Response (Radial mycelial growth)	
	X1(A): Temperature	X2(B): Water Activity	X3(C): pH	Actual	Predicted
	degree celcius	aw	No unit	mm	
1	25	0.88	7.5	43.72	43.77
2	30	0.98	6.5	41.35	41.28
3	25	0.98	5.5	30.5	29.87
4	20	0.88	6.5	41.65	41.96
5	25	0.98	7.5	35.05	35.10
6	30	0.93	5.5	32.45	32.41
7	25	0.93	6.5	40.63	40.63
8	30	0.88	6.5	44.8	44.93
9	20	0.98	6.5	40.1	40.12
10	25	0.93	6.5	40.65	40.63
11	25	0.93	6.5	40.65	40.63
12	25	0.88	5.5	30.5	29.87
13	25	0.93	6.5	40.61	40.63
14	25	0.93	6.5	40.61	40.63
15	20	0.93	7.5	38.3	38.31
16	30	0.93	7.5	37.35	37.39
17	20	0.93	5.5	29	28.92

Table 3. Analysis of Variance (ANOVA) for the quadratic model on mycelial growth maximization

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	372.07	9	41.34	77.59	< 0.0001	significant
A-Temperature	5.95	1	5.95	11.17	0.0124	
B-Water Activity	23.36	1	23.36	43.84	0.0003	
C-pH	127.76	1	127.76	239.79	< 0.0001	
AB	0.9025	1	0.9025	1.69	0.2343	
AC	4.84	1	4.84	9.08	0.0196	
AC	18.79	1	18.79	35.27	0.0006	
A^2	0.4832	1	0.4832	0.9068	0.3727	
B^2	4.26	1	4.26	8.00	0.0255	
C^2	188.66	1	188.66	354.09	< 0.0001	
Residual	3.73	7	0.5328			

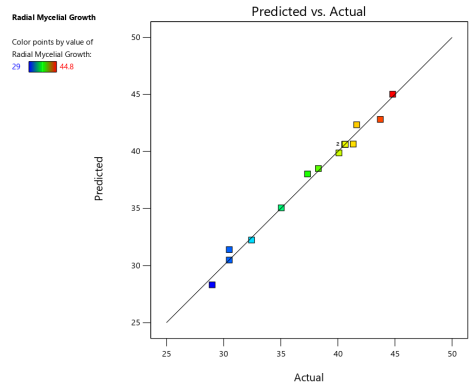

Fig. 1. Validation of mycelial growth optimization for *C. rosea*: Predicted vs. Actual.

The F value of 77.59 indicated that the quadratic model was highly significant. An R^2 value of 0.9901 signified that 99.01 % of the variability in growth was captured by the model. A standard deviation of 0.7299 and an average growth measurement of 38.11 mm demonstrated excellent statistical reliability. With a coefficient of variation of 1.92 %, the model showed high precision and the adjusted R^2 (0.9773) confirmed its robustness even with multiple predictors. Additionally, the predicted R^2 (0.8413) demonstrates reliable predictive capability and the adequate precision value of 29.8394 indicates a strong signal-to-noise ratio, validating the model's reliability for predicting growth under varying conditions (Table 4).

Table 4. Statistical data for ANOVA analysis

Std. Dev.	**0.7299**	**R^2**	0.9901
Mean	38.11	**Adjusted R^2**	0.9773
C.V. %	1.92	**Predicted R^2**	0.8413
		Adeq Precision	29.8394

These findings align with recent findings in optimizing fungal biocontrol agents, particularly in agricultural applications. Research has investigated the impact of culture conditions, including pH, temperature, and water activity, on beneficial fungi such as *Trichoderma* and *Beauveria*, which are commonly used for biocontrol (23, 24). Similar optimization techniques, such as RSM, have been applied to increase antifungal metabolite production and conidial growth, improving biocontrol efficiency (25, 26). For instance, used RSM to optimize conditions for *Trichoderma lixii* TvR1, predicting maximum spore production (19.1245 × 10^7 spores/g) at 30 °C and 68.87 % moisture following 31 days of incubation period (27). Furthermore, It was also confirmed from a study that the growth of *C. rosea* IG119 and its chitinolytic enzyme production under polyextremophilic conditions (28). Growth and enzyme production profiles across varying pH and salinity levels were generated using a D-optimal model employing response surface methodology (RSM). Furthermore, an innovative solid fermentation reactor that features light transparency and ventilation on both the top and bottom (29). Using RSM, they optimized the cultivation conditions for *C. rosea* in solid-state fermentation. This fermenter offered a spore growth surface that was twice the size of traditional models. Thus, RSM has been successfully applied to a range of antagonistic fungal species, showing that precise control over growth conditions can significantly improve both experimental outcomes and the reliability of model predictions across various strains and conditions.

