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Endophytic establishment of the fungal entomopathogen, *Beauveria bassiana* (Bals.) Vuil., in cucumber plants

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Abstract

Background: The fungus, *Beauveria bassiana* (Bals.) Vuil., is one of the most important entomopathogenic fungi (EPFs). Recently, its new role was discovered in nature, to be an endophyte in plants. It has been reported as an endophytic fungus in many monocotyledonous and dicotyledonous plants.

Main body: The study was conducted to evaluate the ability of the fungus, *B. bassiana*, to colonize and persist in cucumber plants under laboratory conditions and to detect its systemic growth inside the plant tissues in addition to pathogenicity in the plant. The isolate, B195, of the fungus, *B. bassiana*, was used. Five different inoculation methods were followed: seed dusting, seed immersion, soil drench, seedling drench, and foliar spray. The fungus, *B. bassiana*, could persist inside different cucumber tissues up to 90 days from inoculation. Soil drench provided the highest recovery rates, while foliar spray gave the lowest rates. Colonization rates reached 94.44 and 73.68% for stem and 68.26 and 37.79% for root, 30 and 90 days post soil drench, respectively, while in foliar spray, it reached 33.51 and 16.45%, after 30 and 90 days post-treatment, for the stem and 9.45 and 0% for the root, respectively. No negative effects were observed in inoculated plants or on fungal pathogenicity.

Conclusion: Results showed for the first time the ability of the fungus, *B. bassiana*, isolate B195, to artificially colonize and survive in different parts of cucumber plants under laboratory conditions by different inoculation methods and to grow systemically in plant tissues. This study is considered a preliminary study to the utilization of the fungus, *B. bassiana*, as an endophyte in cucumber plants to reduce the density of insect pests.

Keywords: *Beauveria bassiana*, Endophyte, Entomopathogenic fungi, Cucumber plants, Colonization, Systemic growth

Background

Entomopathogenic fungi (EPFs) play an important role in the biological control of plant pests, and now many species are available as commercial products (Shahid et al. 2012). Recently, some of the EPFs have shown an ability to colonize tissues of a number of plants endophytically, which provide protection against various insect pests, and the term "endophytic entomopathogenic fungi" (EEPFs) was introduced (Ownley et al. 2010; Vidal and Jaber 2015).

The fungus, *Beauveria bassiana* (Ascomycota: Hypocreales), is one of the most important EEPFs. Although it has been described as an entomopathogen by Agostino Bassi since 1835, it has not been recognized as an endophytic fungus until 1991 by Bing and Lewis, who demonstrated its ability to colonize corn plants (*Zea mays* L.) endophytically (Bing and Lewis 1991, 1992; Wagner and Lewis 2000).

In recent few years, more interests about the role of the fungus, *B. bassiana*, as an endophyte in plants, have been shown. It has been reported as an endophytic fungus in many monocotyledonous and dicotyledonous plants. It can colonize different parts of the plant such as roots, stems, leaves, flowers, and seeds either as

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occurring naturally or as a result of inoculation (Vega et al. 2008; Ownley et al. 2010; Vidal and Jaber 2015; Card et al. 2016; Pus 2017).

B. bassiana has been recorded as an endophyte in several plant species such as corn (Wagner and Lewis 2000), cacao (Posada and Vega 2006), date palm (Gómez-Vidal et al. 2006), opium poppy (Quesada-Moraga et al. 2006), banana (Barta 2011), coffee (Posada et al. 2007), sorghum (Tefera and Vidal 2009), jute (Biswas et al. 2013), bean (Mutune et al. 2016), grapevine (Jaber 2015), radiate pine (Brownbridge et al. 2012), cotton (Griffin 2007), wheat (Sánchez-Rodríguez et al. 2018), strawberry (Dara and Dara 2015), cabbage (Pus 2017), tomato (Omukoko and Turoop 2017), and potato (Alsaoud et al. 2017). It was also able to colonize some cucurbitaceous plants such as pumpkin (Gurulingappa et al. 2010), squash (Jaber and Salem 2014), and melon (Resquín-Romero et al. 2016). Recently, its ability to colonize cucumber plants has been shown, where Hassan et al. (2019) recorded a natural endophytic isolate of the fungus, *B. bassiana*, from cucumber leaves in Iraq. In addition, Shaalan and Ibrahim (2018) demonstrated an endophytic establishment of *B. bassiana* in cucumber plants, 10 days post seed immersion, and achieved about 50–60% recovery.

