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M.Sc. in Biology

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DETERMINATION OF SUNFLOWER (*Helianthus annuus*) AND SOYBEAN (*Glycine max*) VARIETY REACTIONS TO *Macrophomina phaseolina* AND CHARACTERIZATION OF THE AGENT

M.Sc. THESIS

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July 2020

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Talap TALAPOV

ABSTRACT

DETERMINATION OF SUNFLOWER (*Helianthus annuus*) VE SOYBEAN (*Glycine max*) VARIETY REACTIONS TO *Macrophomina phaseolina* AND CHARACTERIZATION OF THE AGENT

TALAPOV, Talap
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Macrophomina phaseolina is a polyphagous phytopathogenic fungus and it can cause serious yield losses on economically important crop plants and the most appropriate control measure is utilization of resistance sources. To achieve this, an efficient, fast, and reliable screening method is prerequisite. The aim of this study was to apply widely used inoculation methods and test effect of inoculum maturity on disease development under fully controlled conditions and to define the phylogenetic analysis of *M. phaseolina* population collected from maize, sunflower and soybean. Fastest disease development on sunflower was observed for cut-stem and stem-tape inoculation methods applied using young hyphal inoculum and both applications had high repeatability rate. However, cut-stem can be applied only for pathogenicity studies due to high predisposition of sunflower seedlings after application of cut-stem inoculation method while opposite situation was observed for stem-tape inoculation method. Cut-stem inoculation technique was proven to be repeatable method for pathogenicity and variety reactions studies on soybean. The 11 TR 077 variety was found moderately resistant among sunflower cultivars tested and no complete resistance was observed for soybean varieties. phylogenetic analysis of ITS region formed 3 clusters with 1 cluster containing only sunflower originated isolates, 1 cluster containing only maize originated isolates and 1 cluster comprised of soybean and maize isolates.

Key Words: *Macrophomina phaseolina*, Phylogenetic Analysis, Inoculation Methods, Variety Reactions, Pathogenicity.

ÖZET

AYÇİÇEĞİ (*Helianthus annuus*) VE SOYA FASULYESİ (Glycine max) ÇEŞİTLERİNİN KÖMÜR ÇÜRÜKLÜĞÜ ETMENİ *Macrophomina phaseolina* REAKSİYONLARININ BELİRLENMESİ VE ETMENİN KARAKTERİZASYONU

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Macrophomina phaseolina, polifag bir fitopatojenik fungustur ve ekonomik açıdan önemli tarım bitkilerinde ciddi verim kayıplarına neden olabilir ve hastalığı kontrol etmek için en uygun çözüm direnç kaynaklarının kullanılmasıdır. Bunu başarmak için hızlı ve güvenilir bir tarama yöntemi ön şarttır. Bu çalışmanın amacı, tam kontrollü koşullar altında yaygın olarak kullanılan inokülasyon yöntemlerini uygulamak ve inokulum olgunluğunun hastalık gelişimi üzerindeki etkisini araştırmak ve mısır, ayçiçeği ve soya fasulyesinden toplanan *M. phaseolina* popülasyonunun filogenetik analizini gerçekleştirmektir. Ayçiçeği üzerinde en hızlı hastalık gelişimi, genç hifal inokulum kullanılarak uygulanan gövde-kesiği ve gövde-bandı inokülasyon yöntemleri için gözlenmiştir ve her iki uygulamada da tekrarlanabilirlik oranı yüksektir. Bununla birlikte, gövde-kesiği inokülasyon yönteminin uygulanmasından sonra ayçiçeği fidelerinin hastalığa yüksek yatkınlığına bağlı olarak patojenisite çalışmaları için uygulanabilirken, gövde-bandı inokülasyon yöntemi için zıt bir durum gözlenmiştir. Gövde-kesiği inokülasyon tekniğinin, soya fasulyesinde patojenite ve çeşit reaksiyonları için tekrarlanabilir bir yöntem olduğu kanıtlanmıştır. Test edilen ayçiçeği çeşitleri arasında 11 TR 077 çeşidi orta derecede dirençli bulunmuştur ve soya fasulyesi çeşitleri için tam bir direnç gözlenmemiştir. ITS bölgesinin filogenetik analizi, ayçiçeği ve mısır kaynaklı izolatlar içeren 2 ayrı küme ve soya fasulyesi ve mısır izolatlarından oluşan 1 küme ile 3 küme oluşturmuştur.

