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Morphological characterization of *Verticillium dahliae* isolated from olive trees in District Mardan

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Abstract

Verticillium dahliae is a soil-borne fungus that causes Verticillium wilt, a destructive disease of Olive trees worldwide. The common symptoms of Verticillium wilt in Olive trees include wilting, leaf rolling, chlorosis, defoliation, and dead brown leaves on sunny days. During the year 2020-21, one field of Olive Arched (17-acre area) was investigated for the incidence of Verticillium wilt located in Mardan District of Khyber Pakhtunkhwa. Root, stem and leaves, samples were collected from suspected plants, and cultured on potato dextrose agar (PDA) media for the isolation of *Verticillium dahliae*. Spores of the isolated fungal colonies were counted using a hemocytometer. The diameter of colonies was measured on PDA and complete media (CM) using a ruler with intervals of three days. *Verticillium dahliae* was isolated only from the olive tree roots, while no isolation of *Verticillium dahliae* was observed in stem and leaves samples. The average colony diameter after seven days on PDA and CM media was 4.6 cm and 2.4 cm respectively. Spores count for the pure colony was 3.44 x 10^7/ml conidia. It was concluded that the wilting observed in Olive trees was due to *Verticillium dahliae* in district Mardan.

Introduction

Verticillium wilt is caused by a soil-borne plant pathogen called *Verticillium dahliae* [1]. Verticillium is a tiny genus of ascomycete fungus presently with just ten species [2]. Verticillium species have hyaline mycelium that is simple or branching, septate, and multinucleate. Conidia are ovoid to elongate and formed on long phialides that are arranged in a whorl or spiral way around the conidiophores. Conidiophores also branch in whorls at different levels. The name of the genus Verticillium comes from the so-called "verticillate" arrangement of the conidiophore branches and phialides [1]. Six plant pathogenic Verticillium species have been identified following a revision of the original genus.

Verticillium wilt of the olive [3], caused by *Verticillium dahliae*, is currently the most devastating olive disease in Spain and, likely, throughout the Mediterranean Basin [3]. Irrigation is blamed for promoting wilt in intensive crops [4] and a large olive harvest [5]. Semicontinuous watering increases the incidence and severity of wilt disease in herbaceous hosts [6]. Wilting, leaf rolling, chlorosis, defoliation, and dead brown leaves that stay connected to the branches are all symptoms [7].

Verticillium dahliae causes severe economic losses to the agriculture sector worldwide. The Nature of the disease and characteristics of this tree crop has complicated the assessment of the actual economic losses. Currently, Verticillium wilt of olive is considered one of the major limiting factors for olive oil production. Losses from Verticillium wilt include high rates of tree mortality and reductions in fruit yield, especially in highly susceptible cultivars [8]. Besides these economic losses, it is recently demonstrated that there is also a negative effect on the commercial value of olive oil because of the poor organoleptic properties from fruits of *Verticillium dahliae* infected trees [9]. Infection of plants leads to a reduction in the synthesis of the main phenolic compounds responsible for the taste sensory characteristics of virgin olive oil. In Israel, yield reductions in irrigated “Picual” olives were estimated to be 75% and 89% at 3 and 5 years after planting, respectively [10]. Thanassoulopoulos et al. (1979) [11] reported 2–3% incidence (1% tree mortality), of Verticillium wilt and harvest losses of 1% of the national Greek olive production. In Morocco, 10–30% of trees in 60% of the inspected olive orchards were affected by *Verticillium dahliae* [12]. Reports are also confirming the presence of the disease in non-traditional olive cultivating countries such as Australia [13].

The infected plants are very difficult to be treated with fungicides because the fungus resides in the vascular bundles and excessive growth result in the blockage of these vascular bundles. The purpose of this study was to investigate the olive field of district Mardan for the prevalence of *Verticillium dahliae*. Several olive trees were found suspected of the Verticillium wilt as the climate of Mardan is favorable for the *Verticillium dahliae* growth in these olive trees.

Materials and Methods

Sample collection

Olive field consisting of five thousand plants with 17 acres of the area was investigated for the presence of wilted plants. Samples were collected from six suspected trees. All samples were sealed in polythene bags and labelled with tree name their root, shoot, and leaf. i.e., T1R1, T1L1, T1S1, etc.

