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Research Article
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Sercan PAZARLAR [1]

Eray ŞİMŞEK [2 *]

[1] Department of Plant Protection, Faculty of Agriculture, Ege University, Izmir, Türkiye

[2] Department of Plant Protection, Faculty of Agriculture, Harran University, Şanlıurfa, Türkiye

***** Sorumlu yazar (Corresponding author):

eraysim@harran.edu.tr

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Anahtar sözcükler: Biyolojik kontrol, fungal endofit, *Fusarium oxysporum* f.sp. *ciceris*, bitki savunması ajanı, *Serendipita indica*

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The endophytic fungus *Serendipita indica* colonization protects chickpea plants against Fusarium wilt disease

Endofitik fungus *Serendipita indica* kolonizasyonu nohut bitkilerini Fusarium solgunluğu hastalığına karşı korumaktadır

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ABSTRACT

Objective: The objective of this study is to assess the efficacy of *Serendipita indica*, a basidiomycete endophyte, as a biological control agent against Fusarium wilt disease caused by *Fusarium oxysporum* f. sp. *ciceris* in disease susceptible chickpea cultivar JG62.

Material and Methods: Chlamydospores of *Serendipita indica* were applied to the roots of the germinated JG62 variety, followed by inoculation with *Fusarium oxysporum* f. sp. *ciceris* race 5 one week after the application. Disease severity and plant fresh weight were measured 25 days after inoculation. The colonization pattern of *Serendipita indica* and *Fusarium oxysporum* f. sp. *ciceris* were monitored by quantifying fungal DNA using qPCR over time. The antagonistic interactions between the fungi were determined using the dual-culture method.

Results: *Serendipita indica* successfully colonized the chickpea roots leading to a decrease in biomass of *Fusarium oxysporum* f. sp. *ciceris* in the roots and diminished the overall symptoms such as wilting and yellowing caused by *Fusarium* infection. Besides, direct antagonistic effect of *Serendipita indica* was found against *Fusarium in vivo* conditions.

Conclusion: These results indicate the potential of *Serendipita indica* as a biological control agent in developing sustainable strategies for managing Fusarium wilt of chickpea.

ÖZ

Amaç: Bu çalışmanın amacı, nohutlarda *Fusarium oxysporum* f. sp. *ciceris* tarafından kaynaklanan Fusarium solgunluğu hastalığına karşı biyolojik kontrol ajanı olarak *Serendipita indica* adlı bir basidiomycete endofit fungusun etkinliğini değerlendirmektir.

Materyal ve Yöntem: *Serendipita indica* klamidosporları çimlendirilen JG62 çeşidinin köklerine uygulanmış ve uygulamadan 1 hafta sonra *Fusarium oxysporum* f. sp. *ciceris* ırk 5 inokulasyonu yapılmıştır. Hastalık şiddeti ve bitki yaş ağırlığı inokulasyondan 25 gün sonra ölçülmüştür. *Serendipita indica* ve *Fusarium oxysporum* f. sp. *ciceris* kolonizasyonu fungal DNA miktarının zamana bağlı olarak qPCR ile belirlenmesiyle izlenmiştir. Funguslar arasındaki antagonistik etkileşimler ikili-kültür yöntemi kullanılarak belirlenmiştir.

Araştırma Bulguları: *Serendipita indica* başarılı bir şekilde nohut köklerini kolonize ederek köklerdeki *Fusarium oxysporum* f. sp. *ciceris* biyomasının azalmasına ve *Fusarium* enfeksiyonu sonucu ortaya çıkan solgunluk ve sararma gibi belirtilerin azalmasına neden olmuştur. Ayrıca, *Serendipita indica*'nın *Fusarium solgunluğu etmenine* karşı doğrudan antagonistik etkisi bulunmuştur.

Sonuç: Bu sonuçlar, nohutta Fusarium solgunluğuna karşı sürdürülebilir stratejiler geliştirmede *Serendipita indica*'nın biyolojik kontrol ajanı olarak potansiyelini göstermektedir.

