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In vitro antifungal activity of barberry fruit extract (*Berberis* spp.) against *Fusarium* spp.

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ABSTRACT

Purpose: *Berberis integerrima* Bunge and *Berberis vulgaris* L. are traditional plants known for their many health benefits. The aim of this study was to investigate the antifungal potential of *B. vulgaris* and *B. integerrima* fruit extracts against *Fusarium* spp. pathogens as an environmentally compatible natural antifungal compound. **Research methods**: The antifungal activity of methanolic fruit extracts of *B. vulgaris* and *B. integerrima* against *Fusarium solani*, and *Fusarium graminearum* was investigated using the microdilution method, growth area measurement, and morphological Changes were studied using scanning electron microscopy analysis. **Findings:** The methanolic fruit extracts of *B. vulgaris* and *B. integerrima* had significant antifungal activity against the studied plant pathogens, with *B. integerrima* exhibiting a stronger effect. The MIC values of *B. vulgaris* fruit extract against *F. graminearum and F. solani* were 150 and 75 mg mL^{-1}, and *B. integerrima* fruit extract had 100 and 75mg mL^{-1}, respectively. *F. graminearum* was the most resistant fungal species. Scanning electron microscopy analysis showed that the extracts of both medicinal plants changed the structure and morphology of mycelia and, dose-dependently, inhibited conidia formation. **Research limitations:** There were no limitations. **Originality/Value**: The study showed that fruit extracts of *B. vulgaris* and *B. integerrima* have the potential to be used as natural and environmentally friendly agents against *Fusarium* species.

INTRODUCTION

Fusarium spp., a widespread filamentous fungus found in soil, plants, and organic substrates, is a significant plant pathogen responsible for various diseases, economic losses on crops, and food spoilage (Nehra et al., 2021), with over 300 species in 22 species complexes (Nosratabadi et al., 2022), and 24 toxic species that have a significant impact on both human and animal health (Adeyeye, 2016). Among the most important ones, which are more destructive, can be pointed out: the *F. graminearum* species complex, responsible for Fusarium head blight in wheat and barley; and the *F. solani* species complex, the cause of destructive foot and root rot (Aoki et al., 2014). Heavy reliance on synthetic pesticides to manage plant pathogens has become an important concern due to their negative effects on human health, the environment, and the emergence of resistant pests and disease-causing species (Lengai et al., 2020). Therefore, new antifungal strategies aim to create fungicides with low production costs, high efficacy, and safe for people, animals, host plants and ecosystems. Biological control is one of the strategies that, due to its effectiveness on target organisms and its biodegradability, has gained global popularity (Pârvu & Pârvu, 2011). Plant extracts and plant-derived compounds have received much attention as a potential alternative to synthetic fungicides for biological control. Plant tissues produce secondary metabolites that are highly active against pathogens and have been tested against various fungal pathogens (Bhandari et al., 2021). Berberis is a genus of plants with 650 species and 15 genera, found in Asia, North Africa, and Europe (Goodarzi et al., 2018). Barberry species, including *B. vulgaris*, are produced worldwide for medicinal purposes. In addition to the pharmaceutical industry, they are also used in the food sector, and ornamental species are used for decoration in different places (Rahimi-Madiseh et al., 2017). The diverse and contentious nature of barberry species identification has prompted numerous studies (Ghahramanlu et al., 2023; Rezaei et al., 2011). Species including *B. vulgaris*, *B. orthobotrys*, *B. khorasanica*, *B. integerrima*, *B. crataegina*, *B. lycium*, and *B. aristata* are frequently utilized in traditional medicine in Iran and other regions (Rahimi-Madiseh et al., 2017; Rezaei et al., 2011). *Berberis vulgaris* L., a variety in Khorasan Province, Iran, is a special fruit with high economic value for farmers and a rich history in folk medicine. Its high antioxidant capacity may increase its popularity. In vitro and in vivo studies have shown barberry's pharmacological activities, making it a valuable addition to the country's diet (Goodarzi et al., 2018). It is known for its health benefits, including fat reduction, anti-cancer, anti-diabetes, liver protection, antioxidant, and anti-inflammatory properties (Ardestani et al., 2015). *Berberis integerrima* Bunge, a wild barberry species, is used in Iran for its antioxidant, anti-diabetic and renal prevention properties. Its pharmacological activity includes antinociceptive, anticonvulsant, antiinflammation, antioxidant, anticancer, antihyperglycemic, antihypertensive, and antibacterial effects (Moein et al., 2020). In order to identify a natural and environmentally friendly anti-fusarium agent, this study investigated the antifungal properties of *B. vulgaris* L. and *B. integerrima* Bunge fruit extracts against *F. graminearum* and *F. solani*, as well as their effects on the morphology of these pathogens.

MATERIALS AND METHODS

Chemicals and media

Potato dextrose agar (PDA), sabouraud dextrose agar (SDA), sabouraud dextrose broth (SDB), and absolute methanol were all obtained from Merck (Germany).