Cucumber plants are exposed to numerous insect pests that decrease productivity, and the fungus, *B. bassiana*, has been used for many years as a biological control agent by traditional methods to reduce insect damage, but in the direct use, its propagules are exposed to the harmful UV radiation, fluctuating humidity, and temperature, reducing its effectiveness (Kim et al. 2013; Omukoko and Turoop 2017). Therefore, endophytic establishment in the plant provides an acceptable approach for avoiding this problem in field application and opens up new horizons for biological pest control.

The aims of the present study were (1) to evaluate the ability of a local isolate of the fungus, *B. bassiana*, to colonize cucumber plants, using different inoculation methods under laboratory conditions; (2) to detect the persistence of the fungus inside the plant; and (3) to demonstrate the potential of its systemic growth from the point of inoculation to other plant parts. Five inoculation methods were used, and the colonization was evaluated every 30 days for 3 months.

Main text

Materials and methods

Fungal isolate

The isolated fungus (B195), *B. bassiana*, was used. It was isolated in 2018 from olive orchard soil at Al-Shabatliyah, Latakia, Syria (35°41'10.6"N 35°49'36.6"E), using the *Galleria* bait method, which was described by Zimmermann (1986) and Meyling (2007). The isolate was identified morphologically as *B. bassiana* based on each of Humber and Steinkraus (1998), Rehner et al. (2011), and Lacey (2012). It was cultured on potato dextrose agar (PDA; Titan Biotech LTD.) to which the antibiotic amoxicillin was added, and stored at 4 °C until use.

The spore suspension was prepared based on Lacey (2012) as the following: 14-day-old colonies grown on PDA were flooded by 10 ml sterile distilled water containing 0.05% Tween 80. The colonies' surface was scraped off by a sterile syringe to ensure maximum conidial harvesting, then filtered through sterile muslin to remove any mycelial fragments, and shaken on a mechanical shaker for 15 min. Suspension concentration was calculated using a Malassez counting chamber, then adjusted to 4.9×10^7 spores/ml.

Viability of the conidia was assessed by germination assessment. A 100 μl from the suspension was spread over the surface of a Petri plate containing PDA and incubated at 25 ± 2 °C for 24 h. After incubation, a drop of lacto-phenol cotton blue was added to each plate, overlaid with a coverslip, and examined under a microscope. The percentage of germination was determined by randomly counting at least 300 spores for each plate. Conidium was considered to be germinated when it had a germ tube at least as long as the smallest diameter of the conidia (Lacey 2012). Viability was up to 90% in all assessment times.

Cucumber plants

Raade F1 hybrid cucumber (Elite Plant-Breeding and Seeds Company, Russia) was used in this study. Prior to application, seeds were immersed in sodium hypochlorite (NaOCl) 2.5% for 3 min for surface disinfection, then in ethanol 70% for 1 min, and rinsed 3 times with sterilized distilled water. To ensure that the surface disinfection process worked, seeds selected randomly were put on PDA (9-cm Petri plate) at a rate of 5 seeds per plate. Three plates were prepared and incubated at 25 ± 2 °C in darkness for 2 weeks. The disinfection process was considered successful when no fungal growth was observed on the disinfected seeds.

Inoculation methods

Five inoculation methods: seed dusting (T1), seed immersion (T2), soil drench (T3), seedling drench (T4), and foliar spray (T5) were tested. For seed dusting treatment, cucumber seeds before planting were placed in contact with conidia of a full-grown colony of the fungus for 2 h in a Petri plate, then placed onto sterilized moist filter paper for 10 min. In seed immersion treatment, seeds were immersed before planting into the fungal conidial suspension (4.9×10^7 spores/ml) for 2 h with hand stirring every 30 min (Omukoko and Turoop 2017). For the soil drench, 5 ml of the spore suspension was applied to the surface of the soil in each pot immediately after

seed planting. Also, in the seedling drench, each seedling was watered by 5 ml of the spore suspension. This treatment was applied on 14-day seedlings according to Pus (2017). The last method, foliar spray, was performed according to Rondot and Reineke (2014), using 14-day cucumber seedlings with a hand sprayer and an average of 5 ml of the spore suspension per plant was applied. To avoid run-off of conidial spores to the soil, each pot was covered with aluminum foil.