INTRODUCTION

Fusarium wilt of chickpea, caused by *Fusarium oxysporum* f. sp. *ciceris (Foc*), is recognized as a significant soil-borne disease affecting chickpea, leading to wilting at various stages, from seedling to podding (Navas-Cortés et al., 2000; Sharma et al., 2005; Jiménez-Díaz et al., 2015; Jendoubi et al., 2017). Fusarium wilt represents a globally significant pathogenic threat to chickpea cultivation, exerting substantial impact on the crop's health and productivity (Kumar et al., 2015; Jendoubi et al., 2017). Chlamydospores as highly infectious asexual spores present in the soil serve as the main source of inoculation for Fusarium wilt in chickpeas. *Foc* penetrates the roots either directly or through the wounds, colonizes intercellular spaces of the root cortex, and ultimately invades the xylem vessels, resulting in mostly wilting, yellowing and necrosis of foliage (Jiménez-Díaz et al., 2015; Bhar et al., 2021).

Foc chlamydospores exhibit a remarkable ability to persist in soil for an extended duration of up to six years, even in the absence of a suitable host (Haware et al., 1996). This resilient characteristic poses a significant challenge to the effective control of the pathogen. Given their nature as soil pathogens, managing these organisms through synthetic fungicides poses considerable challenges and is not applicable most of the time (Dubey et al., 2007). Furthermore, their colonization predominantly within the xylem and root cortex of the plant impedes effective chemical delivery to these specific regions. Despite the demonstrated efficacy of certain fungicides in managing this disease *in vivo*, it is imperative to acknowledge the potential risks associated with their practical application, particularly concerning human and environmental health (Jamil & Ashraf, 2020). Meanwhile, Fusarium wilt is primarily managed through the cultivation of resistant cultivars and the most cost-effective and environmentally friendly approach to control Fusarium wilt is the utilization of resistant cultivars, whenever they are accessible (Landa et al., 2007; Li et al., 2015; Bharadwaj et al., 2022; Yadav et al., 2023). However, it should be noted that the resistance, especially controlled by a single R gene, can be easily overcome by different *Foc* races.

The challenge of managing Fusarium wilt, along with similar soil-borne wilt diseases, has prompted researchers to investigate biological control methods mainly in the last decade (Soltanzadeh et al., 2016; Bubici et al., 2019; Kumari & Khanna, 2020; Poveda, 2021; Akbaba & Genc, 2024). Plant-associated endophytic fungi, constitute a biodiverse group of microorganisms typically present asymptomatically within plant tissues or intercellular spaces. It has been showed that endophytic fungi play a crucial role in promoting the growth of host plants by directly producing secondary metabolites, thereby enhancing the plants' resistance to both biotic and abiotic stresses (Phurailatpam & Mishra, 2020; Fontana et al., 2021; Lu et al., 2021; Chaudhary et al., 2022).

Serendipita indica (S. indica), formerly known as *Piriformospora indica* is a basidiomycete that colonizes plant roots as an endophyte and it has gained significant attention in recent decades due to its broad host range and the various beneficial effects on its host in terms of protection and growth support (Verma et al., 1998; Singhal et al., 2017; Saleem et al., 2022; Roylawar et al., 2023). *S. indica* can colonize a wide range of plants including mono- and dicotyledonous species and establish symbiotic associations with them (Qiang et al., 2012). *Serendipita indica* has a capability to suppress the immune system, which is crucial for its biotrophic root colonization (Daneshkhah et al., 2018). This feature may specifically account for its remarkably wide range of host compatibility (Jacobs et al., 2011). The most agriculturally significant advantages include promoting growth, increasing seed yield, and enhancing resistance or tolerance to both biotic and abiotic stresses (Singhal et al., 2017; Boorboori & Zhang, 2022; Saleem et al., 2022). *Serendipita indica* exhibits similarities to typical arbuscular mycorrhizal fungi (AMF) in terms of growth promotion, morphological features, and functional characteristics (Aslam et al., 2019).

Serendipita indica has proven to be successful against fungal pathogens including necrotrops such as *Botrytis cinerea* (Narayan et al., 2017) and *Fusarium culmorum* (Harrach et al., 2013), and biotrophs such as *Blumeria graminis* f. sp. *hordei* (Felle et al., 2009) and *Golovinomyces orontii* (Stein et al., 2008). However, it is noteworthy that a significant part of the studies is against soil-borne root rot or wilt diseases, especially caused by *Fusarium* species. Many studies from independent groups have shown