Sample preparation

The diseased plant components (roots, stems, and leaves) were rinsed completely with sterile distilled water after being washed with running tap water for 10 minutes to remove soil, mud, and debris particles. Samples surfaces were disinfected by immersing them in 75 % ethanol for 5 minutes, followed by 3 minutes of 1 percent sodium hypochlorite (leaves) and 5 minutes of 1 percent sodium hypochlorite (branch and root), 75 percent ethanol for 1 minute, three rinses with sterile distilled water, and finally drying on sterile tissue paper. The plant samples (roots, stems, and leaves) were then cut into 5-10 mm lengths aseptically using a sterile scissor. Cross-sectional and longitudinal cuts were made on the root and branch samples.

Culture plate preparation

Disinfected tissues were plated on PDA (Potatoes (200 g), Glucose (20 g), Agar (10 g), Distilled Water (1 L)) media using sterile forceps. Plates were sealed with parafilm tape and labelled with the name of the field and the cultured date. One plate is used as a control and has no samples cultured on it. The plates were incubated for 7 days at 26˚C.

Sub culturing

Plates with obvious growth were sub-cultured to isolate pure colonies. In a centrifuge tube, 50 µl of sterile distilled water was added. Pipette tips were used to streak each colony on the plates and then dipped in distilled water in a centrifuge tube. The water from the centrifuge tubes was pipetted into the PDA plates' centre. The plates are allowed to dry before sealing with parafilm and incubated for 7 days at 26˚C.

Microscopic analysis

Slides were made from plates with pure white colonies for identification and morphological characterization. On the glass slide, one drop of water (40 µL) was poured. Pipette tips were used to streak colonies on the plates. On the slide, the pipette tip was dipped in water and dropped. The glass slide is then covered with a coverslip. A light microscope was used to examine the slide. All slides are prepared using the same method.

Spores counting

Conidial suspension of isolates was prepared by adding 3 ml distilled water to plates. A pipette tip was used to scrape the colony on the plate. The water containing spores was collected after scratching and transferred to Eppendorf tubes. The spore suspension was diluted by dissolving 10 µL of spore suspension in 990 µL of water. Spores were counted by putting 20 µL of spore suspension on hemocytometer slides and observed under a light microscope.

Spores' preservation

Glycerol (30 %) was used to preserve the spores. 30 mL glycerol was dissolved in 70 mL distilled water and autoclaved for 15 minutes at 121 °C. Half of the spore suspension and half of the 30 % glycerol solution were taken in Eppendorf tubes. All of the tubes were stored at -20°C in the freezer.

Colony Diameter

To measure colony diameter, spores at a concentration of 1×10^7 /ml were cultured on PDA and CM media (Yeast extract (6 g), Casein acid hydrolysate (6 g), Sucrose (10 g), Distilled water (1 L) On each plate, the diameter of colonies was measured using a ruler at three-day intervals for 7/9 days.

Results and Discussion

Verticillium dahliae is a soil-borne fungus affecting more than 400 plant species including Olive trees [14]. In order to determine the prevalence of this fungus, plant samples from a single olive field in the region of Mardan, Pakistan were collected. Infected plants displayed varying degrees of symptoms such as stem yellowing, leaf chlorosis, and leaf necrosis, which are all frequent signs of *Verticillium dahliae* infection (**Fig. 1**). The samples were collected and sealed in the polythene bags (**Fig. 2**) and shifted to the lab and kept in the freezer at -20 °C. After seven days of incubation on PDA media, some plates showed evident growth (**Fig. 3**) of various fungal colonies, while others showed no growth (**Table 1**). A total of six suspected trees were evaluated for the presence of *Verticillium dahliae*. Out of which *Verticillium dahliae* was isolated from the roots of one tree. No growth of *V. dahliae* was recorded from other trees (**Table 2**). *Verticillium dahliae* was isolated from different tissues of the trees (**Table 3**). Single colony isolation from the root of the T4 tree was performed on PDA medium and the colony was designated "Z" and used for morphological characterization. Pure colonies were isolated by sub-culturing on PDA plates (**Fig. 4**).

