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On the virulence of the entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* (Ascomycota: Hypocreales), against the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae)

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Abstract

Background: The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is a noxious pest of cruciferous crops all over the world causing serious economic damage. Management of insect pest generally depends on chemical control; however, due to development of resistance against all types of insecticides, alternative approaches especially utilization of a microbial agent is inevitable.

Results: Potential of 2 entomopathogenic fungi (EPF), viz., *Beauveria bassiana* and *Metarhizium anisopliae*, was evaluated against 2nd and 3rd larval instars of *P. xylostella* by adopting leaf dip and direct spraying methods under laboratory conditions. Significant mortality rate was achieved by each fungus under adopted methodologies. However, *B. bassiana* was found to be more effective in both conditions than *M. anisopliae*. Highest mean corrected mortality (77.80%) was recorded, when spores of *B. bassiana* were sprayed on the 2nd instar larvae (LC₅₀= 1.78×10⁴/ml) after the 6th day of treatment. Similarly, in case of *M. anisopliae* LC₅₀ for the 2nd instar at the same methodology was 2.78×10⁴/ml with a mortality percentage of 70.0%. Offspring sex ratio was non-significantly related to treatment concentration and methodology, except for the control.

Conclusion: *Beauveria bassiana* and *M. anisopliae* had potential to suppress *P. xylostella* infestations when applied appropriately. Present findings suggested that *B. bassiana* and *M. anisopliae* when sprayed on immatures of host insect had more effect as compared to leaf dip procedure. Furthermore, no significant effect of concentrations was observed on sex ratio.

Keywords: *Beauveria bassiana*, *Metarhizium anisopliae*, *Plutella xylostella*, Foliar application, Potential

Background

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is a widely distributed lepidopteran insect pest of many crops (Li et al. 2016). This pest preferably feeds on cruciferous crops causing up to 80% crop losses globally (Javed and Mukhtar 2017).

Application of highly toxic, broad-spectrum synthetic insecticides is the primary management strategy against insect pests; however, *P. xylostella* is on the frontline in developing resistance to every class of insecticides (Ridland and Endersby 2011). Another drawback of overuse of insecticides is the environmental hazards and adverse effects on natural enemies (Peng et al. 2010). Therefore, exploration of eco-friendly management strategies to cope with resistant DBM population is inevitable. Alternatively, entomopathogenic fungi (EPF), like *Beauveria*

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bassiana (Balsamo-Crivelli) Vuillemin (Ascomycota: Hypocreales), *Metarhizium anisopliae* (Metschn.) Sorokin (Hypocreales: Clavicipitaceae), *Isaria fumosorosea* Wize (Hypocreales: Cordyciptaceae), *Verticillium lecanii* (Zimm.) (Deuteromycotina: Hyphomycetes), and *Nomuraea rileyi* (Farl.), have been found to be promising tools for controlling several agricultural insect pests (Trdan et al. 2020). The most efficient and well-studied EPF are the soil-borne fungi mainly *B. bassiana* and *M. anisopliae* that have a wide range of insect hosts (Javed et al. 2019). These 2 fungi are compatible with each other and showed significant results against lepidopteran insect pests (El Husseini 2019). Several studies have been conducted to evaluate efficiency of EPF against DBM immatures either alone or in combination with other EPF (Correa-Cuadros et al. 2016). However, to obtain sufficient results in insect pest management by EPF, proper methods of application are mandatory. Therefore, this study was conducted to evaluate the bioefficacy of *B. bassiana* and *M. anisopliae* against *P. xylostella* under the best feasible method of their application.

Methods

Insect culture

Plutella xylostella culture was reared on natural diet (cabbage leaves) in cages (60×60×60 cm) at 25°C, 65±5% RH, and 16L:8D photoperiod. Emerging adults were fed on 20% honey solution soaked in cotton balls. To obtain homogenous population, moths were collected and placed in separate cages for mating and oviposition on cabbage plants. After 6 h, plants were removed and shifted to new cages for egg hatching. Neonate 2nd and 3rd larval instars were collected for pathogenicity trials.

