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Original article

Investigation of the Effectiveness of Inoculation Methods of *Macrophomina Phaseolina*, the Causative Agent of Coal Rot Disease, in Soybean

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Abstract

Although soybean is affected by many fungal diseases from seed to harvest, charcoal rot caused by *Macrophomina phaseolina* is one of the most important diseases of soybean. It is important to grow resistant varieties because microsclerots can remain alive in the soil for many years, and there is currently no effective chemical control. Currently, many inoculation methods are used to determine the resistance of soybean plants to *M. phaseolina*; however, their effectiveness under field conditions has not been fully determined. In this study, soybean plants were inoculated with *M. phaseolina* during the flowering period using agar-disc, toothpick, and microsclerot injection methods, and the efficiency of these methods was investigated. In the disease observations made close to harvest, the disease index value was 3.3 in the agar disk method, 1.9 in the toothpick method and 1.3 in the microsclerot method. The results of the study showed that the symptoms in all methods were similar to those in field conditions, the agent could be transmitted by seeds and had an effect on seed quality, and the agar-disc method was the most successful method among the methods used to inoculate soybean plants with *M. phaseolina* under field conditions.

Keywords: Soybean, Disease, Macrophomina, Charcoal rot, Resistance.

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INTRODUCTION

Soybeans are an ancient plant species that have been cultivated by humans for thousands of years. They are considered one of the oldest crop species and are known for their cultural significance. In Turkey, soybean cultivation began during the First World War and was initially grown as a secondary crop in the Black Sea region (Ilisulu, 1983). Later, during the 1968-1970 period, soybeans were also started to be grown as a secondary crop in the Aegean and Mediterranean regions (Deniz, 1988). Soybeans (Glycine max. Merr.) are a legume that belongs to the family Fabaceae and are an annual crop that produces a single annual yield of oil. They contain high levels of protein (36-40%) and 18-24% oil, 26% carbohydrates, and 18% mineral elements. Their reputation as a "wonderful plants " is due to the richness of their nutrient content (Arıoğlu, 2007). Additionally, soybean contains a compound called "genistein," which has been reported to inhibit prostate cancer growth (Anonim, 2002). Soybean oil contains significant amounts of calcium, iron, zinc minerals, and vitamins A, B1, B2, C, D, E, and K, making soy products an important part of both human and animal nutrition (Arıoğlu, 2007). Soybeans can be grown in soil that has a high demand for nutrients and is not suitable for growing wheat or cotton. Soybean farming improves soil fertility by increasing the organic matter content of the soil, which leads to better soil structure. Soil pH is very important for soybeans and should be between 6.2 and 6.8. When the pH of the soil is lower than optimal, it can lead to nodule formation problems, and the excessive intake of molybdenum, which is required for nodule formation, can be harmful. Additionally, when the pH is too high, deficiencies in copper, zinc, and iron can be observed (Arıoğlu, 1999).

The *M. phaseolina* fungus, which resides in the soil and causes root rot, is a significant source of damage in terms of crop yield. *Macrophomina phaseolina* (Tassi) Goid. is a fungus that has a very wide host range and can cause root rot in more than 500 plant species, including cultural crops and weeds. The economic losses caused by this fungus are significant (Hussain et al., 1990). The first report of this disease in Turkey was in 1942, and since then, the fungus has been isolated from soil, sugar beets (Yorgancı & Turhan, 1988), peppers (Yıldız, 1989), tomatoes (Arca & Yıldız, 1990), carrots (Karcılıoğlu et al., 1990), soybeans (Karcılıoğlu & Yıldız, 1991), cotton (Yücel & Güncü, 1991), eggplants (Onan et al., 1992), beans (Temizel & Ertunç, 1992), chickpeas (Tezcan & Yıldız, 1993), and sunflowers (Tezcan et al., 1994). In addition, the fungus has been isolated from beans (Maden & İren, 1984), sugar beets (Esentepe et al., 1985), and cotton (Maden, 1987) seeds. The fungus is more prevalent in warm, tropical, and subtropical regions, where it thrives in areas with high temperatures and low rainfall, and it is known to spread unfavorable conditions in the soil by causing microsclerotia. *M. phaseolina* can cause diseases such as root and stem rot, which can lead to symptoms such as brown lesions on the stems and pods, as well as wilting and chlorosis. Such symptoms may not appear until the latter stages of the growth cycle of the host plant, which can develop normally until the first signs of infection appear. In some cases, the infected plants may not exhibit any symptoms after flowering. The

