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Identification and Pathogenicity of the Soybean Root Rot Pathogen in Arid Conditions

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Abstract: Soy is an important oilseed crop in Kazakhstan. However, its yield is significantly reduced due to an increase in the development of fungal diseases with soil infection. This study aims to identify the root rot pathogen in soybean varieties in Western Kazakhstan and assess its pathogenicity. Samples of soybean roots to determine the root rot pathogen were selected at the experimental plots of the Aktobe agricultural experimental station in 2021. Genetic analysis of isolates observed on potato dextrose agar medium from soybean root slices was carried out using the sequencing reaction of internal transcribed spacer region nucleotide sequences using the Big Dye Terminator protocol (applied biosystems) with a further comparison of data with the Gen bank database. A test for the pathogenicity of the identified isolates of the fungus was carried out. Statistical data processing was carried out using the R-studio software according to the nonparametric Kruskal Wallis test. Phylogenetic analysis of samples of soybean affected by root rot showed that the main biotic agent causing root rot was the imperfect fungus *Fusarium equiseti*. Sequencing of the highly informative internal transcribed spacer region of fungal isolates allowed identifying strains 1 and 2 belonging to the species *Fusarium equiseti*. The pathogenicity test established that isolates of the *Fusarium equiseti* fungus caused discoloration of seedlings and plant roots and necrosis in soybean seedlings.

Keywords: *Fusarium equiseti*, ITS, Pathogenicity, Root Rot, Soybean

Introduction

Kazakhstan is an agrarian country with huge areas of land used for growing crops and animal husbandry (Kashina *et al*., 2022). Soy is one of the important agronomic crops attracting increasing attention in Kazakhstan, due to its growing demand as an oilseed crop and animal feed to ensure food security (Makulbekova *et al*., 2017; Kuldybayev *et al*., 2021; Suleimenova *et al*., 2021). The global experience of successful crop cultivation implies seasonal crop alternation, i.e., rotation with other grain or feed crops (Rodriguez *et al*., 2021; Bome *et al*., 2022), as well as the use of cover crops (Wen *et al*., 2017) to avoid the emergence of fungal and bacterial diseases transmitted through the soil. Fungal diseases, under favorable conditions, can significantly reduce the yields of important crops (Karakotov *et al*., 2020). Thus, in Brazil, the world leader in soybean production (121.8 million Metric Tons (MT), food and agriculture organization corporate statistical database (FAOSTAT, 2020), due to rust that affected 90% of the country's territory, crop losses in only two states in 2003 amounted to 2.2 million tons (Yorinori *et al*., 2005). In the USA, the average annual crop losses due to diseases are estimated at 11% of total production (Mueller *et al*., 2016; Dutbayev *et al*., 2006), where the soy nematode (heterodera glycines ichinohe) accounts for more than 30% of crop losses, which can occur without noticeable above ground symptoms (Mueller *et al*., 2016). Argentina, the third largest producer, and exporter with 6.9 million tons (Gale *et al*., 2019), is also significantly

affected by soil pathogens and especially oomycetes, which results in crop losses of up to 20% (Lerch-Olson and Robertson, 2020). Among the most common diseases, germination diseases, sclerotiniosis stem blight, and phytophthora root rot are the most characteristic ones of the top soybean producers in Canada (Lerch-Olson and Robertson, 2020) and China (Yang *et al*., 2020). In turn, *Fusarium* damping off and root rot, Alternaria leaf spot, seed rot, and anthracnose are more often mentioned in the soybean regions of Ukraine (Mykhalska *et al*., 2019), Russia (Kurilova, 2010) and India (Nataraj *et al*., 2020). The above-mentioned rust and charcoal rot are most often noted in Bolivia (Yorinori *et al*., 2005) and Paraguay (Orrego Fuente *et al*., 2009).

Soybeans experience biotic stress from extensive exposure to *Fusarium* pathogens affecting their seeds, seedlings, leaves, and roots (Bienapfl, 2011). Symptoms of soybean *Fusarium* all over the world are the death of the growth point, leaf spot, and bean and seed rot, but the most common ones are root rot and tracheomycosis related plant damping off (Kurilova, 2010).