Fungi culture

Already established cultures of *B. bassiana* and *M. anisopliae* were used in the bioassays. To check the hyphal viability, the obtained cultures were grown on potato dextrose agar (PDA) media in Petri plates (9-cm diameter) and incubated at a temperature of 28°C, 65±5% relative humidity (RH), and a photoperiod of 12:12 (L:D) h for 15 days. Conidia were extracted from each culture plate by adding 20 ml sterile distilled water with 0.05% Tween-80 suspension. Conidia in each Petri dish were harvested from the suspension with the help of 90-µm mesh sieve, the number of conidia/ml were determined, using Neubauer chamber, and concentrations of 10⁸, 10⁷, 10⁶, 10⁵, 10⁴ conidia/ml were prepared.

Pathogenicity of entomopathogenic fungal isolates

The virulence of EPF against 2nd and 3rd larval instars of *P. xylostella* was determined using direct spraying of fungal inoculum (methodology I) and leaf immersion method (methodology II) on host insect

immatures. Each applied treatment was replicated 3 times. Thirty larvae of *P. xylostella* were used for each experimental unit. In the 1st methodology, 30 individuals of 2nd or 3rd larval instars were placed in Petri plates, and 2 ml of fungal suspension from each concentration was sprayed directly onto the larvae, using a sprayer with droplet size about 200–300 µm. After that, larvae were air dried, shifted into the Petri plates having moistened filter paper provided with cabbage leaf disc. Afterwards, treated larvae were placed in growth chamber under the abovementioned conditions.

In leaf immersion method, leaf discs of cabbage (*Brassica oleracea* L.) cv. Asha (approx. 8-cm diameter) were prepared. Leaf discs were dipped in 2-ml fungal suspension from each concentration (10⁸, 10⁷, 10⁶, 10⁵, 10⁴ spores/ml of water) for 1 min. After air drying, individual leaves were placed in Petri dishes having moistened filter papers at their bottoms (8-cm diameter). In control treatment, leaves were dipped in distilled water only. A soft brush was used to transfer 30 individuals of 2nd or 3rd larval instars of *P. xylostella* to each leaf disc, and the dishes were placed in a growth chamber at 25°C, 65±5% RH, and a photoperiod of 16L: 8D h. Mortality rates were recorded after every 24 h, starting 48 h of post-application up to one week. As significant mortality occurred after 120 h and 144 h of post treatment application, therefore data was analyzed according to these time intervals. The larvae, unable to move on poking, were considered dead and were placed in a humid chamber to confirm whether the larvae were killed by the entomopathogen or not. The humid chamber consisted of sterilized and moistened filter paper disc (8-cm diameter) and foam, placed on Petri dishes (9-cm diameter). Confirmation of mortality was performed after 10 days of incubation in the humid chamber by observing the conidial structures of the fungus with the help of a microscope.

Sex ratio of emerged adults

The larvae that remained viable and developed to pupal stage were recorded, and afterward number of emerged moths was counted. On the basis of emerged adults, sex ratio of the progeny was assessed as percentage of females as described by Maia & Alfredo (2000).

Statistical analysis

Total mortality rates were corrected, using Abbot's formula (Abbott 1925). The experiment was conducted in a completely randomized design, and the data were subjected to analysis of variance. Means were compared by Tukey's test ($P < 0.05$; PROC GLM, SAS Institute 2002). The lethal concentrations (LC₅₀) were determined using Probit analysis ($P > 0.05$) by the R statistic software.

Results

Pathogenicity of *B. bassiana* on the 2nd and 3rd instars of *P. xylostella*

A direct relationship was found between concentration and mortality in case of both methods adopted ($P \leq 0.05$) (Table 1). Mortality induced showed positive relationship to time after treatment application. In the case of the 2nd instar larvae, significant pathogenicity (63.3%) was recorded, when *B. bassiana* spores 10^8 were sprayed directly on larvae (methodology 1) after 120 h (5 days). However, the mortality rate reached the maximum value of 77.78% after the 6th day at the highest concentration (10^8). In case of low concentration, 10^7 spores /ml, fungal-induced pathogenicity was corresponded to the mortality rate of 61.32% after 144 h. Results revealed that in case of low spore concentrations, i.e., 10^6 , 10^5 , and 10^4 spores/ml, the lethal rates decrease to 52.3%, 42.3%, and 32.3%, respectively (Table 1). However, in case of the leaf dip method (methodology 2), virulence induced in the 2nd instar larvae were low compared to spraying method. Results indicated that a significant difference in mean-corrected mortality exists between the methodologies corresponding to time interval and concentration. Percentage mortality (51.3%) was observed at high concentration after the 5th time interval, and 70% was the maximum lethal effect induced after the 6th day