Response surface 3D and contour plots

The relationships among the variables, including temperature, water activity and pH of the PDA medium, were explained via a regression equation and illustrated through response surface 3D graphs and contour plots. These plots depicted how two of the variables influenced growth while the third variable was held constant at a zero level.

The results from the 3D and contour plots demonstrated significant interactions among temperature (A), water activity (B) and pH (C) on the radial growth of mycelia (Y). As shown in Fig. 2a, d, the interaction between water activity and temperature is significant (i.e., increased temperature promotes growth, whereas increased water activity reduces it). This outcome matches the findings a previous study who examined *Aspergillus flavus* and identified an inhibitory effect on conidial germination at minimal water activity and high temperatures, concluding that fungi necessitate a precise equilibrium of moisture and heat for optimal growth (30). It was also reported that water activity (aw) has a stronger effect than temperature on fungal growth. It was also showed that changes in environmental conditions, especially water availability, often reduce the viability of conidia and slow mycelial growth (31).

Fig. 2b, e indicates that growth increases with pH, reaching its peak at 6.5 and then declining beyond this point. However, temperature does not significantly interact with pH to affect growth. This adaptability implies that *C. rosea* could perform effectively across diverse environments with varying

temperatures, as long as the pH remains favorable. This finding agrees with the outcome of a previous study which explored the effect of pH on the biological control activity of *Trichoderma atroviride* against *Rhizoctonia solani* and confirmed that pH levels of approximately 6.5-7.5 create a favorable environment for antagonistic fungal growth by promoting enzyme activity and nutrient availability in the medium. Chitinases, glucanases, proteases, cellulases and lipases from *Trichoderma atroviride* break down pathogen cell walls and organic matter, facilitating nutrient absorption and enhancing its biocontrol effectiveness, particularly at this optimal pH. They also reported that culture conditions are interdependent factors that influence each other (32). It was also identified that a pH of 6.5 as optimal for the highest chitinase secretion from *Bacillus pumilus* MCB-7, using response surface statistical analysis (33).

Fig. 2c, f indicates a significant interaction between water activity and pH, with growth being optimized at higher pH values, while lower water activity further increases growth. This aligns with the findings of of a study which demonstrated that in *Beauveria bassiana*, temperature and pH exert independent influences on fungal growth (24). Temperature primarily governs the metabolic activity of fungi, while pH plays a key role in regulating enzyme production and expression, with each factor having distinct but critical effects on the overall growth process.

RSM model validation

To achieve maximum mycelial growth, the factors of temperature, water activity and pH were optimized using 'in range' criteria for the factors and 'maximum' criteria for the response. All factors were equally weighed, resulting in the optimal solution (45.17 mm) at 30°C, with a water activity of 0.88 and a pH of 6.5. The observed experimental result, *i.e.,* radial mycelial growth (44.80 mm) (Fig. 3), is closely aligned with the predicted response (44.93 mm), demonstrating a robust model fit, as depicted in Table 2 and Fig. 1. The strong agreement between the observed and predicted values attests to the model's precision and practical effectiveness, validating its accuracy.