Table 1: Fungal Isolates on PDA media

Plates	Fungal Colonies
T1p1	5 white and 1 reddish
T1p2	5 white
T1p3	4 white
T1p4	4 white
T1p5	3 white
T1p6	4 white
T2p1	2 white
T2p2	3 white
T2p3	2 white
Control	Nill

Table 2: Percentage of Verticillium positive samples in various trees

Sr. No.	Tree	Pure colony	Percentage
1	T1	0/6	0%
2	T2	0/7	0%
3	T3	0/5	0%
4	T4	6/6	100%
5	T5	0/7	0%
6	T6	0/7	0%

Table 3: Isolation of *Verticillium dahliae* from different tissues of plants.

Sr. No.	Tissue type	Culture positive	Percentage
1	Root	9/9	100%
2	Steam	0/8	0%
3	Leaves	0/5	0%

After subculture on PDA medium, a pure colony of *Verticillium dahliae* was isolated. Conidia, resting structures, and colony colour were all studied in the isolate. Under a light microscope, the isolate was recognized as having a white colony, septate mycelium with phialides in a distinctive arrangement, and oval conidiophores. Microsclerotia were also seen on the cultures as globose to oblongate dark melanised structures throughout the colony, with no dark mycelia, indicating that the tested isolate was morphologically identical to *Verticillium dahliae*. These characteristics were similar as described earlier [15].

Conidial morphology of Verticillium isolates was observed under the microscope. Conidia were unicellular, oval, hyaline, and borne singly on whorl conidiophore [16]. **Fig. 5** shows a high quantity of conidia on 40 x magnification.

The fungus mycelium was hyaline, septate, multinucleate, and branched. The phialides of the isolate were sharp at the apices, and the short branching is grouped in whorls. Conidia were produced individually, terminally as spore balls at