The purpose of our study was to identify the root rot pathogen in European soybean varieties in Western Kazakhstan using modern Deoxyribonucleic Acid (DNA) sequencing techniques and to assess its pathogenicity.

Materials and Methods

Samples of soybean roots affected by root rot were taken at the Aktobe agricultural experimental station, in Western Kazakhstan (50°21′22.2″ N, 57°23′47.2″ E). Twelve soybean varieties from the Eurasian collections were examined for the presence of root rot: Samer 1, Samer 2, Samer 3, Samer 5, Svapa, Tanais, Belor (Russia), Toury (Czech Republic), Isidor (France), Maple Ridge (Canada), Anastasiya, Cheremosh (Ukraine). Root samples were taken during soybean ripening at the end of August 2021 to determine the impact of the disease on crop yields.

One hundred and twenty samples of soy roots of each variety with a size of 1 × 1 cm were collected randomly. To obtain the *Fusarium* fungus inoculate, these soybean samples were disinfected by washing in 70% ethanol for 10 sec, followed by immersion in a 1% solution of sodium hypochlorite for 3 min. According to the methods developed by the Kazakh institute of plant protection and quarantine, after three rinses in sterile water, the roots were placed in aseptic conditions in wet boxes on sterile pre-moistened filter paper. The wet boxes (plastic containers), in turn, were sterilized with a 95% ethanol solution. Under these conditions, the roots were incubated at 25°C in the dark until the fungal mycelium was well expressed. The formed conidia of the fungus were used to isolate the DNA. Pure *Fusarium* fungus cultures were obtained by re-seeding the initial culture with four varieties for 7-9 days on Potato Dextrose Agar (PDA) medium under thermostat conditions at a temperature of 25°C (Dutbayev *et al*., 2006).

Three-seven daily strains of fungi were used in the study. The mycelium was frozen at 20°C. Then it was ground with a pestle in a 1.5 mL Eppendorf test tube to a powdery state. After grinding, fungi samples with a pure culture were transferred for further sequencing. The DNA concentration was determined on a double strained DNA (dsDNA) High Sensitivity (HS) scale. After amplification, the resulting Polymerase Chain Reaction (PCR) product was purified. The universal primers of the fungi Internal Transcribed Spacer (ITS) region were used in the study.

Phylogenetic analysis of DNA sequences was performed using MEGA6 software to compare the regularities of the molecular evolution of genes through a graphical interface in real time (Tamura *et al*., 2013). The alignment of nucleotide sequences was carried out using the ClustalW algorithm as an operational generation of the tree and its sequential process (Li, 2003). The neighbor joining method was used to restore the correct phylogeny (Elias and Lagergren, 2005).

To understand the pathogenic nature of the isolated and identified *Fusarium* isolates, a pathogenicity test was conducted using three soybean varieties characterized by the lowest, average, and severe susceptibility to root rot (Cummings *et al*., 2018; Dutbayev *et al*., 2021).

Statistical data processing was carried out using the R studio software according to the nonparametric Kruskal Wallis test. The significance of the calculations was evaluated using the p-value (Aphalo, 2017).

Results and Discussion

Pathogenic fungi of the *Fusarium* genus are very widely represented in all cultivated plants of the agricultural sector. The morpho structural features of many groups are quite similar and cause some difficulty in their morphological identification (Dutbayev *et al*., 2021). The emerging need for rapid and accurate diagnosis of fungal species was successfully solved by molecular identification of nucleotide sequences based on PCR technology (Raja *et al*., 2017). In recent years, the diagnosis using the recombinant DNA (rDNA) ITS region has found the greatest use for this identification of fungi, as it determines the sequence of nucleotides in the segment between two large and small subunits of rRNA genes (Tedersoo *et al*., 2015), although according to Nilsson *et al*. (2019), the ITS method has several limitations, such as the presence of polymorphism and low-quality sequences. At the same time, there are also highly informative identification methods, such as Translation Elongation Factor 1-alpha (TEF-1α) and β-Tubulin (BT), which overcome the ITS limitations and are recommended as secondary barcoding for the vast majority of Ascomycota genera (Dutbayev *et al*., 2022). Nevertheless, Zhou *et al*. (2018) extracted 102

Fusarium isolates from infected soybean seedlings in Canada, of which 10 species of fungi of the *Fusarium* genus were identified благодаря the joint use of sequencing methods based on ITS and EF-1α regions. Pure cultures of *Fusarium* fungi were obtained after the incubation of soybean root samples on PDA under thermostat conditions for 9 days (Fig. 1).