rapid growth of the disease can be attributed to the structure of the host plant, soil conditions, and climatic factors, with the latter having a significant impact on the development of symptoms (Karaca, 1974). High soil temperature, low humidity, and unfavorable environmental conditions can cause stress in plants infected with *M. phaseolina*, resulting in more severe disease symptoms. Infections can reach their maximum level after flowering, in the post-flowering period, when the stress caused by drought conditions is at its peak. The use of excessive irrigation and fertilization, as well as the presence of biotic and abiotic stress sources such as insects or mechanical damage, can contribute to the development of the disease. The pathogen can infect plants within a wide temperature range of 20–35°C depending on the soil moisture conditions of the host plant (Diourte et al., 1995).

Inoculation methods used for pathogenicity and population screening studies under field conditions include toothpick, stem-tape, and cut-stem inoculation techniques, some of which have been widely applied to a wide range of crops (Zazzerini & Tosi, 1989). 1998; Twizeyimana et al., 2012). The cut-stem inoculation method is widely applied to members of the Fabaceae family, and the stem-tape method can be applied to crops with thick stems, such as sunflowers. The toothpick inoculation method is not limited to any particular plant part or plant species but is usually used on plants near the end of vegetative development (Avilés, 2008; Pickel, 2020). In this study, in addition to the toothpick method, which has been reported in the literature and reported to be successful, agar disk application under the bark and microsclerot injection into the stem, which have not been previously reported for *M. phaseolina*, were used for the first time in this study. Although many studies have been conducted under controlled conditions, this study was conducted under field conditions. Therefore, this study aimed to standardize inoculation techniques for soybean plant screening under field conditions.

MATERIALS AND METHODS

Isolation and identification of *M. phaseolina*

To identify the disease agent, survey studies were carried out in soybean cultivation areas in Hatay and Adana provinces during the September 2021 soybean growing season. Root collar, stem and seed samples were collected from plants in the field that showed symptoms of charcoal rot disease such as stunting, yellowing, graying and necrosis and black spots in the stem. Root and stem parts brought from the field were washed thoroughly under tap water, and soil residues were removed and dried using paper towels. PDA (Potato Dextrose Agar (Merck) medium was used to isolate *M. phaseolina* from soybean plants (Seifert, 1996). Plant parts were cut into 6-7 mm tissue pieces from the root, root collar and vascular tissues, and the seeds were used whole after surface disinfection. Plant parts and seeds were surface-disinfected with sodium hypochlorite (1%) solution and 4-5 seeds were transferred to each petri dish. After 5 days of incubation at room temperature, the fungal colonies were purified and species identification of *M. phaseolina* was carried out using the diagnostic criteria proposed by Booth (1977);

Gerlach & Nirenberg (1982) and Malone & Muskette (1964) (Figure 1). All isolates were stored as agar disks and microsclerot suspensions in a 15% glycerol solution at +4°C until use.