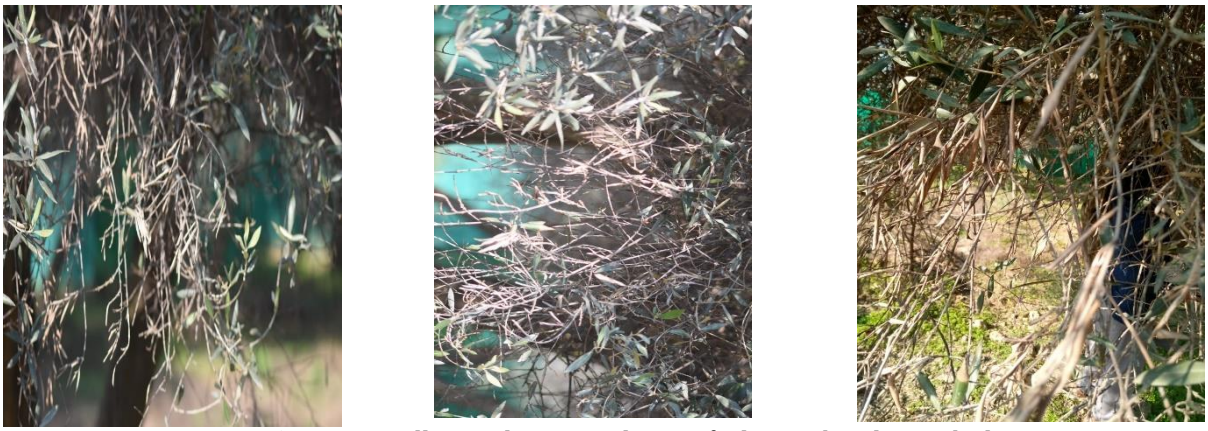

Fig. 1: Verticillium wilt suspected trees of Olive in the Olive arched

Fig. 2: Plant samples collected from Olive trees sealed in polythene bags

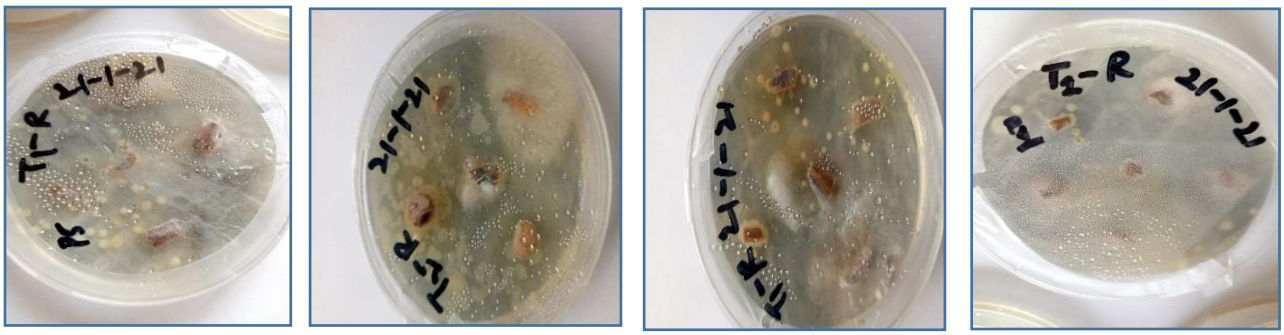

Fig. 3: Fungal Isolates on PDA media

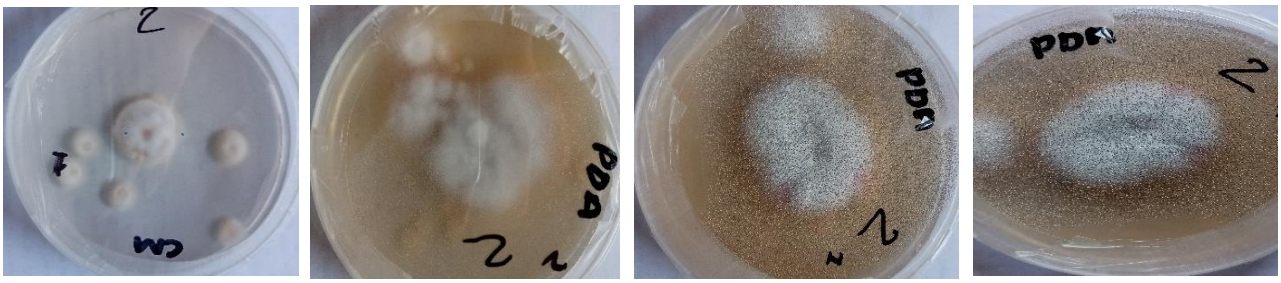

Fig. 4: Pure white colony of *Verticillium dahliae*

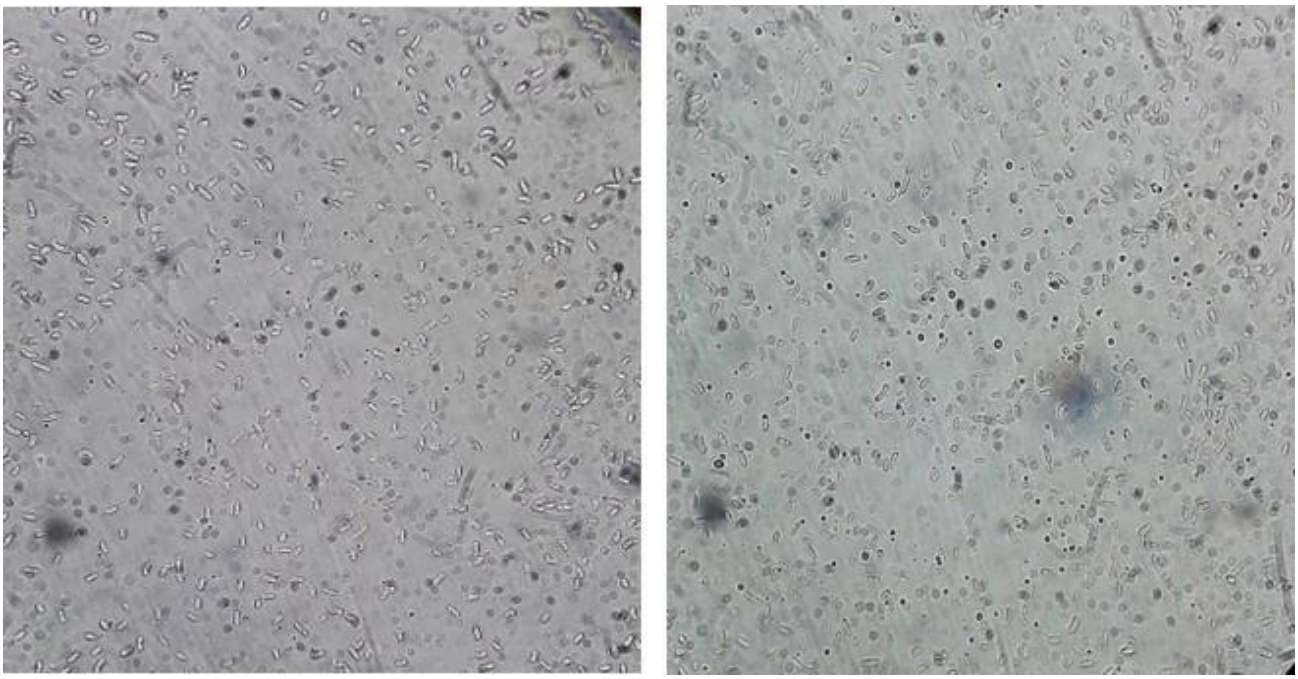

Fig. 5: Spores morphology of Verticillium dahliae isolate from Olive tree

the ends of phialides. The colony has a lot number of spores and mycelia (**Fig. 6**). Earlier studies have described similar morphology of *V. dahliae* for its mycelium morphology [17]. Based on this morphology, we concluded this fungus to be *V. dahliae*.

Microsclerotia were observed after culturing the fungus for 15 days on PDA media. *Verticillium dahliae* microsclerotia were dark brown to black in colour (**Fig. 7**) and consisted of inflated, nearly spherical cells that arose from single hyphae by recurrent budding as reported earlier [18].