at the highest concentration of 10^8 . For other concentrations applied of 10^7 , 10^6 , 10^5 , and 10^4 spores/ml, insect pathogenicity ranged between 53.3, 44.4, 37.7, and 28.9%, respectively (Table 1). In case of the 3rd instar larvae, results proclaimed that conidial pathogenicity is relatively lower as compared to earlier instar. Maximum mortality rate (71.3%) corresponded to the highest concentration after the 6th day of treatment application under method 1. However, at the 2nd method, the peak mortality rate (60%) has been recorded after same time interval. Results elaborated significant differences among the lethal rates induced by the 2 different methods. Direct spraying yielded good results than the leaf-dip bioassay.

Pathogenicity of *M. anisopliae* on the 2nd and 3rd instars of *P. xylostella*

In case of *M. anisopliae*, the highest concentration yielded a maximum lethal effect for both larval instars. Results of the first method were higher than the second on both exposed larval instars. Significant mortality rates were induced at the 1st method in the 2nd and 3rd larval instars (70% and 64.7%), respectively. However, for other concentrations, the corrected mortality rates were low and varied from 58.9 to 31.1% at the 2nd and 3rd instars, respectively (Table 2). However, in case of the leaf dip

Table 1 Mortality rates of *Plutella xylostella* larval instars (2nd and 3rd) at different concentrations of *Beauveria bassiana* at various time intervals by direct spraying and leaf immersion methods

	Time intervals	Concentrations	Larval instar-II	Larval instar-III
Methodology I	120h	1×10^8	63.3bc	57.8bc
		1×10^7	50def	45.3def
		1×10^6	36.7ghi	35.7fghi
		1×10^5	22.2jk	23.3jkl
		1×10^4	20jk	21.1kl
	144h	1×10^8	77.8a	71.3a
		1×10^7	61.3bcd	56.7bcd
		1×10^6	52.3cde	47.7cde
		1×10^5	42.3efgh	41.3efgh
		1×10^4	32.3hij	31.1hijk
Methodology II	120h	1×10^8	51.3cde	44.7efg
		1×10^7	45.5efg	39.7efgh
		1×10^6	32.3hij	31.3hijk
		1×10^5	20jk	17.8l
		1×10^4	18.9k	17.8l
	144h	1×10^8	70ab	60b
		1×10^7	53.3cde	47.7cde
		1×10^6	44.4efgh	41.3efgh
		1×10^5	37.7fghi	33.3ghij
		1×10^4	28.9ijk	27.8ijk

Mortality (\pm S.E) assigned with similar letters within each column is statistically similar ($\alpha=0.5$)

Table 2 Percentage mortality of *Plutella xylostella* larval instars (2nd and 3rd) at different concentrations of *Metarhizium anisopliae* at various time intervals by direct spraying and leaf immersion methods

	Time intervals	Concentrations	L2 larval instar	L3 Larval instar
Methodology I	120 h	1×10 ⁸	56.7bc	45.5bcd
		1×10 ⁷	45.7def	42.3cde
		1×10 ⁶	35.7fgh	34.3efg
		1×10 ⁵	21.1ij	22.2hi
		1×10 ⁴	20j	18.9i
	144 h	1×10 ⁸	70a	64.7a
		1×10 ⁷	58.9bc	51.2bc
		1×10 ⁶	50cde	46.7bcd
		1×10 ⁵	41.1efg	38.9def
		1×10 ⁴	33.1ghi	31.1fgh
Methodology II	120 h	1×10 ⁸	50cde	42.3cde
		1×10 ⁷	45.3def	37.7defg
		1×10 ⁶	32.3gh	31.3fgh
		1×10 ⁵	20j	17.8i
		1×10 ⁴	18.9j	16.8i
	144 h	1×10 ⁸	62.3ab	55.7ab
		1×10 ⁷	53.3bcd	47.7bcd
		1×10 ⁶	44.3def	41.3cdef
		1×10 ⁵	36.7fgh	33.3efg
		1×10 ⁴	28.9hij	27.8ghi

Mortality (±S.E) assigned with similar letters within each column is statistically similar ($\alpha=0.5$)

bioassay 62.3% was the maximum mortality that was occurred in 2nd instar larvae, 55.7% was the peak mortality corresponding to later instar (Table 2).