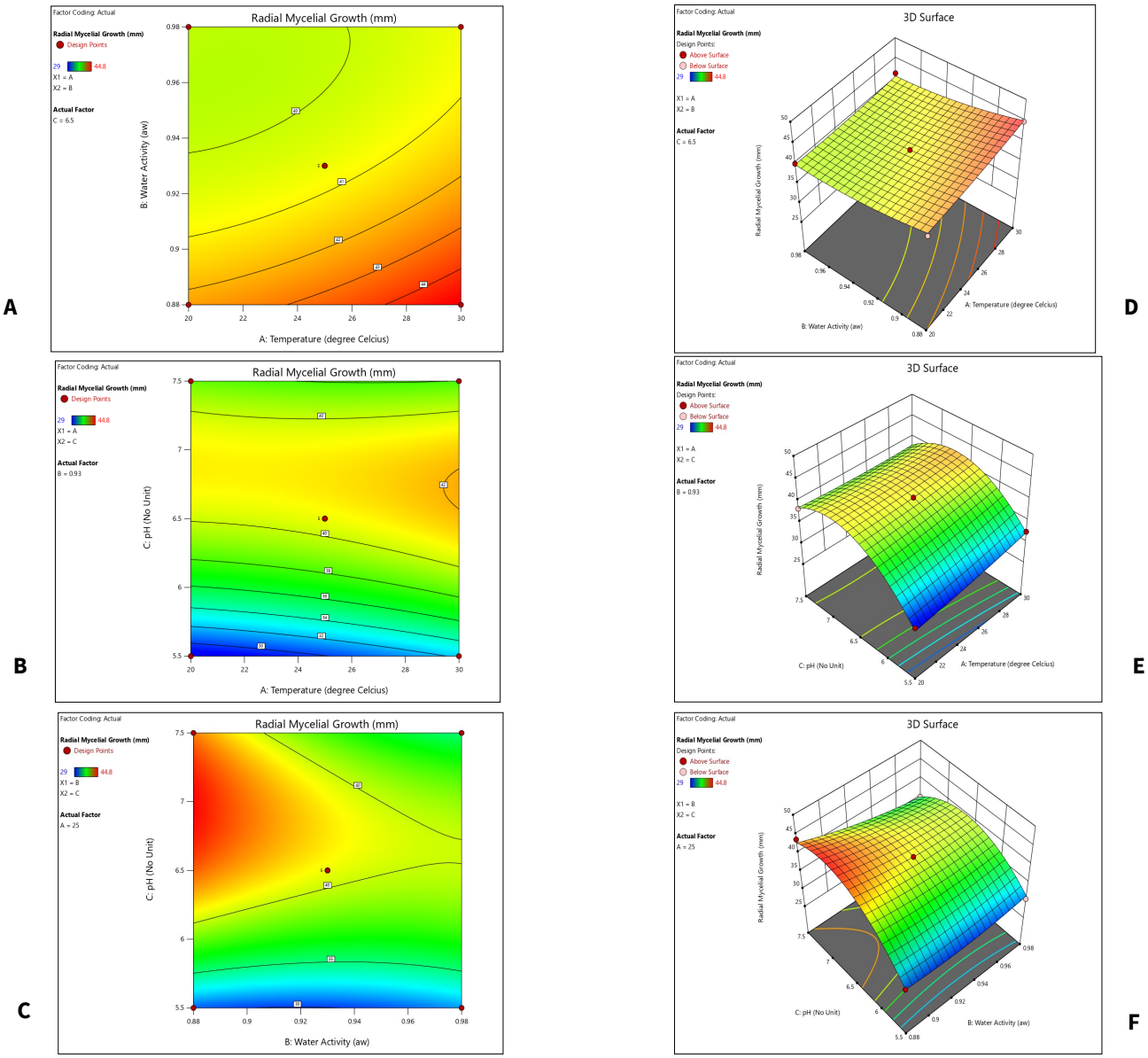

Fig. 2. Influence of temperature, water activity and pH on radial mycelial growth of *C. rosea* **A, D:** Contour and response surface 3D plot of temperature and water activity interaction **B, E:** Contour and response surface 3D plot of temperature and pH interaction **C, F:** Contour and response surface 3D plot of water activity and pH interaction. Other variables were held constant at zero in coded units. Values 40-44 indicate radial mycelial growth (mm) of *C. rosea.*

Fig. 3. Mycelial growth of *Clonostachys rosea* in optimized condition

Conclusion

This research represents an important advancement in understanding the growth dynamics of *Clonostachys rosea* TNAU CR04, using response surface methodology (RSM) to optimize temperature, pH and water activity. The application of a Box-Behnken design facilitated a comprehensive understanding of the interactive effects of these variables on mycelial development. By advancing our understanding of its growth dynamics and optimizing its application, *C. rosea* can contribute significantly in reducing the use of synthetic pesticides, enhancing crop health and promoting long-term sustainability in agriculture. However, the applicability of the model is limited to a controlled experimental context, making extrapolation to real-world conditions challenging due to variables such as ultraviolet radiation, nutrient dynamics and microbial interactions. Future research should focus on validating the model under field conditions and investigating the impacts of environmental factors on other *C. rosea* strains *in vivo*, ultimately facilitating the development of effective bio fungicide formulations and enhancing sustainable agricultural practices.

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Authors' Contributions

AA carried out investigation, experimentation and analysis. KR carried out project administration, supervision and methodology. SV and HS carried out conceptualization, validation, reviewing and editing. SU and SN carried out resources, data interpretation and analysis. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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