DNA concentrations measured in ng/µl according to the Qubit 2.0 fluorimeter readings based on previously and further crushed isolate samples are shown in Table 1.

As a result of amplification with its primers, PCR products with a size of about 550 bp were obtained.

As a result of cleaning, we obtained the pronounced PCR products shown in Fig. 2 that corresponded to the required purity. The concentration of the purified product is shown in Table 2.

Based on the capillary foresis of the PCR product after the second purification, the clarity, and purity of the peaks in Fig. 3, indicate the absence of cross contamination of cultures and the good quality of the analysis.

A phylogenetic tree was constructed with the closest related strains (Fig. 4), which allowed taxonomic identification of the studied strains.

Nucleotide sequence of sample No. 1.

TACCTATACGTTGCCTCGGCGGATCAGCCCGC
GCCCTGTAAAAAGGGACGGCCCGCCCGAGGACCC
TAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAAC
AAACAAATAAATCAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCAAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACATTGCGCCCGCC
AGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
TCAACCCTCAAGCTCAGCTTGGTGTTGGGACTCG
CGGTAACCCGCGTTCCCCAAATCGATTGGCGGTC
ACGTCGAGCTTCCATAGCGTAGTAATCATACACC
TCGTTACTGGTAATCGTCGCGGCCACGCCGTAAA
ACCCCAACTTCTGAATGTTGACCTCGGATCAGGT
AGGAATACCCGCTGAACTTAAGCAT.

Taxonomic identification of strain 1 shows that isolate MK562068 *Fusarium equiseti* 09 is the closest related strain. The degree of homology was 100%, which shows that the studied strain can be attributed to this species.

Table 1: Fungal DNA concentration

Item no.	Sample name	Concentration in ng/mL
1	No. 1	110.0
2	No. 2	118.0

Table 2. PCR product concentration after purification

Item no.	Sample name	Concentration in ng/mL
1	No. 1	71.2
2	No. 2	102.0

Fig. 1: *Fusarium* genus fungus mycelium is grown on a PDA medium

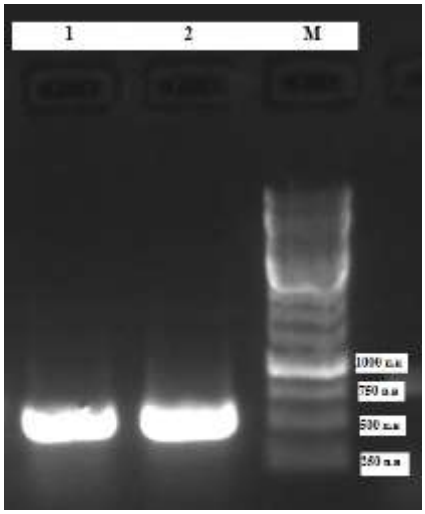

Fig. 2: PCR product after DNA amplification with ITS primers

Nucleotide sequence of sample No. 2.

TACCTATACGTTGCCTCGGCGGATCAGCCCG
CGCCCTGTAAAAAGGGACGGCCCGCCCGAGGAC
CCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAA
AACAAACAAATAAATCAAAACTTTCAACAACGG
ATCTCTTGGTTCTGGCATCGATGAAGAACGCAG
CAAAATGCGATAAGTAATGTGAATTGCAGAATT
CAGTGAATCATCGAATCTTTGAACGCACATTGC
GCCCGCCAGTATTCTGGCGGGCATGCCTGTTCG
AGCGTCATTTCAACCCTCAAGCTCAGCTTGGTG
TTGGGACTCGCGGTAACCCGCGTTCCCCAAAT
CGATTGGCGGTCACGTCGAGCTTCCATAGCGT
AGTAATCATACACCTCGTTACTGGTAATCGTCG
CGGCCACGCCGTAAAACCCCAACTTCTGAATG
TTGACCTCGGATCAGGTAGGAATACCCGCTGA
ACTTAAGCAT.