that colonization of tomato roots with *Serendipita* species showed strong potential to decrease the Fusarium wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* by enhancing the plant defense mostly (del Barrio-Duque et al., 2019; Ghezel Sefloo et al., 2019; Hallasgo et al., 2022). The findings of Cheng et al., (2020) suggested that *S. indica* colonization may enhance the resistance of banana to *Fusarium oxysporum* f. sp. *cubense FocTR4* which is undoubtedly the biggest threat to global banana production by regulating the activity of antioxidant enzymes. No direct effect was observed between *S.indica* and *FocTR4,* indicating that improved protection depends on indirect interaction rather than antagonistic effect. More recently, significant improvement in morphophysiological parameters of maize plants was reported with the application of *S. indica*-inoculated compared to non-inoculated plants under conditions of drought stress, *Fusarium proliferatum*, and combined of them. Moreover, inoculation with *S. indica* led to an increase in chlorophyll content, suggesting enhanced photosynthesis in maize plants (Noori et al., 2023).

In this study, it is aimed to test the plant protection activity of *S. indica* against Fusarium wilt disease, as well as plant growth promoting features in chickpea under growth chamber conditions. The colonization levels of both *Foc5* and *S. indica* in a time dependent manner was monitored. The study provides evidence that colonization of chickpea roots with *S. indica* prevents the colonization of *Foc* resulting the reduction in disease severity.

MATERIAL AND METHODS

Plant materials, *S. indica* inoculations and growth conditions

The wild-type chickpea (*Cicer arietinum* L.) genotype ´JG62´ seeds were kindly provided by Assoc. Prof. Dr. Abdullah Kahraman (Harran University, Türkiye). The seeds were surface sterilized by soaking 2% sodium hypochlorite for 3 min, followed by rinsing 5 times with sterile water. Seeds were then germinated between sterile filter paper layers moistened with 1 mM calcium sulfate solution in the dark conditions for 5 days at 25ºC.

Serendipita indica (IPAZ-11827) was kindly provided by Dr. Jafargholi Imani (Justus Liebig University Giessen, Research Centre for BioSystems, Land Use and Nutrition, Institute of Phytopathology, Giessen, Germany) and grown on PDA (potato dextrose agar) medium for 8-10 days at 25ºC. Mycelial plugs transferred to the PDB (potato dextrose broth) medium were further cultivated at 25ºC and 120 rpm. Chlamydospores were harvested from 7 days of cultures, washed three times with sterile water and adjusted to a final concentration of $1x10^5$ chlamydospore mL^{-1} in sterile water supplemented with Tween-20 (0.002%).

Roots of 5-day-old seedlings were inoculated with *S. indica* chlamydospores by the root dipping method, followed by the planting to the pots filled with soil:perlite:peat mixture (1:1:1 v/v/v). Plants were grown in walk-in growth chambers with a 16-hours light/8- hours dark photoperiod with 24±1ºC. Mock plants were treated with sterile water containing Tween-20.

Pathogen inoculation and disease assessment

Fusarium oxysporum f. sp. *ciceris* strain Foc5 was kindly provided by Prof. Dr. Canan Can (Gaziantep University, Türkiye) and cultured on PDA at 25ºC in the dark for 7 days. Mycelial plugs were transferred to the PDB medium for spore production and cultivated at 25ºC for 5 days under constant shaking at 120 rpm at dark conditions. The conidial suspension was filtered through several layers of cheesecloth, washed several times with sterile water and the concentration was adjusted to $1x10^7$ conidia mL^{-1}. One week after *S. indica* treatment, plants were uprooted and dipped into *Foc* spore suspension for 5 min. Inoculated plants were re-planted to the same pots. Control plants were treated with sterile water by using the same method.

The level of disease was scored based on the external symptoms such as wilting, yellowing and necrosis (0-4 scale) 25 days after inoculation: 0= no lesions and 1= 1 to 33%, 2= 34 to 66%, 3= 67–100 and 4= dead plants (Navas-Cortés et al., 1998). Demonstration of scale used in this study was given in Figure 1. Shoot fresh weight was also taken after disease assessment.

Figure 1. Disease scoring scale used for the assessment of *Fusarium oxysporum* f.sp. *ciceris* (race5) infection.