For adequate seed attachment, 2% of carboxy methyl cellulose (CMC) was mixed with spores before seed treatment by dissolving it first into 35–40 °C pre-gelatinized water. After treatment, each pot was covered with a plastic bag for 24 h to maintain a high level of humidity. Thirty plants were cultivated for each treatment, 10 replicates for each sampling date. Control treatments received sterile Tween water which was applied in the same method as each treatment mentioned above. The soil used for planting was previously autoclaved at 121 °C for 20 min. All pots were placed on benches at room temperature. The experiment lasted 3 months, and the recovery of *B. bassiana* was evaluated every 30 days.

Endophytic colonization assessment

Endophytic establishment of *B. bassiana* in cucumber plants was evaluated under laboratory conditions according to Barta (2011) as follows: for each sampling date, 10 plants of each treatment were uprooted. Different parts of each plant (leaves, stems, petioles, and roots) were washed out with running tap water. These parts were surface disinfected in a 2.5% sodium hypochlorite for 3 min, 70% ethanol for 1 min, and rinsed 3 times in sterile distilled water, 2 min for each time, then dried on sterile filter paper. A 100 μl of the final rinsed water was incubated on PDA for 2 weeks to determine whether the surface disinfection process was successful. It was considered successful when no fungal growth was found.

After surface disinfection, sections of the leaves, stems, petioles, and roots were cut into similarly small pieces (about 4-mm pieces), using a sterile scalpel. Randomly, 6 pieces of each part for every plant were cultured on PDA plates (9 cm dia.) and incubated at 25 ± 2 °C in darkness for 21 days and examined regularly to observe fungal growth. When fungal growth was observed, it was removed to a new Petri plate containing PDA medium, incubated, and studied morphologically using a light microscope. The colonization of different parts by *B. bassiana* was calculated as the following: % colonization = [number of plant pieces showing fungal growth/the total number of plant pieces] × 100 (Petrini and Fisher 1986), and the ability of the studied fungus to grow systemically to non-inoculated parts of the plant was detected. All plants were monitored throughout the experimental period. Physiological damages or disease symptoms of inoculated plants relative to the control plants were recorded.

Endophytic effects on fungal pathogenicity

For this purpose, the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), larvae in the last instar were used. It was reared in the laboratory as described by Bhatnagar and Bareth (2004). Before endophytic establishment experiments, *G. mellonella* larvae were placed in contact with full-grown colonies of *B. bassiana* for a few minutes and then moved to sterile Petri plates (12 cm diameter) containing moist filter paper, kept in darkness at 25 ± 2 °C for a week. Mortality (%) was recorded daily. Five replicates and 5 larvae/replicate were prepared. The control treatment was carried out without the fungus. The same steps were repeated after endophytic establishment experiments, using fungal colonies that were isolated from plant tissues to compare the fungal pathogenicity before and after fungal establishment in the plant.

Statistical analysis

Data were statistically analyzed by CoStat program, using a completely randomized design. For each sampling date, the variation test one-way ANOVA was used, the means were compared, using the LSD test at $p \leq 0.05$. To determine the best method of inoculation, one-way randomized blocks with repeated measures were used.

Results and discussion

Presence and persistence of the endophyte in cucumber plant

All fungal inoculation methods resulted in an endophytic establishment of the fungus, *B. bassiana*, in cucumber plants under the laboratory conditions (Table 1), and the fungus survived inside the plant up to 90 days of the inoculation (Fig. 1), whereas no presence of the fungus was observed in the control plants, indicating the absence of natural endophytes and contamination. No fungal growth was recorded from the last rinsed water and in plates with disinfected seeds, which demonstrated the effectiveness of surface disinfection procedure and confirmed that the fungi growing from the plant pieces were endophytes.