Anahtar Kelimeler: *Macrophomina phaseolina*, Filogenetik Analiz, İnokülasyon Metotları, Çeşit Reaksiyonları, Patojenisite

"Dedicated to my family"

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LIST OF ABBREVIATIONS

AUDPC	Area Under Disease Progression Curve
VCG	Vegetative Compatibility Group
ITS	Internal Transcribed Spacer
PDB	Potato Dextrose Broth
PDA	Potato Dextrose Agar
CTAB	Cetyl trimethylammonium bromide
EDTA	Ethylene diamine tetra acetic acid
TE	Tris-base EDTA
EtBr	Ethidium Bromide
DAI	Days After Inoculation
SPSS	Statistical Package for Social Sciences
PCR	Polymerase Chain Reaction
µl	Microliter
mM	Millimolar
µm	Micrometer
bp	Base pair
kb	Kilobase

CHAPTER I

INTRODUCTION

Oilseed plants are one of the most important crops because of its oil content that can be used for nutritional and industrial purposes. Oil and protein content of oilseeds are between 18-45% and 16-40% respectively and depends on crop and cultivar (González-Pérez and Arellano, 2009; Ahmad, 2017). As the main product is the seed meal left after extraction of the oil, the rest can be used as food source for human and animals because of high protein and fiber content. However, some oilseed crops such as soybean are grown for their seed meal and other components and the oil obtained during extraction process is just a byproduct of the procedure (Mailer, 2016).

Table 1.1 Production quantity of oilseed crops

Crop	Yield (ton)	Percentage %
1. Soybeans	352,643,548	34.68**%**
2. Oil palm fruit	317,571,421	31.23**%**
3. Rapeseed	76,238,340	7.5**%**
4. Cotton seed	74,352,809	7.31**%**
5. Coconuts	60,773,435	5.98**%**
6. Sunflower seed	47,863,077	4.71**%**
7. Groundnuts, with shell	47,097,498	4.63**%**
8. Olives	20,872,788	2.05**%**
9. Sesame seed	5,531,948	0.54**%**
10. Oilseeds nest	4,744,203	0.47**%**
Others	9,274,957	0.91**%**

Benefits like various irrigation options, high input into economy and raw material for ecofriendly fuels are being supported by advantage of some members from Fabaceae family like soybean and groundnut to soil community by the way of symbiotic nitrogen fixation and diverse in oil composition of oilseed crops (Ahmad, 2017; Mailer, 2016; Singh, 2006). According to FAOSTAT data, over 1.016 billion tons of oilseed was produced all over the world in 2017 and 99 % of the produced oilseed crops composes first 10 most produced crops (Table 1.1).