Şekil 2. *Fusarium oxysporum f.sp. ciceris (ırk5)'in enfeisyonunun değerlendirilmesinde kullanılan hastalık skorlama skalası.*

Quantification of *S. indica* and *F. oxysporum* f. sp. *ciceris* colonization by real-time PCR

The relative quantification of fungal colonization was done by calculating the ratio of either *S. indica* or *Foc* DNA to chickpea DNA. Plant roots were carefully harvested, washed several times gently with tap water to remove growth medium particles and flash-frozen in liquid nitrogen. Genomic DNA was isolated from roots by applying CTAB method described by Doyle & Doyle, (1987), and diluted to 20 ng μl^{-1}. qPCR was performed with primers specific for *S. indica Internal Transcribed Spacer* (*ITS*) gene, *Foc5 5.8 ribosomal DNA* (5.8 rDNA) gene or *C. arietinum* L. *Elongation factor 1*α (*Ef1*α) using PikoReal 96 real-time PCR system (Thermo Scientific, Burlington, Canada) and RealQ Plus 2x Master Mix Green without ROX (Ampliqon, Denmark) according to instructions from the manufacturer. Primer sequences used in this study as follows: *S. indica ITS* forward 5′-CAACACATGTGCACGTCGAT-3′ and *S. indica ITS* reverse 5′-CCAATGTGCATTCAGAACGA-3′ (Deshmukh et al., 2006), *Foc5 5.8 rDNA* forward 5′-GTTGAAATGACGCTCGAACA-3′ and *Foc5 5.8 rDNA* reverse 5′- GCCAGAGGACCCCTAAACTC-3′ (Chakraborty et al., 2020). Cycling conditions were 15 min at 95ºC followed by 35 cycles of 20 s at 95ºC, 30 s at 60ºC, 30 s at 72ºC.

In vitro confrontation assay for direct antifungal activity

The study conducted an *in vitro* assessment to investigate the potential antagonistic capability of our *S. indica* isolate against *Foc* using a dual culture assay (Sun et al., 2014). Mycelial plugs (6 mm diameter) of *S. indica* and *Foc* were obtained from the edges of 7-d-old actively growing colonies on PDA and were placed opposite to each other at the edge of 9 cm diameter sterile petri dishes containing PDA. Plates were incubated at 25ºC in the dark for 7 d.

Statistical analysis

The statistical analysis of the data was conducted utilizing GraphPad Prism version 9 software (GraphPad Software Inc., San Diego, CA, USA). Data points are presented as the mean values accompanied by standard error (SE). A minimum of 15 biological replicates were utilized for disease assessment and fresh growth analyses. Additionally, for biomass determination of *S. indica* and *Foc*, a

minimum of 5 biological replicates were used. The assessment of normality and homogeneity of variance was conducted utilizing the Shapiro-Wilk test. Subsequently, the comparison of mean values for the measured parameters was performed using Student's *t*-test.

RESULTS and DISCUSSION

The mechanism of action of most biological plant protection products is based on direct of the antimicrobial products on phytopathogens. Utilizing biocontrol agents that directly colonize plants and enhance plant defense represents a potentially more sustainable strategy, which may exhibit more resilience to external environmental influences. *S. indica* as a fungal endophyte, has gained significant attention recently due to its capability to colonize a wide range of plant species and enhance plant performance against diverse stress factors. Here, it is evaluated that the efficacy of *S. indica* in managing Fusarium wilt in chickpea, a particularly challenging task as Fusarium wilt invades the roots and vascular systems of the plants.

To verify and monitor the root colonization of *S. indica*, its DNA was quantified by qPCR. Relative biomass data indicated that *S. indica* successfully colonized chickpea roots and although no significant difference was observed between 7 and 14 days, the colonization ratio of *S. indica* increased over time (Figure 2). Successful colonization of chickpea roots by *S. indica* was also shown before by using histochemical analysis (Narayan et al., 2017).

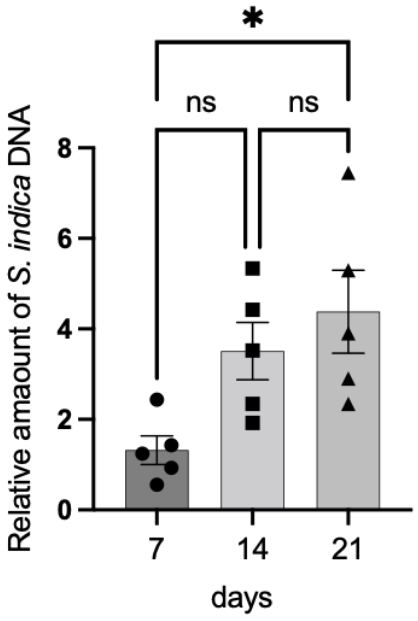

Figure 2. Relative amount of *Serendipita indica* DNA obtained from plant tissue.