Taxonomic identification of strain 2 (Fig. 5) shows that isolate MK562068 *Fusarium equiseti* 09 is the closest related strain. The degree of homology was 100%, which shows that the studied strain can be attributed to this species.

Another representative of this genus was also identified in the studied mixture of *Fusarium sp* fungus isolate.

GAGGTCACATTCAGAAGTTGGGGGTTTAACG
GGTTGGCCGCGCCGCGTTCCAGTTGCGAGGGGT
TAGCTACTACGCAATGGAGGCTGCAGCGAGACC
GCCACTAGATTTCGGGGCCGGGCTGGAGTACAG
CCCGATCCCCAACACCAAACCCGGGGGCTTGAG
GGTTGAAATGACGCTCGAACAGGCATGCCCGCC
AGAATACTGGTGGGCGCAATGTGCGTTCAAAGA
TTCCATGATTCACTGAATTCTGCAATTCACATTA
CTTATCGCATTTTGCTGCGTTCTTCATCGATGCC
AGAACCAAGAGATCCGTTGTTGAAAGTTTTGAT
TTATTTGTTTTTTAGACTCACAAGTTACACTAAA
ATCAGAGTTTTGTGGTTCCTGCGGCGGGCCGTCC
CGTTTTACGGGGCGCGGGCTGATCCGCCGAGGC
AACATAAAGGTATGTTCACAGGGGTTTGGGAGT
TGTAAACTC.

According to Fig. 6, it can be determined that MG267121.1:25-497 *Fusarium brachygibbosum* is the closest related strain of the species. The degree of homology was 100%, which shows that the studied strain can be attributed to this species.

Simultaneously isolated isolate of the fungus *Fusarium brachygibbosum* is characterized by a similar white color and abundant aerial mycelium, like *Fusarium equiseti*. However, it differs from the latter by the presence of red pigmentation in the middle of the isolated colony. In turn, the central part of the mycelium in isolates of *Fusarium equiseti* 09 was predominantly light yellow.

A similar coloration of *Fusarium brachygibbosum* isolates was previously noted by Wang *et al*. (2021).

In the process of field and laboratory studies, based on the visual symptoms of the roots and the presence of a colony of fungi during their initial isolation, we determined that the varieties Maple Ridge, Samer 5, and cheremosh were more often susceptible to fungal diseases. In the varieties as Samer 1, Svapa, and Toury, the lowest fungal presence was detected, thus characterizing the latter as the most resistant to pathogens.

An earlier study conducted in the Caspian water area by Khapilina *et al*. (2011) isolated from the soil and identified an isolate of the *F. sporotrichioides* species by using the Integrated Genome Sizing (IGS) sequencing method of the DNA region. At the same time, in the Almaty region, the work of Ignatova *et al*. (2013) showed the common presence of *Fusarium* fungi such as *F. equiseti* and *F. oxysporum* in the soil of such crops as *alfalfa*, *rapeseed*, *esparcet*, mille, and soybean. In general, the southeastern region, known as favorable for soybean growth, is characterized not only by the presence of the representatives of *Fusarium*, *Penicillium*, and *Aspergillus* genera but also by pathogens such as *Cercospora sojina*, *Cercospora kikuchii* and *Septoria glycines* (Zatybekov *et al*., 2018). Soybeans, which grow mainly in the warm southern regions, are now actively cultivated in the northern and eastern regions of Kazakhstan, representing the most adapted varieties with a short growing season (Abugalieva *et al*., 2016). There is practically no information on these or other pathogens typical for legumes from western Kazakhstan.

In the last decade, the technique of DNA sequencing by the ITS region has been widely used in phytopathology as a relatively fast and reliable method for the procedure of identifying microorganisms (Parikh *et al*., 2018). Thanks to this technology, the obtained nucleotide sequence of isolates according to the comparisons made with the Gen bank database and phylogenetic tree alignments allowed us to identify the isolate as *Fusarium equiseti*.

Determination of the infectious nature of diseases in leguminous crops is of paramount importance in Kazakhstan as an initial level of pathogen control.