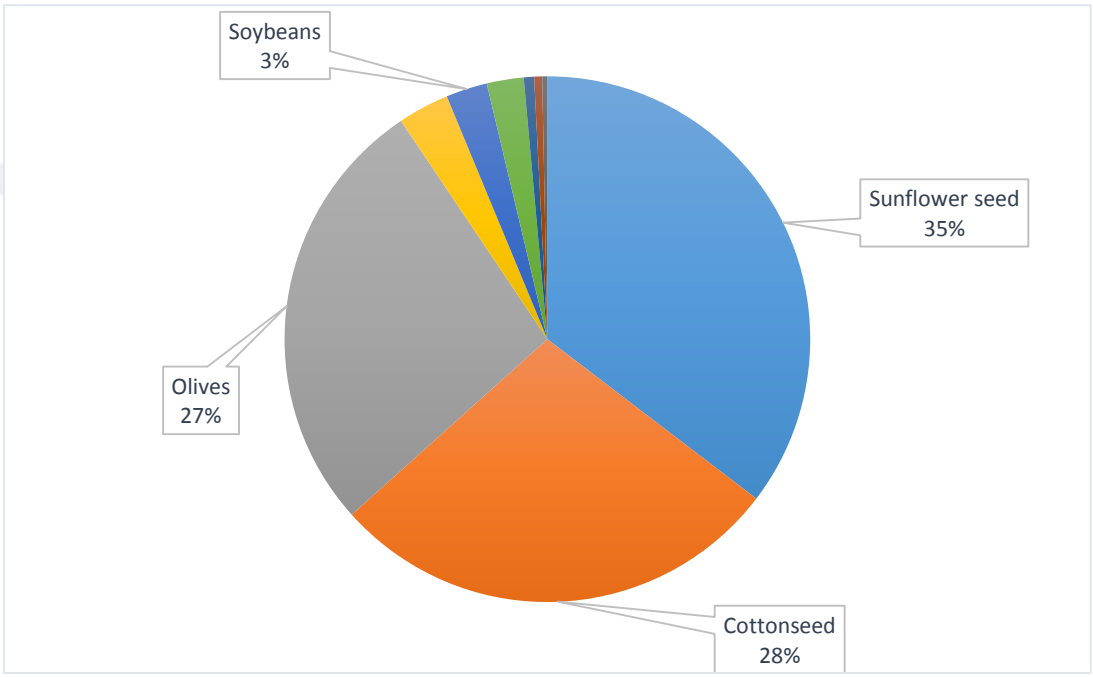

Figure 1.1 Most produced oilseed crops in Turkey

Due to geographical and ecological diversity of Turkey most of the main oilseed crops can be produced in our country. Since 1965 oilseed crop production have boosted dramatically and, sunflower (*Helianthus annuus* L) and soybean (*Glycine max* Merril) production increased for 618% and 3200% respectively. However only 4% of agricultural fields are used for production of oilseed crops while in USA and European countries this value is near 30% (Onat et al., 2018). Sunflower, cottonseed and olive are the main oilseed crops produced in Turkey (FAOSTAT, 2018).

1.1 Sunflower

Sunflower, member of Asteraceae family, is an important crop cultivated worldwide for different purposes such as biodiesel production, oil industry and nutrition due to

its high oil content. It was first domesticated in the North American continent. One of the unique features of this crop is breeding aiming for improvement of one feature. Each industry has its own bred cultivar starting from oil industry and ending with ornamental uses for gardens. When the explorers first introduced sunflower to Europe, it was mainly used for garden design. As the time passed, sunflower seeds were used for nutritional purposes. But the first industrial use of sunflower oil was dated to 1917 in England. The rapid boost of sunflower cultivation was due to long term fasting of Russian orthodox church that prohibited consumption of animal derived oils (Jan and Seiler, 2006).

Oil content of sunflower seeds varies between 47-53% and fatty acid content of sunflower oil mainly depends on cultivar and can be improved by breeding and

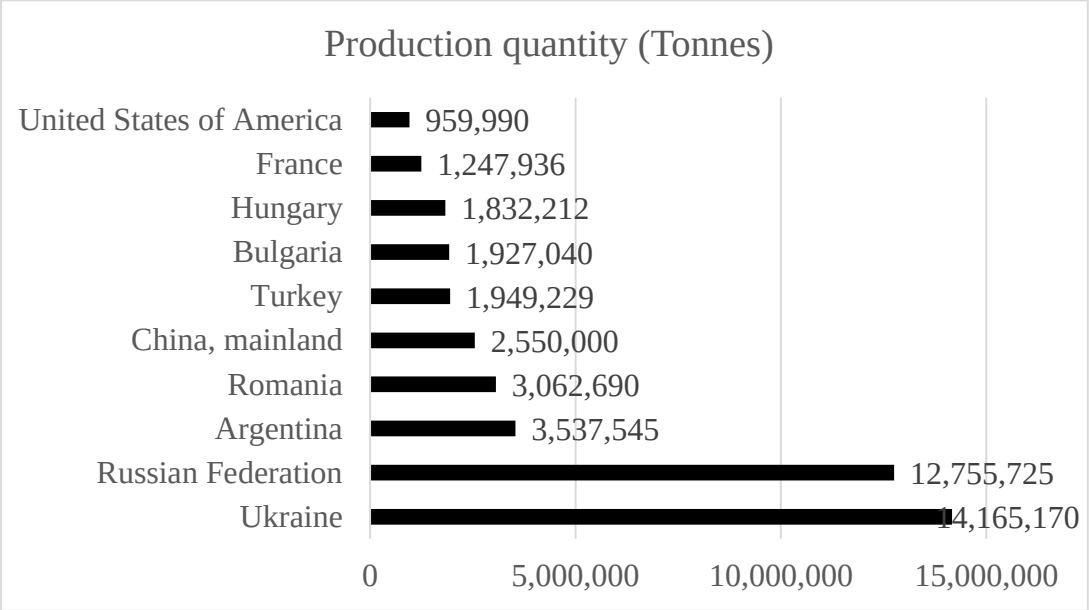

Figure 1.2 Top sunflower producers (**FAOSTAT, 2018)**

mutagenesis. Oleic and linoleic fatty acids comprise the main fraction of fatty acid content (up to 90%) and stearic and palmitic acid as a minor fraction (Martínez-Force et al., 2015).