Although all inoculation methods were effective in introducing the isolate B195, into the plant, they were at different levels of efficiency. Soil drench provided the highest colonization of various cucumber parts, followed by seed immersion and seed dusting, while foliar spray gave the lowest values. This may be attributed to the plant's growth stage when it was infected with the fungus. The maximum colonization was achieved, 30 days after treatment, then it decreased gradually ($F = 37.43$, $p < 0.0001$ for inoculation methods; $F = 147.21$, $p < 0.0001$ for days; and $F = 3.49$, $p = 0.0007$ for days × methods). Table 1 also shows the

Table 1 Colonization of the fungus, *Beauveria bassiana*, in cucumber plant under laboratory conditions, 30 days post different inoculation methods

Treatment	Mean of colonization % ± SD			
	Stem	Leaf	Petiole	Root
Seed dusting	73.61c* ± 5.69	77.5a ± 10.16	73.5a ± 9.7	31.57c ± 10.53
Seed immersion	83.33b ± 10.08	81.97a ± 9.53	57.8b ± 13.3	56.54ab ± 22.16
Soil drench	94.44 ± 7.32	80.25a ± 6.64	61.3b ± 13.15	68.26a ± 28.19
Seedling drench	29.83d ± 16.94	37.98c ± 23.02	37.95c ± 11.94	41.66bc ± 13.34
Foliar spray	33.51d ± 9.83	63.58b ± 14.2	55.3b ± 11.18	9.45d ± 19.96

*Different letters in the same column refer to significant differences (one-way completely randomized ANOVA), LSD test at $p \leq 0.05$