In the case of the 2nd instar larvae, *M. anisopliae* spores were sprayed directly on the larvae. The maximum mortality rate of the 3rd instar larvae at same method was 55.7%, which was lower than at the 2nd instar. Overall results have showed that *B. bassiana* performs better in both the methodologies adopted against both larval instars as compared to *M. anisopliae*.

Sex ratio of emerged adults

Sex ratio, in term of females, was estimated from the emerged adults. Results revealed that progeny sex ratio is independent of treatment application even at high concentration. Therefore, it can be inferred from results that there exists a non-significant relationship among all the treatments including control in terms of sex ratio. Survived adults under control treatment relatively showed higher percentage of females as compared to other treatments in both the methodologies and under both fungal applications. In the case of the 1st method, a significant low percentage was recorded in adults that emerged from 10⁴ spores/ml dilution, followed by the 10⁸ spores/ml concentration. However, at other treatments, a relatively less number of females were observed.

At the 2nd method, non-significant difference in percent female ratio of emerged adults in both the larval instars has been observed ($\alpha=0.05$) and was found (Fig. 1).

In case of *M. anisopliae*, the percent of sex ratio of emerged adults showed that a significantly high number of females emerged from the control treatment of both methods and on both larval instars. Results of the 1st method, however, depicted that relatively less percent of females was recorded from 10⁸ spores/ml concentration, survived adults of 2nd as well as 3rd instars. At In the 2nd method, female percentage was significantly low in the adults that emerged from treated 3rd instar larvae at high concentration. However, survived females from other treatments were non-significantly different. Generally, less female number was observed at the highest concentration (Fig. 2).

Median lethal concentrations LC₅₀ of the two EPF showed that *B. bassiana* caused higher pathogenicity comparative to *M. anisopliae* as its estimated value of LC₅₀ is lower while *M. Anisopliae* had more LC₅₀ value for both instars (Tables 3 and 4).

Discussion

The present study highlighted the virulence of *B. bassiana* and *M. anisopliae* against 2nd and 3rd larval instars of *P. xylostella* under 2 different application methods. In