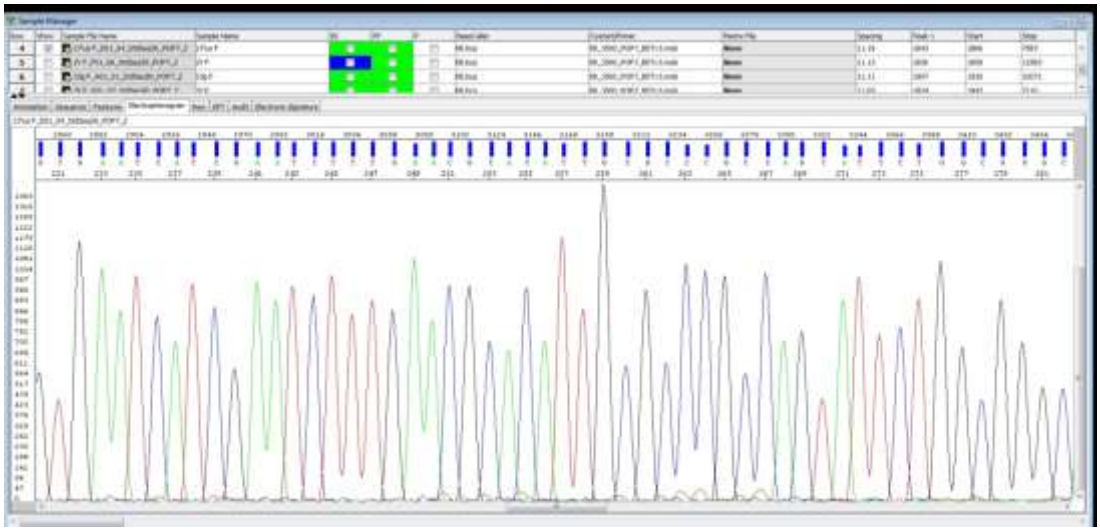

Fig. 3: An electropherogram of capillary phoresis during the fungal sequencing

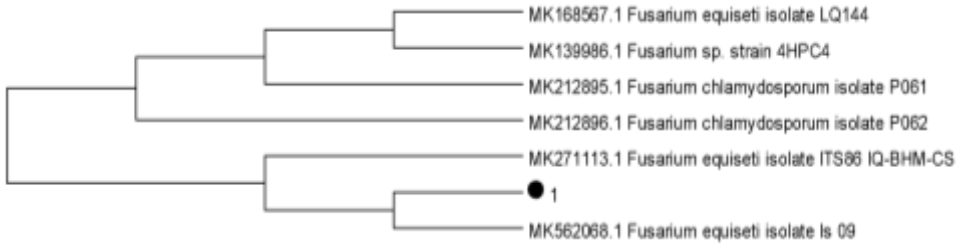

Fig. 4: The phylogenetic tree of sample No. 1

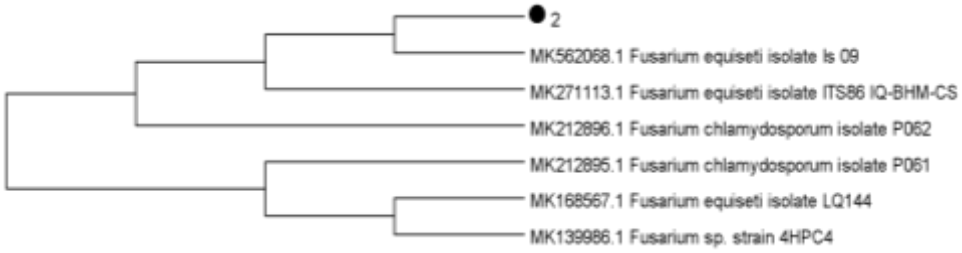

Fig. 5: The phylogenetic tree of sample No. 2

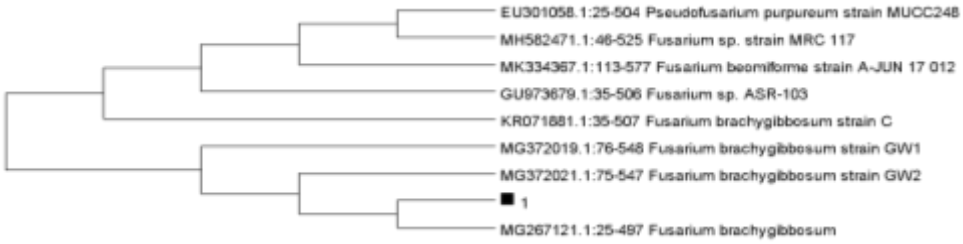

Fig. 6: The phylogenetic tree of *Fusarium brachygibbosum* isolate sample

In the last decade, the use of genetic methods at the sites of the southeastern, eastern, and northern regions of Kazakhstan has allowed Kazakh specialists to genotype this material, including for the identification of DNA markers of resistance to certain fungal diseases, identifying genes or Quantitative Trait Loci (QTL) associated with disease resistance, which is one of the most effective preventive measures to overcome biotic dependence. In this case, they identified the QTL associated with soybean productivity and resistance to *Fusarium*, cercosporosis, *septoria*, and *purpurea* cercosporosis (Zatybekov *et al*., 2018). Moreover, to adapt the culture to the arid conditions of western Kazakhstan, the influence of some species of fungi of the *Fusarium* genus on photosynthesis, plant productivity, and appropriate measures to combat pathogens was clarified (Kuldybayev *et al*., 2021). Works confirming the positive effect of a mixture of fungi or a combination of fungal and bacterial isolates have acquired special ecological significance as measures to combat such soybean diseases as root and stem rot (Chang *et al*., 2018; Zhang *et al*., 2013). This fact of preference for the use of biocontrol agents in the regulation of pathogenic organisms in both soybeans and other crops is beyond doubt. This is especially relevant due to the extreme limitations of the studies conducted on legumes in Kazakhstan concerning the adaptation of the crop to various environmental conditions of the country against the background of its increasing export and internal demand from year to year (Makulbekova *et al*., 2017).

According to the pathogenicity test and Koch's Postulate, repeated isolation and identification of the isolate, *F. equiseti* 09 caused discoloration of seedlings and plant roots and necrosis (Table 3). The highest values of the seedlings damping off, the spread, and the development of the disease was noted in the Maple Ridge variety (80.0, 84.0, and 71.0%, respectively) corresponding to a susceptible variety