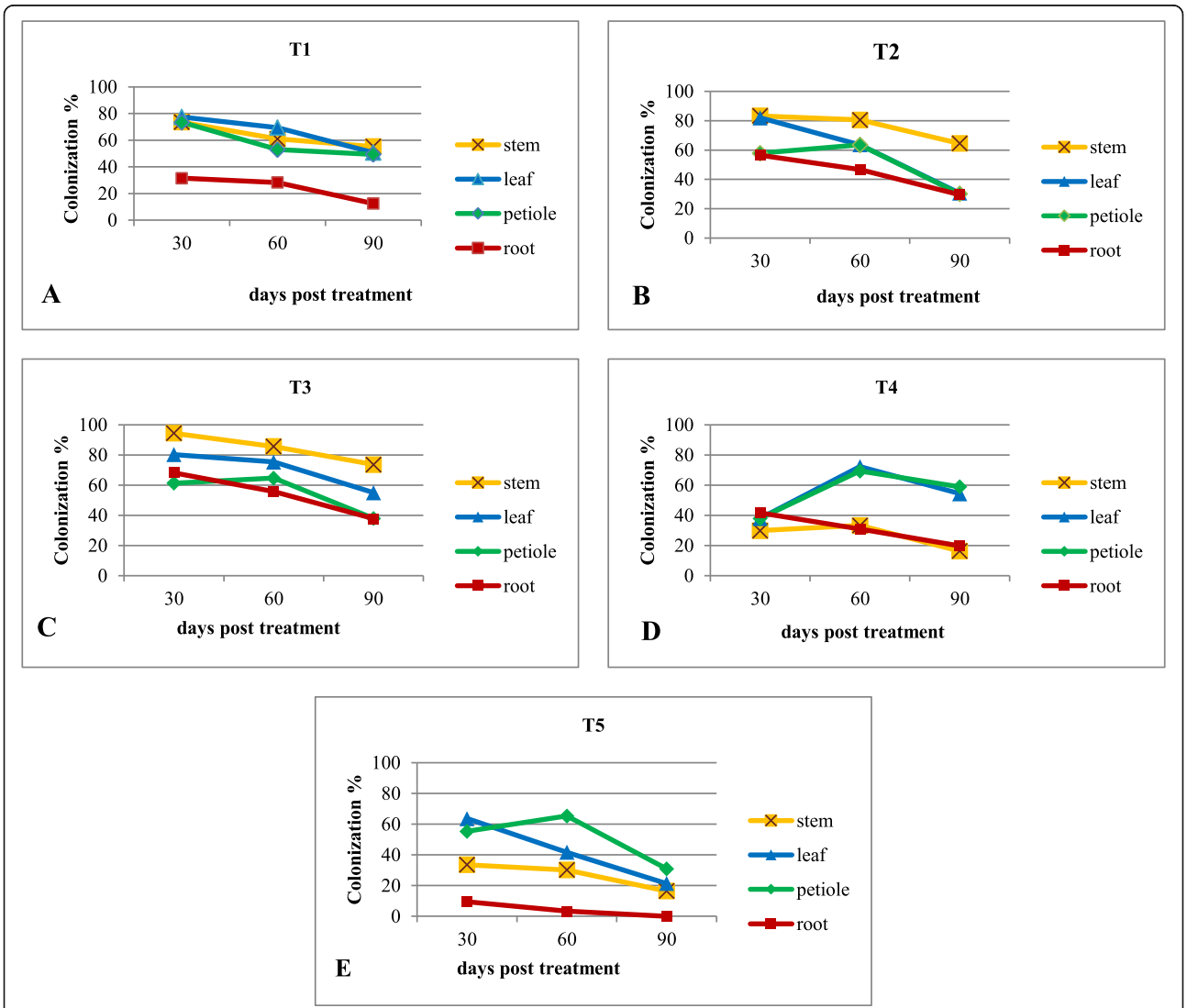

Fig. 1 Re-isolating of the fungus, *Beauveria bassiana*, from cucumber tissues under the laboratory conditions after different periods of treatment (**a** seed dusting, **b** seed immersion, **c** soil drench, **d** seedling drench, **e** foliar spray)

differences in colonization rates among the methods in the first sampling date of the experiment. The fungal colonization in the upper parts of cucumber plants (leaves, stems, and petioles) was higher than that in the roots (F = 36.540416, p < 0.0001 for different parts; F = 88.88, p < 0.0001 for days; and F = 2.608, p = 0.0172 for days × parts).

Stem colonization reached 94.44 ± 7.32, 85.67 ± 9.07, and 73.68 ± 14.39% (± SD), after 30, 60, and 90 days post soil drench, respectively, while it reached 16.45 ± 12.3 and 16.31 ± 2.87% (± SD) in foliar spray and seedling drench, respectively, after 90 days of treatment (Fig. 1).

Colonization of leaves reached 77.5 ± 10.16, 81.97 ± 9.54, 80.25 ± 6.65, 37.98 ± 32.02, and 63.58 ± 14.21% (± SD), respectively, for each of T1, T2, T3, T4, and T5, after 30 days of inoculation (F = 17.46, p < 0.0001, LSD 5% = 12.549). However, it decreased to 50.75 ± 25.7, 30.63 ± 9.05, 54.99 ± 19.64, 54.36 ± 26.63, and 21.36 ± 13.17% (± SD), respectively, at the end of the experiment (F = 5.92, p = 0.0006, LSD 5% = 18.0688) (Fig. 1).

Also, root colonization reached its maximum value after 30 days of inoculation, and it was 68.26 ± 28.19 and 56.54 ± 22.16% (± SD), for soil drench and seed immersion, respectively (F = 13.10, p < 0.0001, LSD 5% = 17.895), while it was in the lowest level at 90 days of treatment (F = 9.89, p < 0.0001, LSD 5% = 13.304), and in foliar spray in general, whereas it was 9.45 ± 19.96, 3.33 ± 10.53, and 0% (± SD) in this method, after 30, 60, and 90 days of treatment, respectively. No physical symptoms of damage were observed in colonized plants.

Endophytic effects on fungal pathogenicity

Results showed no negative effects of the establishment of the fungus, *B. bassiana*, in cucumber plants under laboratory conditions on its pathogenicity. On the 4th day of the experiment, mortality reached 100% against the last instar of *G. mellonella* larvae before and after introducing the fungus to the plant, while it was 0% in the control. As a result of this study, the presence of the fungus, *B. bassiana*, inside cucumber plants was confirmed by re-isolating it from stems, leaves, petioles, and roots under laboratory conditions, and the persistence of the fungus in the inner tissues was proved by its presence in all sampling dates.