Sunflower is sixth most produced oilseed crop in the world and its yield continues to grow worldwide and particularly in Turkey. Cultivation area and production quantity increases every year. Turkey stands in sixth place in sunflower production and yield of sunflower that is above the average continues to grow (FAOSTAT, 2018) (Figure 1.2).

1.2 Soybean

Soybean (*Glycine max*) yield forms 34.68% of world oilseed production and sixth most produced crop worldwide (FAOSTAT, 2018). It belongs to the Fabaceae family, that is third largest family of the Angiosperms that live in mutual symbiosis with Rhizobium bacteria that form nodules on the roots of the plant and takes necessary metabolites in exchange for fixed nitrogen molecules (Long, 1989) and due to this almost unique feature they are used in crops rotation systems to restore soil nitrogen content (Barnes, 2010).

Soybean was firstly domesticated in China and populations of its wild relatives of the soybean still presents in East Asia. Protein composition of the soybean is relatively similar to that of wild soybean but have higher oil concentration (Singh et al., 2006) and cultivated soybean have oil and protein content equal to 18-24% and 36-40% respectively (Tayyar and Gül, 2007). 80% of total protein content consists of globulins (Mailer, 2016) and difference types of flour and organic concentrates are made from soybean (Jideani, 2011). High oil and protein content make it ideal nutrition source. Besides that consumption of the meals containing soybean was reported to protect against some cancer forms, heart diseases, diabetes and obesity (Omoni and Aluko, 2005; Manavalan et al., 2009). Isoflavones that also present in soybean seeds was reported to have antibacterial, antifungal and estrogenic activity (Eldridge and Kwolek, 1983). Due to oil and protein content it is widely used in Asia and other regions as a nutrition source and it is also industrially important (Barnes, 2010).

Main soybean producers in the world are USA, Brazil, and Argentina. As mentioned before soybean is main oilseed crop produced in the world and its production and cultivation area in Turkey grows exponentially (FAOSTAT, 2018; Onat et al., 2018) (Figure 1.3).

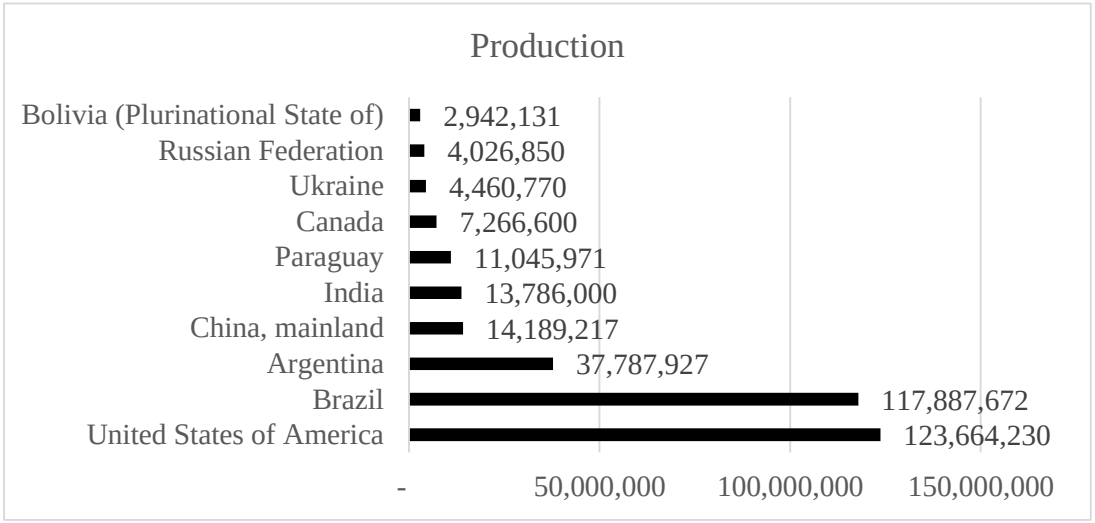

Figure 1.3 Top soybean producers worldwide (**FAOSTAT, 2018)**

1.3 Sunflower and Soybean Pathogens

There are a lot of different biotic and abiotic stress factors that limits yield of soybean and sunflower. Among the most important biotic stress factors that causes disease of soybean pathogens that belong to kingdom fungi. *Phytophthora sojae, Fusarium virguliforme and Macrophomina phaseolina* are just a few fungal pathogens but the most important ones (Tyler, 2008).