Plant material and preparation of berberis extracts

Berberis vulgaris L. from Qain city in South Khorasan province and *Berberis integerrima* Bunge species from Shahr-e-Babak city in Kerman province were collected in 2022 and deposited in the herbarium of the Faculty of Natural Resources and Environment of Birjand University with voucher numbers 2670 and 2911, respectively. The barberry fruits from Birjand were seedless, while those from Kerman had one to three small spindle-shaped seeds. The fruits were washed, dried in an oven (CE.FH.151.4, Germany) for two days at 50 °C, milled to a fine powder, and stored at -20 °C until extraction. The *Berberis* spp. powders were added in a ratio of 1:10 with 80% methanol (methanol: water, 80:20 vv^{-1}) at 50 °C for 24 h with stirring at 150 rpm in a shaking incubator (Lab Tech, South Korea). The extracts were filtered twice using Whatman No. 1 filter paper. A rotary evaporator (IKA, RV 10, DS 99. Germany) was used to evaporate the solvent from the extracts at 50 °C until the thick syrup was collected. The syrups were entirely dried using a freeze-drying device (VaCo 5-D, Zirbus Technology, Germany), and the dried extracts were kept at -20 °C to do tests.

Preparation of fungal spore suspension

*Fusarium solan*i and *Fusarium graminearum* were obtained from the microorganism collection of the Department of Plant Medicine, Faculty of Agriculture, Shiraz University, Shiraz, Iran. Spores were prepared by soft scraping and pipetting sterile normal saline solution or sterile distilled water onto a seven-day PDA culture at 25–28 °C. The spore number was measured using a hemocytometer and adjusted to 2×10^6 spores per millilitre.

Assessment of antifungal activity of berberis fruit extracts against *Fusarium spp.*

Antifungal activity of *B. vulgaris* and *B. integerrima* fruit extracts on the growth of *F. solani, F. graminearum*, was determined by the micro-well dilution technique, and growth area in agar media.

Micro-well dilution technique

The minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of *Berberis vulgaris* and *Berberis integerrima* fruit extracts were determined by the micro-well dilution applied by Kumar et al. (2016) with a slight modification. A volume of 100 µL of concentrations of 400, 300, 200, 150, 100, 75 mg mL^{-1} of extracts were added to the wells of a 96-well plate containing 100 µL of SDB and 10 µl of spore suspension (2×10^6 spores mL^{-1}), incubating at 25°C for 5-7 days. Wells containing 200 µl of SDB and 10 µl of spore suspension were considered positive controls. The lowest concentration of extracts that caused complete inhibition of fungal growth after seven days was considered the MIC. To distinguish fungistatic and fungicidal activity and determine the MFC, after reading the MIC, 20 µl of culture wells with no growth of fungal cells and also a positive control was subcultured onto SDA (incubation at 25°C for five days). The lowest concentration without a fungal colony was considered MFC. The experiments were performed twice, with three replications for each treatment.

Determination of the fungal growth zone

The inhibitory effect of the extracts by the method of Salem et al. (2021) with minor modifications in the 70 mg mL^{-1} concentration of both plant fruit extracts on agar culture medium on the growth of *F. solani* and *F. graminearum* spores was investigated by the spotting method in SDA medium in three replicates for each treatment inoculated with 10 µl spores (2×10^6 spores mL^{-1}) and incubated at 25°C). The growth area was calculated on various days, including 3, 5, 7, 9, 12, 16, 20, 25 and 30. The percentage of growth inhibition

(PGI) (%) was calculated by the formula: PGI (%) = [C-T] × 100/C, where C is the diameter of the control colony and T is the diameter of the treated colony. Three replicates were carried out for all of the treatments.

Scanning electron microscopy (SEM) analysis of the effect of berberis fruit extracts on mycelial morphology

The effect of the *B. integerrima* and *B. vulgaris* extracts on the mycelial structure of *F. solani* and *F. graminearum* was investigated by SEM with some modifications to the Sellamani et al. (2016) method. A volume of 20 µ of spores (2×10^6 spores mL^{-1}) was added to the SDA culture at MIC50 amounts of methanolic extracts and incubated at 25 °C. With the appearance of mycelium on the culture medium, the blocks of mycelium (1×1), were separated and dried with a freeze-dryer to stabilize and prepare for SEM imaging. The mycelia were sputter coated with gold (Q150R ES, Quorum Technologies, United Kingdom), and the morphological feature was observed by SEM (TESCAN-Vega3, Czech Republic) at 20.0 kV in environmental mode. Mycelia grown in cultures without extract were considered as control. (Sellamani et al., 2016)

Statistical analysis

The study utilized a generalized linear model (GLM) for ANOVA, the Statistical Analysis System (SAS), Version 9.3 for examining the significant differences between species using the least significant difference (LSD) test, and Graphpad Prism 8.2.1 for creating graphs.

RESULTS

Assessment of antifungal activity of berberis fruit extracts against *Fusarium spp.*

Microdilution method

The antifungal activity of *B. integerrima* and *B. vulgaris* fruit extracts against *Fusarium* spp. was studied using the microdilution method. The results showed that *B. vulgaris* fruit extract had MIC values against *F. graminearum* and *F. solani* of 150 and 75mg mL^{-1}, respectively, and MFC values of 400 and 300 mg mL^{-1}, respectively. At MIC values of 100 and 75mg mL^{-1} and MFC values of 200 and 100 mg mL^{-1}, respectively, *B. integerrima* fruit extract effectively inhibited *F. graminearum* and *F. solani* (Fig. 1). The results showed that *F. graminearum* was more resistant fungus and *B. integerrima* fruit extract had a stronger anti-fusarium effect.