Şekil 2. *Bitki dokusundan elde edilen Serendipita indica DNA'sının bağıl miktarı.*

Twenty-five days after pathogen inoculation, the disease symptoms of chickpea seedlings, inoculated either with *S. indica* or mock treatment, were assessed. It was found that plants pre-inoculated with *S. indica* exhibited significantly lower disease symptoms such as wilting and yellowing compared to those in the mock group (Figure 3A, 3C). Besides, *S. indica* contributed to the increase in average plant fresh weight. Similarly, plants pretreated with *S. indica* showed higher biomass than mock-treated plants in *Foc* inoculated groups (Figure 3B, 3C).

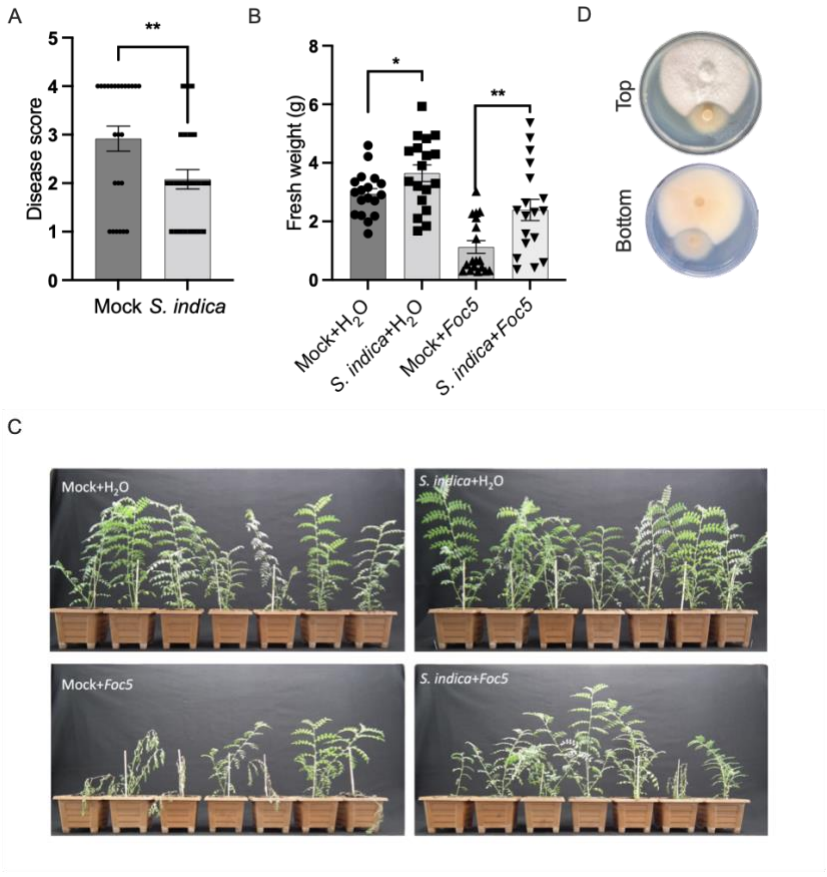

Figure 3. Disease score of mock and *S. indica* inoculated chickpea plants (A), Fresh weight of experimental groups (B), Representative pictures of the experimental groups (C), *In vitro* confrontation plate (D), (**P*<0.05, ***P*<0.01).

Şekil 3. *Mock ve S. indica ile aşılanmış nohut bitkilerinin hastalık skoru (A), Deney gruplarının yaş ağırlıkları (B), deney gruplarının temsili fotoğrafları (C), In vitro karşılaştırma testi (D), (*P<0.05, **P<0.01).*

The *Foc* colonization of chickpea roots was also monitored. The progression of Fusarium colonization was comparable in roots treated with either mock or *S. indica* after 7 days post-inoculation (dpi). However, the *Foc*'s progression was notably found suppressed by *S. indica* 14 and 21 dpi (Figure 4).