The same situation is seen for sunflower, that is subjected to different pathogenic and parasitic agents. Fungal, bacterial and viral pathogens, insects and parasitic plants limits its yield and production (Vear, 2010). It was reported that Most important fungal pathogens of sunflower are S*clerotinia sclerotiorum, Diaporhte helianthi, Macrophomina phaseolina, Plasmopora halstedii* that causes white rot, Phomopsis, charcoal rot and downy mildew respectively (Vear, 2004).

1.4 *Macrophomina phaseolina*

Recently due to global warming and water availability pathogens that are adapted to hot weather and drought conditions such as *M. phaseolina* had become more important constraint affecting yield of major crops those adversely affected by hot and dry conditions.

M. phaseolina is an anamorphic fungus that causes charcoal rot disease on over 500 plant species. Important crop plants such as sunflower, soybean, clover, mung bean,

maize etc. are among the important ones due to their economical and industrial importance (Gupta et al., 2012).

Table 1.2 Taxonomy of *Macrophomina phaseolina*

Kingdom	Fungi
Division	Ascomycota
Subdivision	Pezizomycotina
Classis	Dothideomycetes
Ordo	Botryosphaeriales
Familia	Botryosphaeriaceae
Genus	*Macrophomina*
Species	*Macrophomina phaseolina* (Tassi) Goidanich

It can survive in the soil for 15 years via microsclerotia that constitutes the main inoculum source. Drought and heat are the main factors that favors the charcoal rot disease and symptoms are observed during flowering period. It is a devastating disease that appears as hotspots of dead plants in the farming area (Papavizas and Klag, 1975; Ijaz et al., 2013). Losses due to charcoal rot disease are varies across different regions and under favorable environmental conditions can cause up to 80% and 90% yield loss for soybean and sunflower respectively (Khan, 2007; Gupta et al., 2012)

A lot of attempts were performed for grouping populations of *M. phaseolina* into races, Vegetative Compatibility Group(VCG) based on phenotypic and pathogenic characterization (Dhingra and Sinclair, 1973; Cloud and Rupe, 1991). With the improvement of molecular based technologies and marker systems a new era in population studies arose. A lot of different marker systems were applied for revealing genetic structure of population of causal agent. Diverse marker systems were employed to reveal genetic complexity of population. RAPD (Random Amplified Polymorphic DNA), SSR (Simple sequence repeat), ISSR (Inter simple sequence repeat), DNA barcoding and SRAP (Sequence related amplified polymorphism) marker systems are just a few examples of various techniques used for population genetic studies (Jana et al., 2005a; Reznikov et al., 2018; Sarr et al., 2014).

Plant pathogens affecting phyllosphere of the plants can be managed to some extent by fungicidal practices however soil-borne plant pathogens, like *M. phaseolina*, are hard to control and the most appropriate control measure is utilization of resistant or tolerant varieties (Tyler, 2008). It is necessary to reveal genetic and pathogenic variation among pathogen population for successful breeding programs and employment of resistance resources.

1.5 Screening Methods

Pathogenic variation one of the important factors that affects breeding programs. Choice of suitable inoculation method for acquiring accurate result during pathogenicity and variety reaction studies is required. A lot of different inoculation methods were employed on different host plants. Although a lot of different inoculation and disease progression assessment methods exist, selection the most suitable methods and their combinations are required for achievement of accurate results.

1.5.1 Inoculation Methods

Main features necessary for successful screening assay are susceptible host and pathogenic agent. Although environmental condition and development stage of host organism known as predisposing factors are secondary but important attributes (Tuite, 1969). Inoculation can be performed under controlled conditions in growth chamber and greenhouse, and in the contaminated field with disease background. In some cases, if pathogen population is low field can be enriched with propagules to increase inoculum density (Pastor-Corrales and Abawi, 1988; Twizeyimana et al., 2012).

Although *M. phaseolina* is soil borne pathogen, due to late development of disease symptoms inoculation is performed on stem of the host using different inoculum sources. Cut stem, stem tape and toothpick inoculation methods on different hosts are widely used by researchers all over the world however disease assessment methods can differ even for the same inoculation method (Zazzerini and Tosi, 1989; Pratt et al., 1998; Twizeyimana et al., 2012; Viteri and Linares, 2017). Despite that those

methods are fast; they can give only approximate information about resistance of germplasm and field trials are necessary at some points.

That one proposed by Manici et al. (1995) is based on the reaction of the seed in petri dish filled with media. It can be used for pathogenicity and variety reaction assays, but it does not take in account environmental and physiological state of the plant.