Determination of the fungal growth zone

The results showed that *B. integerrima* fruit extract had a stronger antifungal effect than *B. vulgaris* on the growth percentage of *F. solani* and *F. graminearum* spores in agar medium (Fig. 2B). The fruit extract of *B. integerrima* effectively inhibited the growth of *F. solani* until the end of the incubation period (day 30) (Fig. 2A). *B. vulgaris* fruit extract had the highest inhibitory effect on *F. solani* (Fig. 2C). In general, *F. graminearum* was more resistant fungal species (Fig. 2B). The results of the ANOVA analysis of the impact of plant and fungal species on the percentage of fungal growth inhibition are presented in Table 1.

SEM analysis of mycelial morphology

Changes in the structure of hyphae were easily visible without the use of a microscope. In contrast to the mycelium mass in the control sample, which was spread out throughout the plate, the mycelium mass in the culture medium treated with the extract grew in the center of the plate (Fig. 3).

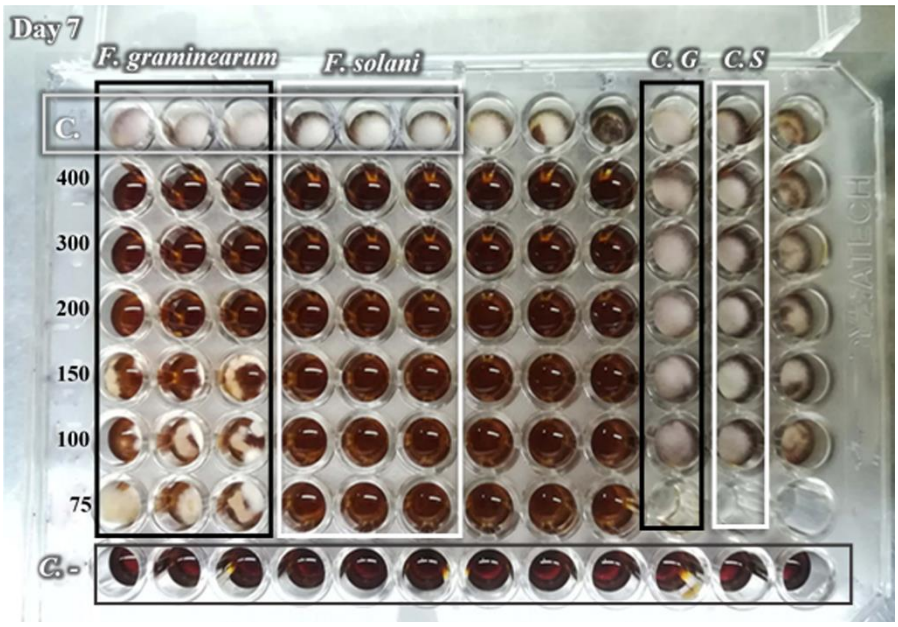

Fig. 1. Seven-old day culture of *F. solani* and *F. graminearum* spores (2×10^6 spores mL^{-1}) in SDB medium at 25°C with 400, 300, 200, 150, and 75 $mgmL^{-1}$ concentrations of methanolic fruit extracts of *B. integerrima*. Control (C.) positive control of *F. graminearum* (C. G); positive control of *F. solani* (C. S); negative control (SDB medium without extract and spores) (C. -).