Comparing the different inoculation methods showed that when the fungus was introduced in the first stage of plant growth, it was able to colonize the tissues more efficiently than when it was applied in the seedling stage. This may be helpful for the control of pests that attack cucumber plants early in the season, especially whiteflies. The absence of any negative effects on the fungal pathogenicity after introducing the fungus in the plant encourages us to study its role as an endophyte against insect pests.

Despite the reduction in colonization rates with time, *B. bassiana* remained present until the last sampling date, either at high rates in soil drench and seed treatment or at low rates in seedling drench and foliar spray. The fungal persistence in plant tissues is considered an important indicator that is taken into consideration in pest control for season-long suppression. Rondot and Reineke (2014) revealed the presence of *B. bassiana* in grapevine plants up to 28 days after inoculation, while Akello et al. (2007) confirmed that it could be re-isolated from banana up to 120 days after inoculation. In addition, Posada et al. (2007) isolated *B. bassiana* from coffee tissues, 120 days post-inoculation by low rates. Brownbridge et al. (2012) re-isolated *B. bassiana* from pine seedlings, 120 days post root dip and seed coating treatments, and after 270 days from treatments, but that was from only one of 30 seedlings. Such studies help us decide whether we need to introduce the fungus more than once.

Colonization rates of the fungus, *B. bassiana*, resulted in this study were higher than those obtained by Shaalan and Ibrahim (2018) in cucumber plants, where recovery of the fungus, *B. bassiana*, reached 58.3% for each of the stems and leaves, and 50% for the roots, when it was applied as seed immersion.

On the other hand, the present study demonstrated that the isolate B195, *B. bassiana*, could grow systemically from the point of inoculation to other parts of the cucumber plant, considering that after the seeds were treated with the fungus, it was isolated from leaves, stems, roots, and petioles. In addition, when foliar spray was applied, the fungus was isolated from the roots. In this regard, Card et al. (2016) explained the growth and development of endophytic microorganisms in numerous plant hosts as either localized or systemic and noted that many species of endophytes were only located within the point of inoculation, whereas other studies have demonstrated the systemic growth of *B. bassiana* in cabbage plants (Pus 2017), wheat (Jaber 2018), and corn (Kuzhuppillymyal et al. 2020). Although Yan et al. (2015) suggested that systemic colonization is not necessary for pest control by endophytes due to the secondary metabolites produced by the fungus that enable the antagonism mode (Begum and Tamilselvi 2016), others thought that the systemic growth allows the fungus to use more than one mode of action (Ownley et al. 2010; Vidal and Jaber 2015). The present study indicated the ability of *B. bassiana* to colonize the upper parts of cucumber plants and that encourages studying its possible effects against foliar pests.

Conclusion

The isolate B195, of the fungus, *B. bassiana*, successfully colonized different parts of cucumber plants under

laboratory conditions. Colonization (%) varied significantly according to the inoculation method used, whereas soil drench and seed immersion gave the highest recovery of the fungus. Results showed that the fungus could grow systemically inside different plant parts and could persist in the cucumber plants for 90 days. No negative effects were observed on the plants or the pathogenicity of the fungus. This study is considered a preliminary research to the utilization of *B. bassiana*, as an endophyte in cucumber plants to reduce the density of insect pest populations.

Abbreviations

EPFs: Entomopathogenic fungi; EEPFs: Endophytic entomopathogenic fungi; PDA: Potato dextrose agar; T1: Seed dusting; T2: Seed immersion; T3: Soil drench; T4: Seedling drench; T5: Foliar spray; CMC: Carboxy methyl cellulose; LSD: Least significant difference; ANOVA: Analysis of variance; SD: Standard deviation

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Authors' contributions

LR conceived the work, designed and performed the experiments, analyzed the data, wrote the paper. MA was a major contributor in writing the manuscript, reviewing & editing the paper. IG reviewed and edited the paper. All author(s) read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in the text.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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