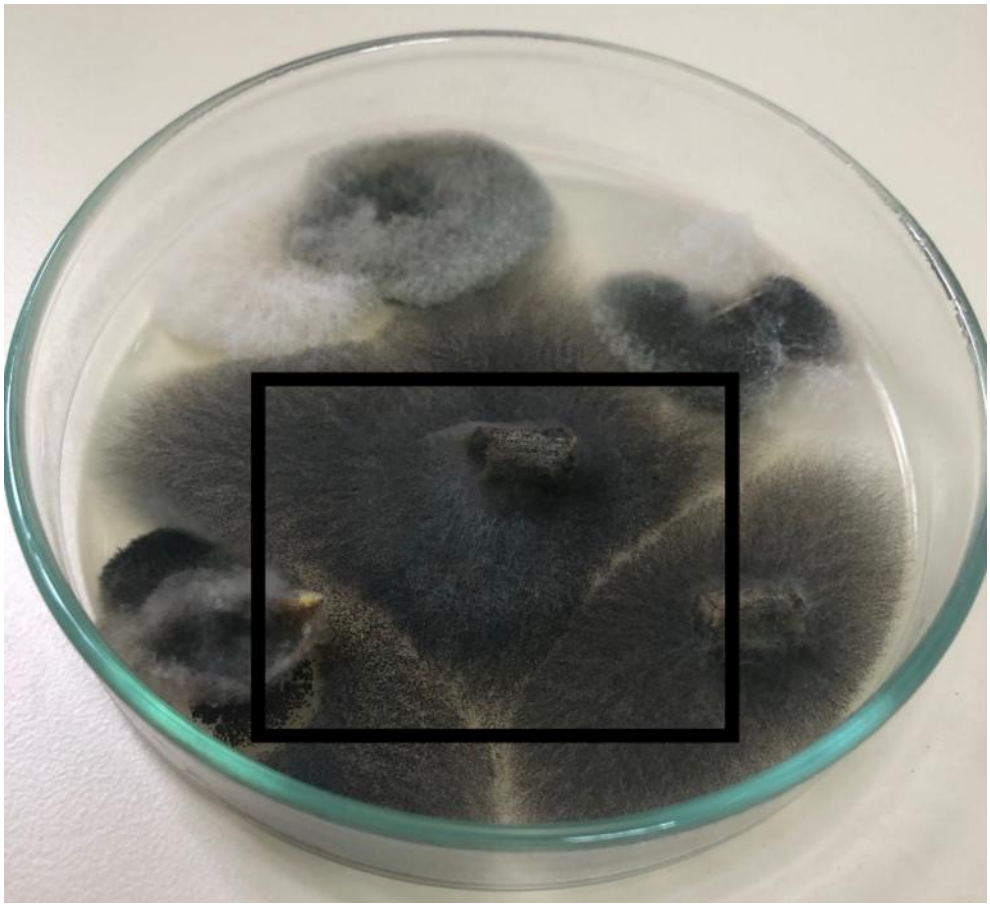

Figure 1. *M. phaseolina* colonies growing on diseased plant parts.

Inoculation methods

Inoculation was carried out under field conditions and at the beginning of soybean flowering in 2020. The experiments were conducted in the testing field of Progen A.Ş. located in the Melekli neighborhood in the Antakya district of Hatay Province (Figure 2).

Figure 2. Testing site of Progen A.Ş. (Melekli/Antakya), where the experiments were conducted.

Agar-disc inoculation method

For this purpose, the isolate, identified as M. phaseolina, was grown on PDA medium for 7 days and prepared for inoculation. When the soybean plants were in the flowering period, the bark tissue was cut at an angle perpendicular to the ground under the first internode and a 5 mm diameter agar disk taken from M. phaseolina culture was transferred under the bark. The bark was then resealed and covered with parafilm and 4 days after inoculation, the parafilm was removed and the tissues were allowed to come into contact with air.

Toothpick inoculation method

Toothpick inoculation was performed as recommended by Cohen et al. (2016). Wooden toothpicks were soaked in water for 24 h, dried slightly on a paper towel, and sterilized in an autoclave at 121°C for 30 min. After cooling, they were placed on M. phaseolina culture for one week to allow the fungus to colonize the toothpicks. The toothpicks colonized by the fungus were transferred to sterile drying sheets, allowed to dry, and inoculated by stabbing the stem under the first node of soybean plants.

Stem-injection inoculation method

Stem injection into soybean plants was performed by modifying the method proposed by Akışcan and Tok (2019). For this purpose, sterile pure water was added to the 7-day-old pure M. phaseolina culture, and the microsclerots were allowed to pass into the water by scraping with a spatula. The

suspension was then filtered through four layers of cheesecloth, and mycelium residues were removed. The density of the resulting suspension was measured with a hemocytometer and adjusted such that the microsclerot density was 10^2. The resulting microsclerot suspension was inoculated immediately below the first node of flowering soybean. The inoculation point was first pierced with a nail in two different parts of the plant, and then the suspension was injected into the plant tissue using a subcutaneous needle.