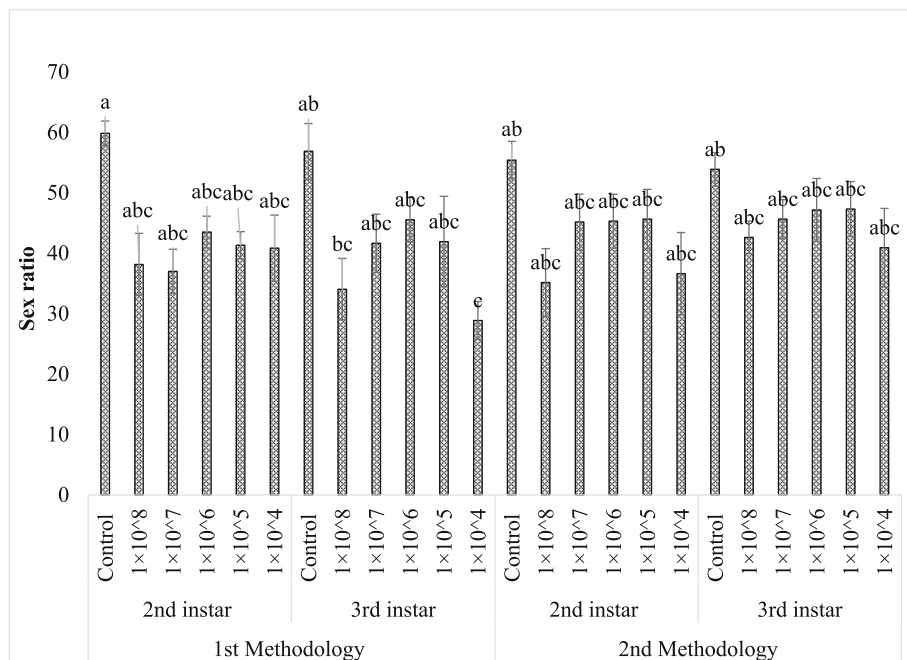


Fig. 1 Mean sex ratio of *Plutella xylostella* after application of *Beauveria bassiana* at both methods tested

the present study, both EPF had lethal effects against *P. xylostella* as it was reported in the previous studies conducted by Loc and Chi (2007) and Zafar et al. (2020). Results had depicted that in addition to method of treatment application, the virulence was strongly related to concentration exposed to immatures, post-treatment time, and fungal species. In the current study, *B. bassiana* had a more pathogenic effect on *P. xylostella* larvae than *M. anisopliae* using both leaf dip method and foliar spray. This difference might be due to variant sporulation capacity of species as some fungal spores may penetrate more frequently than others (Mannino et al. 2019).

Furthermore, production of bioactive enzymes and other metabolites responsible for the death of the host is also a vital factor toward fungal pathogenicity (Shakeel et al. 2017). It can be inferred from current findings that method 1 showed higher toxic effect than the second one. This suggests that foliar application of spores can significantly suppress *P. xylostella*. Findings of Beris et al. (2013) and Gabarty et al. (2014) support our findings suggesting that different application methods pose different virulent effects in insect pests including DBM respectively. Another study conducted by Sáenz-Aponte et al. (2020) also testify our results that different

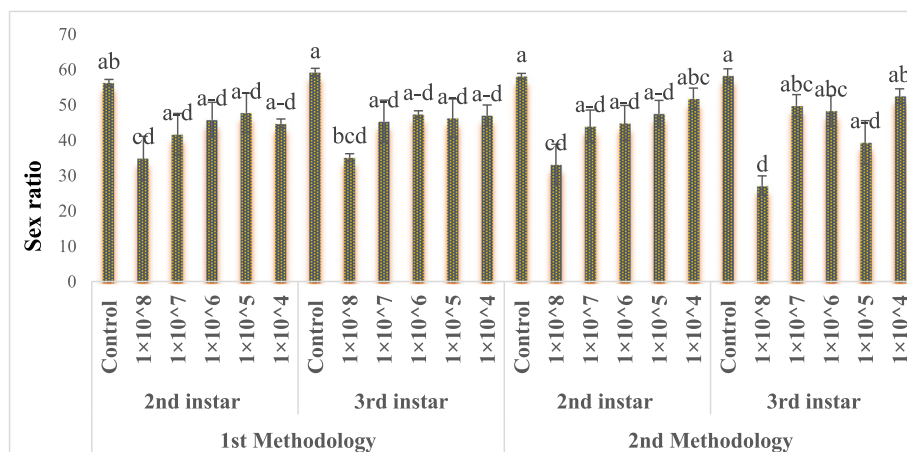


Fig. 2 Sex ratio of *Plutella xylostella* after application of *Metarhizium anisopliae* at both methods used ($\alpha=0.5$)

Table 3 Pathogenicity of *Beauveria bassiana* against larval instars of *Plutella xylostella*

Fungi	Method 1			Method 2		
	LC ₅₀ (95%)	Slope±S.E	χ ²	LC ₅₀ (95%)	Slope±S.E	χ ²
<i>B. bassiana</i>						
2 nd instar	1.782 × 10 ⁴ (4.864 × 10 ³ –3.984 × 10 ⁴)	0.26 ± 0.034	0.809	1.14 × 10 ⁵ (3.754 × 10 ⁴ –6.584 × 10 ⁵)	0.30 ± 0.044	0.609
3 rd instar	2.56 × 10 ⁵ (5.864 × 10 ⁴ –2.584 × 10 ⁵)	0.31 ± 0.028	0.710	3.08 × 10 ⁵ (5.45 × 10 ⁴ –5.684 × 10 ⁵)	0.33 ± 0.038	0.642