to *Fusarium* root rot, and the lowest values were noted in the Toury variety (15.0, 23.0 and 14.0%, respectively). The Toury variety is classified as resistant to root rot. In turn, the symptoms of the disease did not appear on the control soybean seedlings. Summarizing the study, it can be assumed that the *F. equiseti* 09 isolate is of soil origin. However, this conclusion can be fully confirmed only after an infectious analysis of the seeds of the culture.

Table 3: A test for the pathogenicity of *Fusarium* isolates according to the indicators of seedlings damping off, the development and spread of root rot symptoms in seedlings of three soybean varieties

	Seedling damping-off, %			Spread, %			Development, %		
Isolate	Maple ridge	Samer 2	Toury	Maple ridge	Samer 2	Toury	Maple ridge	Samer 2	Toury
F. equiseti 09	80.00	49.00	15.0	84.00	52.00	23.00	71.00	38.00	14.00
Control (without infection)	0.00	0.00	0.0	0.00	0.00	0.00	0.00	0.00	0.00
P-value	<0.01	<0.01	<0001.0	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

It was previously reported (Chittem *et al*., 2015) in the study of field pea roots that *F. equiseti* (6.5%) and *F. redolens* (6.8%) were weakly pathogenic species. In turn, in the work of Japanese experts (Horinouchi *et al*., 2007), it was noted that *Fusarium equiseti* was the most effective organism for controlling and reducing the stem and root rot of tomatoes caused by *Fusarium oxysporum f. sp. radicis-lycopersici*. Thus, as a result of the pathogenicity test, it was found that isolates of the *Fusarium equiseti* fungus could cause discoloration of seedlings and plant roots and necrosis in soybean seedlings.

Conclusion

The purpose of our study was to identify the root rot pathogen in soybean varieties in Western Kazakhstan and assess its pathogenicity. Sequencing of its region of fungi isolated from soybean roots identified strains 1 and 2 belonging to the *Fusarium equiseti* species. The presence of *Fusarium brachygibbosum* fungus was also detected in small concentrations. As a result of the pathogenicity test, it was found that isolates of the *Fusarium equiseti* fungus could cause discoloration of seedlings and plant roots and necrosis in soybean seedlings.

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Author's Contributions

All authors equally contributed to this study.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

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