Evaluation of disease severity and statistical analysis

Near the harvest, the disease index of the plants was measured using a scale. Accordingly, 0: no disease symptoms; 1: lesion development up to 24% of the plant; 2: lesion development up to 25-50% of the plant; 3: lesion development up to 51-75% of the plant; 4: more than 75% of the plant covered with lesions, dead, or dying plants. Disease index values were subjected to analysis of variance and Duncan's multiple comparison test using the JMP statistical package.

RESULTS and DISCUSSION

Observations were made close to harvest, and disease index values were calculated. In general, it was observed that all inoculation methods caused disease symptoms, and no disease symptoms were observed in the control plants used in the experiment. The agar-disc method yielded the earliest and the most homogeneous symptoms. The method in which the microsclerot suspension is injected into the stem results in the latest symptoms. In all inoculation methods used in this study, yellowing and stunting of the leaves, development of a lesion starting from the inoculation point and progressing towards the upper parts of the plant, initially in the form of a line and then expanding, gray coloration of the plant stem, black microsclerot formation in gray areas in the later stages (Figure 3), and finally microsclerot formation on the stem section (Figure 4). All symptoms were consistent with those of charcoal rot disease under field conditions. In addition, seeds from inoculated and non-inoculated soybean plants were harvested manually, and it was found that seeds from inoculated plants had black lesions and reduced seed quality (Figure 5). Re-isolations were made from the inoculated plants, and M. phaseolina was re-isolated from these plants, whereas no pathogen was isolated from the control plants.

Figure 3. Gray areas indicate plants and microsclerots on the stems of infected soybeans.

Figure 4. Microsclerots of *M. phaseolina* in the stem tissues of infected soybean plants.

Figure 5. Lesions and quality loss in seeds obtained from infected soybean plants.

Although all disease symptoms were the same across the methods used, disease severity varied. Accordingly, the average disease index for 1.3 microsclerot injection method, 1.9 in toothpick method and 3.3 in agar-disk method (Table 1). The agar-disc inoculation method was again the method in which disease symptoms appeared first. In the analysis of variance using JMP statistical package program, it was determined that there was a significant difference between the methods (P<0,001).

Table 1. Inoculation methods and the average disease index they produced

Method	Average Disease Index*
Agar-disk Method	3.3 a
Toothpick Method	1.9 b
Microsclerot Injection Method	1.3 c

*Values with different letters are statistically different according to Duncan's multiple comparison test.

The cut stem and toothpick inoculation methods are among the most commonly used inoculation methods in pathogenicity studies under field and greenhouse conditions in various plant species (Zazzerini & Tosi, 1989; Pratt et al., 1998; Twizeyimana et al. 2012; Viteri & Linares, 2017). There is no satisfactory information regarding the applicability of inoculation methods other than cut-stem

inoculation under controlled environmental conditions. Furthermore, the stem-band inoculation method is considered a difficult method for screening genotypes against diseases. The toothpick inoculation method also has some practical difficulties due to the inability of seedlings to form a thick stem during the vegetative development stage. Therefore, the toothpick inoculation method is not suitable for rapid screening studies on soybean and sunflower seedlings under weak plant conditions, such as in a climate chamber. The toothpick inoculation method is more commonly used for pathogenicity studies on 6–8-week-old mature plants under greenhouse or field conditions (Pratt et al. 1998; Avilés et al. 2008). Although many inoculation methods have been used in soybean plants worldwide, the injection of a microsclerot suspension into stem tissues was used for the first time in this study. In addition, the application of the agar disk method under the bark was also used for the first time in this study. Based on the results of this study, it was determined that the agar-disc method is suitable for pathogenicity studies conducted under field conditions for the selection of soybean plants resistant to charcoal rot disease caused by *M. phaseolina*.

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