methods of fungal spore's application had different virulent activities against host insect. This might be due to the fact that successful sporulation of fungi on *P. xylostella* larvae depended on spores' adhesion and penetration to the host cuticle, therefore spraying fungal suspension on larvae confirmed a maximum probability that more number of conidia had adhered to insect surface (Qayyum et al. 2019). Overall, an increase in concentrations was found to be more potent in case of both EPF used against *P. xylostella*. Results obtained by Mehinto et al. (2014) are in compliance with our findings, claimed that 1 × 10⁸ spores/ml showed peak mortality against *Maruca vitrata* (Lepidoptera: Crambidae) insect pest. Furthermore, Freed et al. (2012) also reported that high EPF concentration against DBM had prompted a significantly higher mortality as compared to low ones. Xia et al. (2013) found the same virulence pattern in *P. xylostella*. However, non-significant mortality had been observed in case of low concentrations. Actually, the virulence of fungal strain depends on number and growth of spores within the host body (Ali et al. 2010). The sporulation had direct relation to host mortality as suggested by Pauli et al. (2018).

Current findings also suggest that after 144 h of post-treatment application, both EPF had instigate the best results oblivious from method of application. Therefore, it could be inferred that fungal spores take time to invade insect cuticle as described by earlier studies of Kim et al. (2002) and Mehinto et al. (2014).

In case of each fungus evaluated for virulence, the mortality prompted in DBM was significantly high against 2nd instar larvae under both the methods of application. This could be because earlier instars are more susceptible to biocontrol tactics. The study conducted by Hafez et al. (1997) is pursuant to our results who

reported that *B. bassiana* was more pathogenic to earlier instar larvae of potato tuber moth as compared to ensuing instars. Another study conducted by Qayyum et al. (2015) reported that the 2nd instar larvae were more vulnerable than the later instars. This might be due to hardening of cuticle in later instars as reported by Wraight et al. (2010). Another aspect covered in current study was the sex ratio of survived adults, and results had showed that there was a non-significant difference between both methods. Overall results regarding sex ratio describe that high concentration applied produces relatively low percentage of females. Results of De Souza et al. (2020) are also in line with our current findings predicting that sex ratio of *Helicoverpa armigera* (Lepidoptera: Noctuidae) is independent to concentration applied. The results of Batcho et al. (2018) also support our obtained results. This fact might be because of that multiple factors in addition to fungal spores plays role in insect sex determination which would be explored after genetic studies and yet to be explored.

Conclusion

Direct spray of the EPF, *B. bassiana* and/or *M. anisopliae*, provided higher pathogenicity to the larvae of *P. xylostella* than the leaf dip method. This method can be recommended to be used under field as well as laboratory conditions for conducting bioassays and successful management of the pest.

Abbreviations

DBM: Diamondback moth; IPM: Integrated pest management; EPF: Entomopathogenic fungi; PDA: Potato dextrose agar

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Authors' contributions

MS and MT planned and designed the research experiments. MS performed the experiments and wrote the research article. TM and AS performed data interpretation and statistical analysis. The authors have read and approved the manuscript.

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Availability of data and materials

The data used and analyzed during this project are available from the corresponding author on reasonable request.

Table 4 Pathogenicity of *Metarhizium anisopliae* against different instars of *Plutella xylostella*

Fungi	Method 1			Method 2		
	LC ₅₀ (95%)	Slope± S.E	χ ²	LC ₅₀ (95%)	Slope± S.E	χ ²
<i>M. anisopliae</i>						
2 nd instar	2.782 × 10 ⁴ (5.334 × 10 ³ –3.201 × 10 ⁴)	0.31 ± 0.028	0.601	1.14 × 10 ⁶ (3.754 × 10 ⁵ –6.584 × 10 ⁶)	0.44 ± 0.029	0.886
3 rd instar	2.56 × 10 ⁵ (3.743 × 10 ⁴ –2.884 × 10 ⁵)	0.41 ± 0.036	0.815	2.15 × 10 ⁶ (3.45 × 10 ⁵ –4.684 × 10 ⁶)	0.33 ± 0.036	0.967

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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