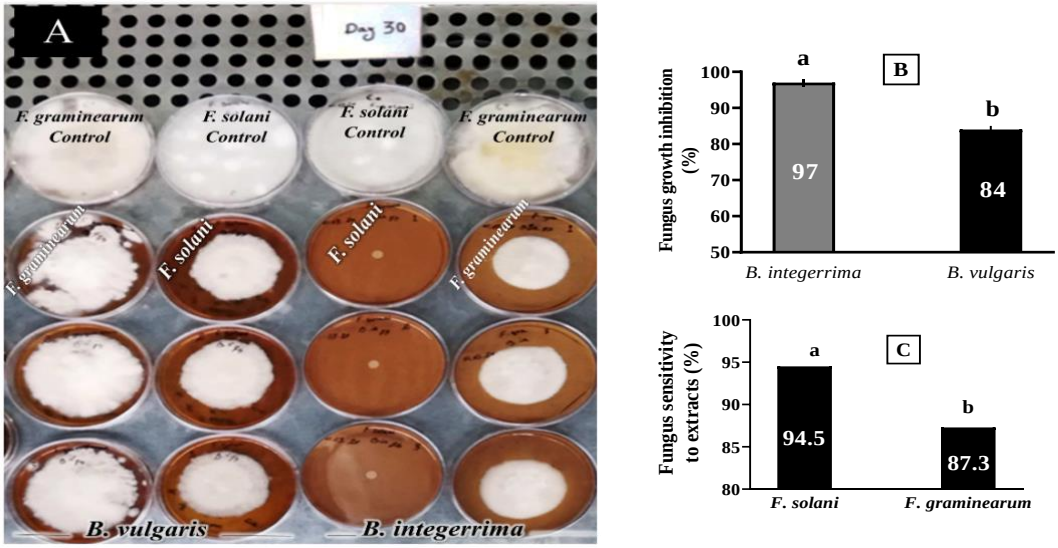

Fig. 2. 30-old-day cultures of spore culture of *F. solani* and *F. graminearum* at 25 °C in SDA medium containing 70 mg mL^{-1} of *B. vulgaris* and *B. integerrima* fruit extracts in three replicates, (A). Comparison of the *B. vulgaris* and *B. integerrima* fruit extracts' ability to inhibit *Fusarium* spp. (B). The sensitivity percentage of *F. solani* and *F. graminearum* to *B. vulgaris* and *B. integerrima* fruit extracts, (C). Controls (no extract); Bars with different letters differ from each other significantly ($P < 0.05$).

Table 1. ANOVA analysis of the impact of plant (*B. vulgaris* and *B. integerrima*) and fungal species (*F. solani* and *F. graminearum)* on the percentage of fungal growth inhibition.

S. O. V	df	Mean Square
Plant	1	5148.3***
Fungi	2	1569.6***
Plant × Fungi	1	142.2 ***
CV (%)		1.92

S.O.V: Sources of variations; df: degrees of freedom;
CV (%): Coefficient of variation. *** Significance at the level of <0.0001 probability.

SEM images of the effect of B. vulgaris on F. graminearum mycelia

The growth rate of *F. graminearum* mycelia in media containing *B. vulgaris* fruit extract decreased significantly compared to the control sample, and the fungal mass was concentrated at the spore inoculation site, unlike in the control sample, which had grown all over the plate (Fig. 3B). Unlike the long, slender and smooth mycelium in the control sample (Fig. 4, A1–A3), the mycelium grown in the extract media was thick, dense, and deformed (Fig. 4, B1–B3). No spores were observed in the extract-containing culture, while many conidia were present in the control sample.

SEM images of the effect of B. vulgaris on F. solani mycelia

In the medium containing *B. vulgaris* fruit extract, thick mycelia with denser texture and structure could be seen (Fig. 5, B1–B3) in contrast to the long, narrow, and smooth mycelia of *F. solani* grown in control cultures (Fig. 5, A1–A3). In the cultures that contained the extract, fewer spores were seen. In comparison to the control sample, the growth rate significantly decreased (Fig. 3B).

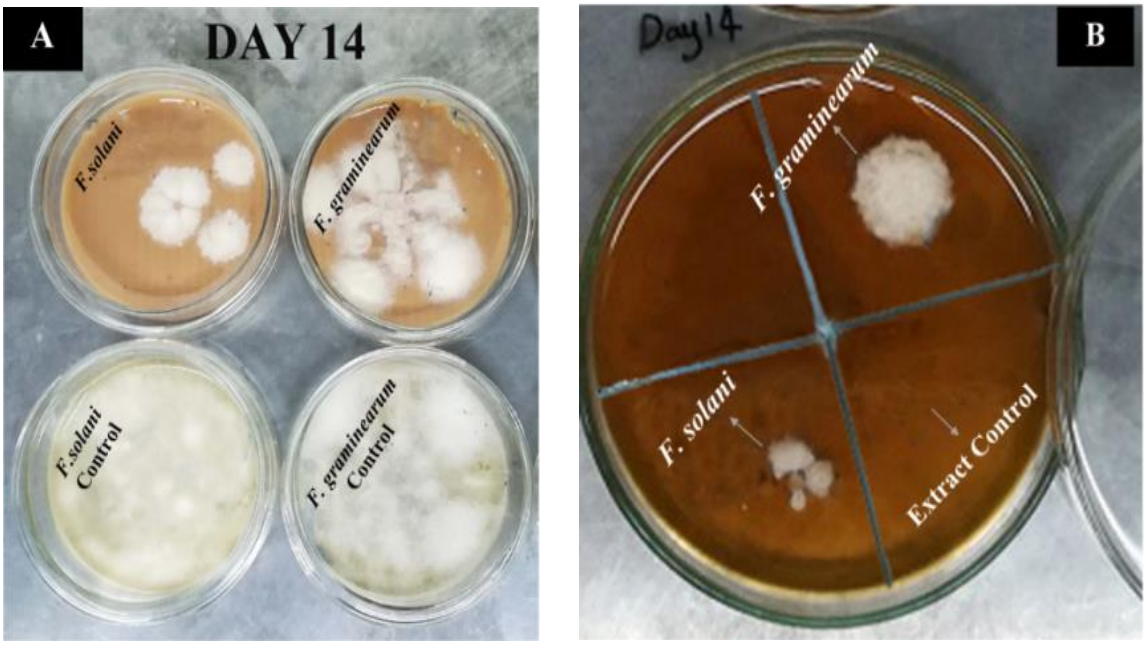

Fig. 3. 14 old-day cultures of *F. solani* and *F. graminearum* in SDA at 25 °C; medium containing the MIC50 value of *B. integerrima* fruit extract with fungus controls, (A); medium containing the MIC50 value of *B. vulgaris* fruit extract, (B).