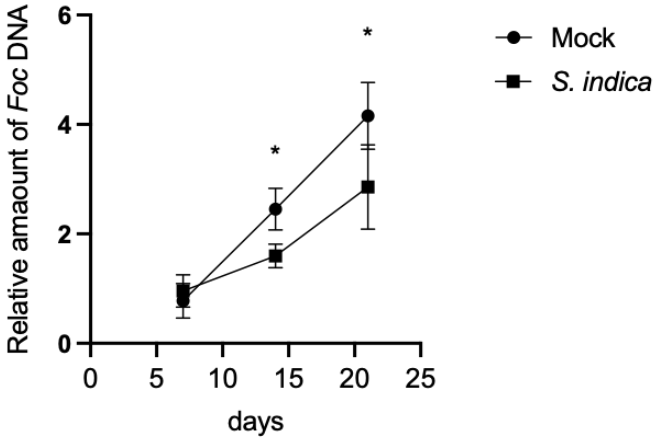

Figure 4. Relative amount of *Fusarium oxysporum* f.sp. *ciceris* (race5) (**P*<0.05)

Şekil 4. *Fusarium oxysporum f.sp. ciceris (ırk5)'in oransal miktarı (*P*<0.05)

Narayan et al. (2017) showed that chickpea plants colonized with *S. indica* exhibited significant increase in plant biomass and root development compared to control treated plants. A significant reduction in disease caused by necrotrophic pathogen *B. cinerea* was also observed in *S. indica* treated plants. In contrast, it has been reported that *S. indica* alone causes a decrease in dry plant biomass in the early period of colonization in chickpea, however when applied in combination with *Pseudomonas striata*, a phosphate-solubilizing bacterium, it has a positive effect on plant biomass (Meena et al., 2009). Furthermore, Combination of *S. indica* with *Mesorhizobium cicer* and *Pseudomonas* spp. has found to have a synergistic effect on bioaugmentation of chickpea productivity (Mansotra et al., 2015).

Serendipita indica does not exhibit a direct antagonistic effect on various Fusarium species such as *F.* oxysporum f. sp. *fragariae* (Huang et al., 2024), and *F. graminearum* (Deshmukh & Kogel, 2007; Li & Guo, 2022), *F. culmorum* (Rabiey et al., 2015). However, it has been previously shown that soil-borne pathogen *Verticillium dahliae* is inhibited by *S. indica in vitro* conditions (Sun et al., 2014). The interactions of *Foc* and *S. indica* were *examined in vitro* conditions using the dual culture method and a weak antagonistic effect against *Foc* was observed (Figure 3D) indicating that improved protection could be related with direct antagonism. *S. indica* delivers its effectors into host cells to manipulate host metabolism for successful colonization (Nizam et al., 2019). These effectors trigger several plant responses leading to enhanced defense against phytopathogens. Induced systemic resistance (ISR) is one of two well-established systemic defense response types of plants known to be mainly regulated by jasmonic acid and ethylene hormones (Pieterse et al., 2014). It has been demonstrated that various plant genes, including vegetative storage protein (VSP) (Stein et al., 2008) and 1-*aminocyclopropane-1-carboxylic acid oxidase* (*ACC oxidase*) (Schäfer et al., 2009), which are associated with the jasmonic acid, and ethylene-related pathways, respectively, exhibit upregulation during the *S. indica* symbiotic interaction, which indicates ISR could be a part of response following *S. indica* colonization. *S. indica* can trigger transcriptional regulation of antioxidant-related genes and/or the production of antioxidant enzymes such as peroxidase and superoxide dismutase (Nassimi & Taheri, 2017; Li & Guo, 2022). Based on these, it is speculated that improved protection against *Foc* in chickpea plants could also be related to the enhanced plant defense responses triggered by *S. indica*.

CONCLUSIONS

The findings reveal a distinct reduction in *Foc* colonization in chickpea inoculated with *S. indica* as compared to mock inoculation. This situation has been consistent with the plant fresh weight and disease scoring data. These results present preliminary evidence supporting the prospective application of *S. indica* fungus as a viable biological control agent against *Foc* in chickpea plants. This study contributes to the expanding knowledge base on the potential utilization of *S. indica* in enhancing resistance against specific pathogens in chickpea cultivation.

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Data Availability

Data will be made available upon reasonable request.

Author Contributions

Conception and design of the study: SP, EŞ; analysis and interpretation of data: SP, EŞ; statistical analysis: SP; visualization: SP, EŞ; writing manuscript: SP, EŞ.

Conflict of Interest

There is no conflict of interest between the authors in this study.

Ethical Statement

We declare that there is no need for an ethics committee for this research.

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