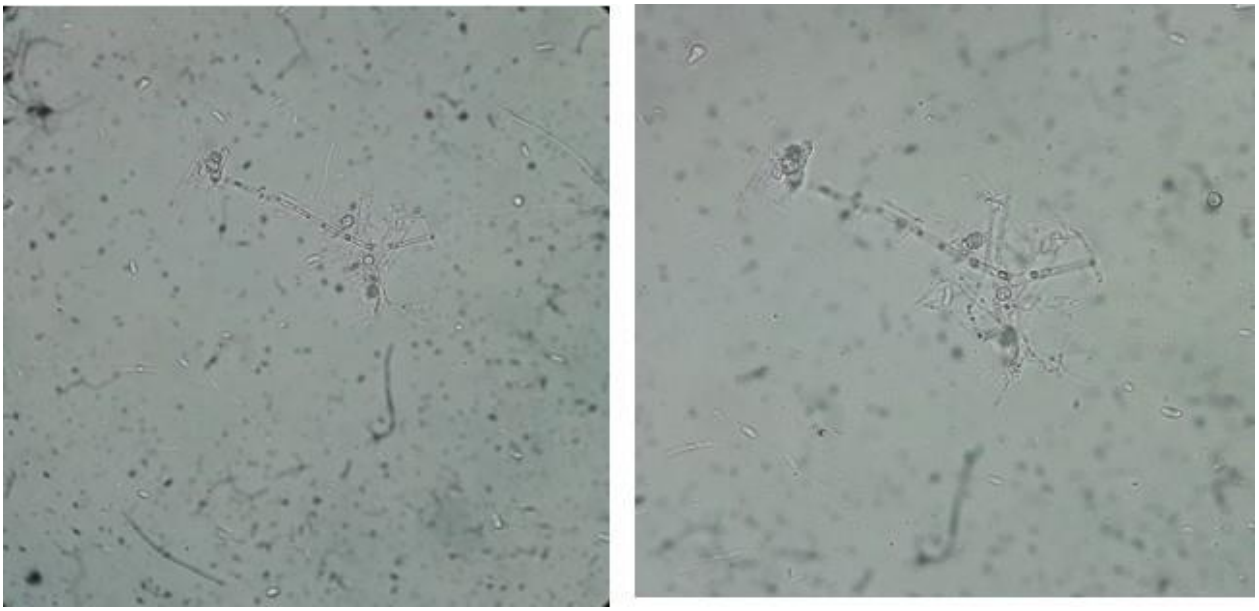

Fig. 6: Mycelium morphology of Verticillium dahlia isolate from Olive tree

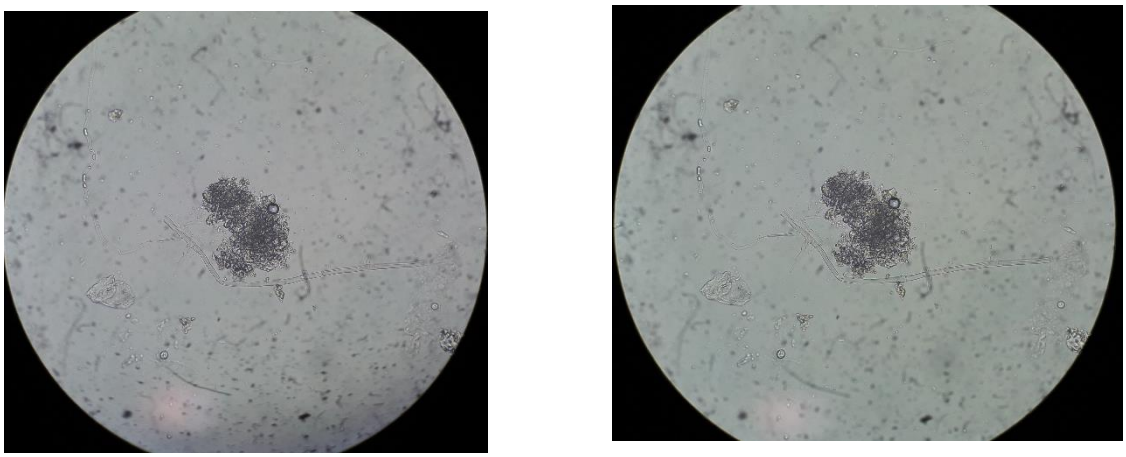

Fig. 7: Microsclerotia morphology of *Verticillium dahliae* isolate from Olive tree

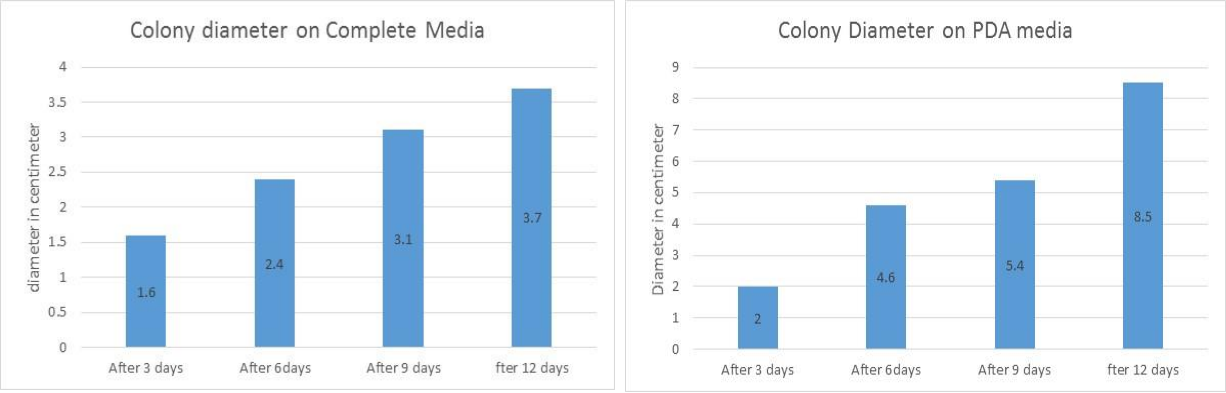

Fig. 8: Graphical indication of colony diameter on PDA and CM media

Known concentration (1×10^7 /ml) of conidial suspension of Verticillium isolate were cultured on PDA and CM media. The Growth efficiency of each isolate on both the media was founded by measuring the colony diameter. On each plate, the diameter of colonies was measured using a ruler at three-day intervals. Figure 8 shows the rapid growth of Verticillium colonies on PDA media as compared to CM media. The colony diameter is a measure of how rapidly a fungus grows and can also contribute to its pathogenicity [19].

Conclusion

Our research was focused on the isolation and morphological characterization of *Verticillium dahliae* from Olive trees. We visited the Olive Orchard located at Mardan KPK region. Samples were collected from suspected plants and *Verticillium dahliae* was isolated from one of the trees. The isolated fungus was identified based on its spore's morphology, mycelia structure, and observation of microsclerotia.

Authors' contributions

LR and HH designed the study. SF, SA SK, SA, and TA collected samples and carried out experimental work. MI, HK and NUQ prepared the draft of the manuscript. LR revised and finalized the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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