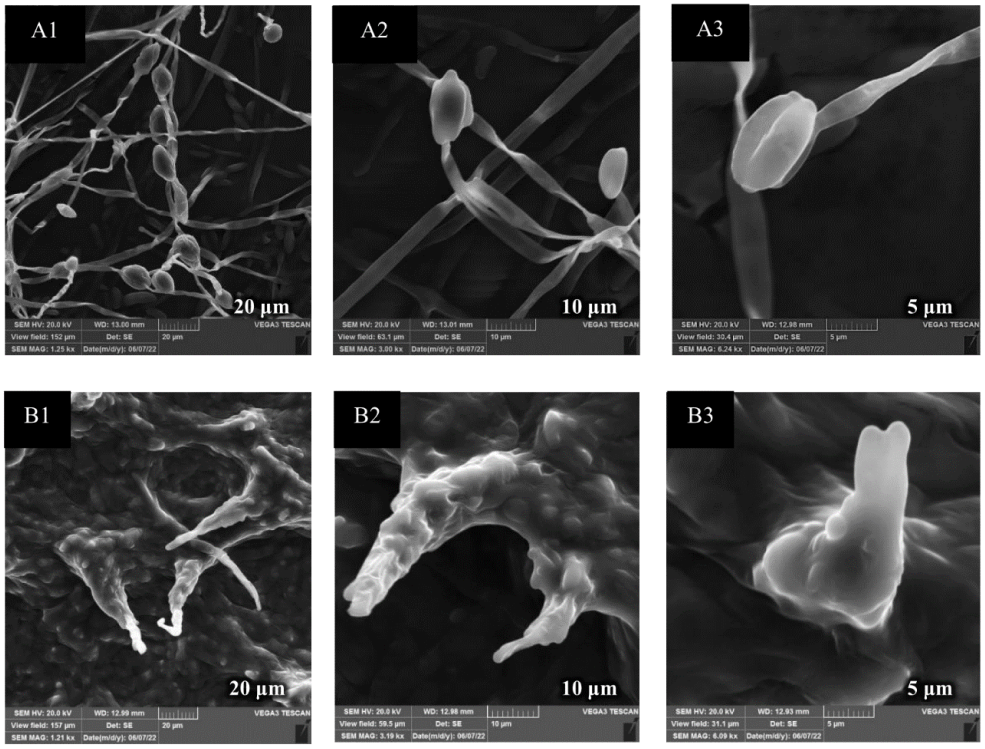

Fig. 4. SEM image of the effect of *B. vulgaris* fruit extract on the structure of *F. graminearum* mycelium in SDA. Control sample (without extract), (A1–A3); MIC50 value of *B. vulgaris* fruit extract, (B1–B3).

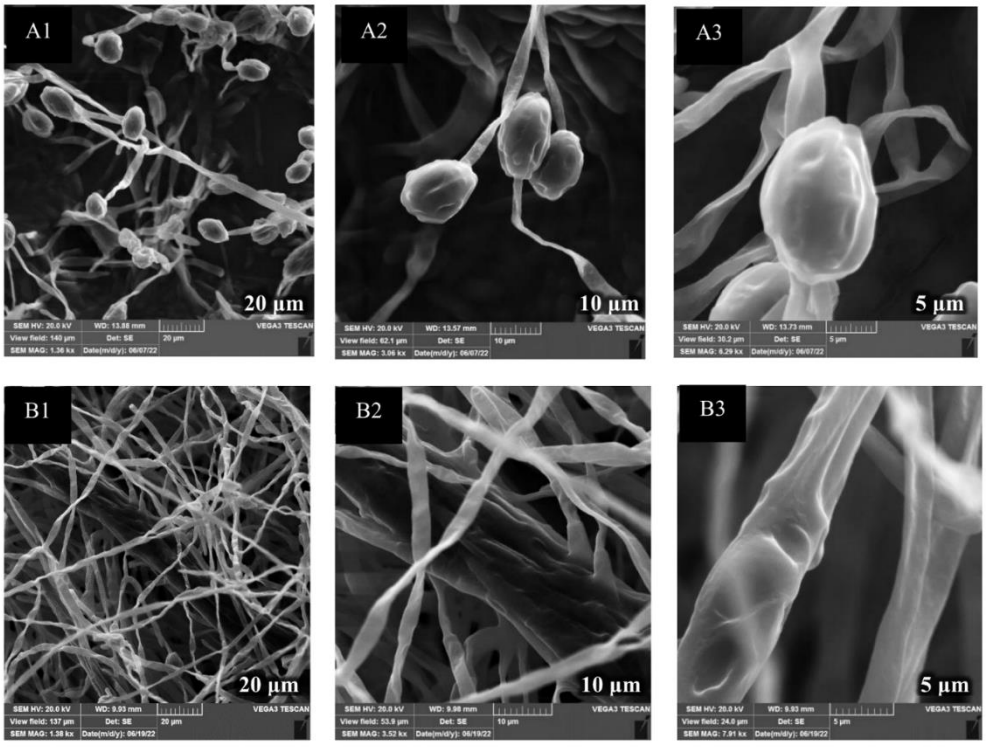

Fig. 5. SEM image of the effect of *B. vulgaris* fruit extract on the structure of *F. solani* mycelium in SDA. Control sample (without extract), (A1–A3). MIC50 value of *B. vulgaris* fruit extract, (B1–B3).

SEM images of the effect of B. integerrima on F. graminearum mycelia

Unlike the control sample's long, thin, and smooth mycelia (Fig. 6, A1–A3), *F. graminearum* mycelia grown in medium containing *B. integerrima* extract were thick, dense, and deformed. The mycelium in the extract-containing medium was networked and interconnected, unlike the control sample's filamentous and separate mycelium (Figs. 6, B1–B3, and C1, C2). The growth rate was significantly reduced, and the mycelium's mass was concentrated at the spore inoculation site (Fig. 3A). No spores were observed in cultures containing the MIC50 value extract and very few in the 25 mg mL^{-1} extract sample, whereas many conidia were present in the control sample (Fig. 6).

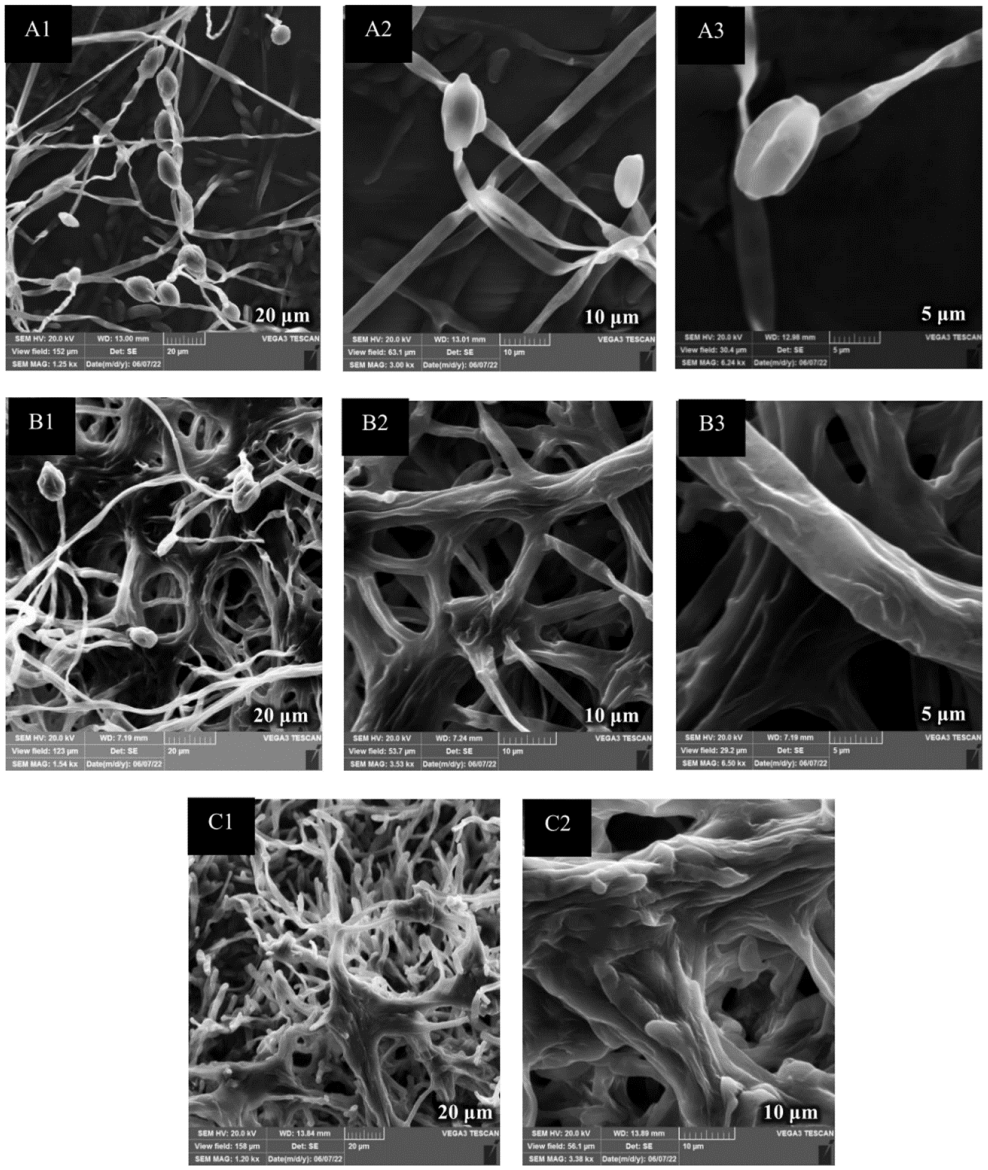

Fig. 6. SEM image of the effect of *B. integerrima* fruit extract on the structure of *F. graminearum* mycelium. Control sample (without extract), (A1–A3); volume of 25 and 50 mg mL^{-1} of *B. integerrima* fruit extract, respectively, (B1-B3 and C1-C2).

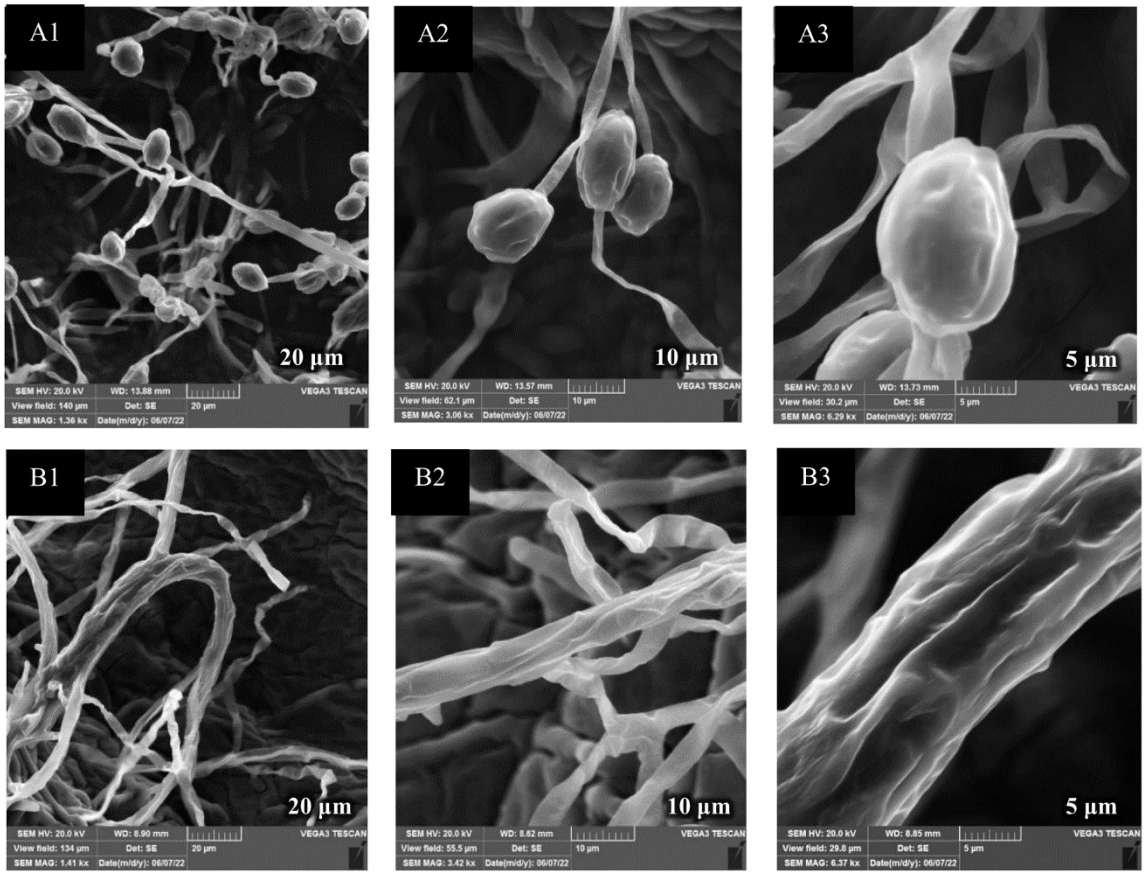

Fig. 7. SEM image of the effect of *B. integerrima* fruit extract on the structure of *F. solani* mycelium. Control sample (without extract), (A1–A3); MIC 50 value of *B. integerrima* fruit extract, (B1-B3).

SEM images of the effect of B. integerrima on F. solani mycelia

The *F. solani* mycelium grown in a medium containing *B. integerrima* extract was thicker, irregular, and dense than the filamentous, thin, and delicate mycelium of control (Fig. 7). Spores were not observed in extract samples. The fungal colony appeared convex and dense, in contact with the culture medium. The growth rate was slower than in the control sample (Fig. 3A).

DISCUSSION

Plant pathogenic fungi, with over 10,000 species, are the most dangerous plant pathogens that cause significant damage to economically important crops (Nazarov et al., 2020). The increasing number of *Fusarium* species exposed to whole-genome sequencing, underscores the significant threat *Fusarium* poses to agriculture and human health (Munkvold, 2017). Over the past decades, numerous research studies have focused on developing an efficient and eco-friendly method for managing phytopathogens (Seo et al., 2013). Several plant families have shown fungicidal activity against *Fusarium* species, such as *Asteraceae* (Rongai et al., 2012), *Oleaceae* (Korukluoglu et al., 2008), and *Lamiaceae* (Yazgi et al., 2015). No reports of anti-*Fusarium* effects were found from barberry fruits of the *Berberidaceae* family. The study demonstrated that *B. vulgaris* and *B. integerrima* fruit extracts effectively inhibited the growth of the studied *Fusarium* spp., with *B. integerrima* exhibiting a stronger inhibitory effect. The phytochemical analysis of the *Berberis* fruit revealed the presence of alkaloids, tannins, carotenoid, vitamin, protein, lipid, anthocyanin, and phenolic compounds (Salehi et al., 2019).

The greater potency of *B. integerrima* fruit extract compared to *B. vulgaris* may be related to the bioactive compounds present in its seeds. Fatty acids (linolenic, linoleic, and oleic acids, as well as omega-3 and omega-6 fatty acids) and phytosterols are also present in the oil found in *B. integerrima* seeds (Tavakoli et al., 2017). This plant is often used in pharmacological studies as a rich source of bioactive substances (Moein et al., 2020). In the present study, *F. graminearum* was the most resistant species both in liquid culture and in agar medium. In the study of Samie and Mashau (2013), *F. graminearum* was more resistant to most plant extracts than other *Fusarium* species. Our results agreed with them. *B. integerrima* fruit extract exhibited MIC values of 75–100 mg mL^{-1}, and *B. vulgaris* fruit extract had MIC values of 100-150 mg mL^{-1} for the fungi that were being examined in this study. *Piper sarmentosum* extract at 1-2 mg mL^{-1} against *F. graminearum* (Zhou et al., 2023), and *Taxus wallichiana* Zucc extract showed inhibitory effects against *F. solani* at MIC values of 0.08–200 mg mL^{-1} (Nisar et al., 2008). Phytochemicals play a crucial role in plant defense against fungal pathogens, either directly by affecting pathogen physiology and morphology (Dang-Minh-Chanh et al., 2013) or indirectly by inducing plant systemic resistance (Al-Wakeel et al., 2013). Studies have linked various bioactive compounds in plants, including alkaloids, organic acids, flavonoids, etc., with antifungal activity (Daradka et al., 2021). Berberine alkaloid, a bioactive compound found in barberry fruit, has been found to have antifungal properties due to its ability to inhibit sterol and cell wall biosynthesis and cell damage by increasing reactive oxygen species production (Xie et al., 2020).The most separated substance from the different parts of *B. integerrima* is also alkaloids (Moein et al., 2020). Both fruit extracts demonstrated inhibitory effects against test microbes, with berberine possibly being an effective compound in this activity. SEM and transmission electron microscopy (TEM) images have shown that the MIC or MFC of some plant extracts caused fungal ultrastructural changes (Dang-Minh-Chanh et al., 2013; Pârvu and Pârvu, 2011). Our study's SEM images revealed significant changes in mycelium structure and inhibition of microconidia production at the MIC50 value of the extract. The mycelia of *F. solani* and *F. graminearum* grown in a culture containing *B. vulgaris* and *B. integerrima* fruit extracts had thick, amorphous, altered, and bulky structures compared to the control mycelium. Bioactive compounds could be responsible for the changes in morphology (Perveen et al., 2022). The presence of various organic acids in barberry fruit extract, including oxalic, tartaric, ascorbic, acetic, malic, and fumaric acids (Ardestani et al., 2015), leads to acidification and lowers the extract's pH. This creates unfavorable environmental conditions for microorganisms and increases the antimicrobial properties of the extract (Khan et al., 2022). The fungal cell wall is a dynamic structure that shields cells from osmotic pressure changes and environmental stress (Gow & Lenardon, 2023). Environmental pH fluctuations and antifungal drug treatments impact gene expression, alter cell wall enzyme expression, stimulate regeneration mechanisms, and induce new cell wall structure changes. A stiffer cell wall, influenced by growth conditions, reduces the risk of cell damage, ultimately increasing cell survival and integrity during hyperosmotic stress. Less-elastic cell walls protect the plasma membrane from rupture during acute osmotic shocks (Ene et al., 2015). These changes were clearly evident when *Fuarium culmorum* was exposed to tebuconazole (a systemic fungicide). Tebuconazole inhibited fungal growth and caused swelling, excessive branching, and cell wall thickening (Kang et al., 2001). Our findings were in line with Kang et al.'s (2001) study. Along with glucan and various glycoproteins, chitin is a vital component in filamentous fungi that contributes to the stiffness, mechanical strength, and structural integrity of the cell wall (Hasim & Coleman, 2019). It has been reported that a reduction in overall chitin synthesis leads to excessive swelling of hyphae and changes in conidiation. The chitin synthase enzyme (ChsE enzyme) is involved in the synthesis of bulky chitin (Bowman & Free, 2006). Bulky mycelia and a lack of conidia

formation in MIC50 extracts observed in our SEM images can be affected by the change in chitin synthesis. The reduction in fungal conidia in the presence of antifungal compounds was reported in the studies of Al-Nazwani et al. (2021) and Perveen et al. (2022), and our findings are consistent with these studies. Our research added new information to the literature regarding the anti-fusarium activity of the fruits of *B. vulgaris* and *B. integerrima* and the effects of their extract on the morphological changes of *F. graminearum* and *F. solani* hyphae. (Al-Nazwani et al., 2021) (Samie & Mashau, 2013)

CONCLUSION

The study explored the potential of *B. integerrima* and *B. vulgaris* fruit extracts as antifungal agents against *F. graminearum* and *F. solani*. Comparative analysis revealed effective inhibitory activities on the mycelial growth of *Fusarium* spp., particularly for *B. integerrima*. SEM analysis of the studied *Fusarium* species showed the ability of the methanolic extracts of *B. integerrima* and *B. vulgaris* fruit to change the structure of mycelia and supported their potential as effective agents to control plant pathogenic fungi. The study emphasizes the need for exploring natural plant compounds as a sustainable and environmentally friendly alternative to synthetic chemicals for disease control. Further research is needed on the antimicrobial components of *B. integerrima* and *B. vulgaris*, their mechanisms of action, and their development into more effective and environmentally friendly solutions.

Declaration of interest

The authors declare that there is no conflict of interest.

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