1.5.2 Disease Progression Assessment Methods

Disease progression assessment methods are divided into 4 groups: direct quantitative, direct qualitative, indirect, and remote sensing methods. Direct quantitative methods are based on disease severity or disease incidence while direct qualitative methods aim to group disease according to symptoms present. Indirect method mainly uses vectors for assessment and remote sensing mainly based on photography (Cooke et al., 2006).

As *M. phaseolina* is cosmopolite and polyphagous pathogen, it is necessary to develop disease assessment methods for every individual host plant. During literature review only direct quantitative were found for different hosts. Disease incidence and disease severity based on lesion length are main scaling systems used for pathogen of interest (Pastor-Corrales and Abawi, 1988; Zazzerini and Tosi, 1989; Pratt et al., 1998; Twizeyimana et al., 2012; Viteri and Linares, 2017).

1.6 Genetic Diversity of Fungi

Accurate identification and characterization of fungal samples or population is important for development and improvement of disease management programs. Conventional characterization methods based on morphology (colony, propagule etc. morphology) and physiology (biochemical and pathogenic variation) of fungal agent are easily affected by environmental and host factors, however DNA based molecular marker systems such as RAPD, ISSR, SSR, AFLP etc. are widely used for phylogenetic and genetic diversity analysis of phytopathogenic fungi (Shamim et al., 2017).

1.6.1 Polymorphism Based Marker Systems

ISSR and SSR are marker systems based distance between microsatellite regions and number of repeats. Codominant nature and high polymorphism rate of those markers made them popular tool for marker assisted selection of plant germplasm and genetic diversity researches of large number of different organisms (Reddy et al., 2002; Shamim et al., 2017). RAPD is polymorphism-based marker system similar to SSR, but primers used for RAPD amplification is randomly synthesized decamers. Polymorphism of this technique originates from range between complementary sequences, but main disadvantage of current marker system is low reproducibility (Shamim et al., 2017). AFLP is fingerprinting technique consisting of 2 steps. Genomic DNA is digested with restriction endonuclease enzymes and adaptor oligonucleotides binding. Conventional PCR reactions with primers complementary to adapter oligonucleotides and adjacent sequences. Polymorphism of AFLP arises from variation of sequences adjacent to restriction site (Blears et al., 1998). All those marker systems have its own advantages and disadvantages, despite that they are widely used during genetic diversity studies.

1.6.2 Sequence Based Marker Systems

In last few decades development of new sequencing methods and improvement of old ones facilitated development of new marker systems and popularized sequence-based studies.

SNP (Single Nucleotide Polymorphism) is widespread in many living things and evolve in a simple manner by nucleotide substitutions. Methods aims to discover single nucleotide substitutions via searching for difference in already presented data in gene banks or sequencing of amplicons obtained via PCR using specific primer pair for previously sequenced region or extended polymorphic primer sets. Second approach mainly performed for population studies of non-model organisms due to lack of information about sequence (Morin et al., 2004). Low mutation rate of SNP compared to microsatellite turns SNP into one of the most suitable methods for evolutionary studies, however, method, approach and number of samples should be planned carefully (Brumfield et al., 2003; Morin et al., 2004).

DNA barcoding is a powerful tool for phylogenetic, taxonomic and population genetics study. Variation in sequences of house-keeping and conserved regions can reveal genetic diversity of population and evolutionary relationship during inter and intra specific studies and form reliable collection that can be shared (Hajibabaei et al., 2007). Different barcode genes are proposed for such studies and there are key features for ideal barcode gene such as sequence length, universality of primers, reproducibility and sequence quality (Stielow et al., 2015). In the light of these features barcode gene selection for studies is the most important part and recently multi locus studies can be exploited as well.

Evolution and mutation rate of genome is different between groups of organisms. Although universal primers that can amplify gene regions were designed, those regions when used alone cannot reveal the genetic variability of some species. ITS, β-tubulin, Elongation factor 1-α, RNA Polymerase big subunit, 18S ribosomal DNA regions are the most widely used ones for fungi kingdom (James et al., 2006).

Aim of our study was to modify widely used plant inoculation and pathogenicity assays in sunflower and soybean seedlings with *M. phaseolina* in controlled growth chamber conditions. In addition, it was aimed to check the utility of multilocus phylogenetic analysis and its value as the discriminant in population studies of *M. phaseolina*. The data obtained through this study would be used in fast screening resistance breeding programs against *M. phaseolina* in sunflower and soybean.

CHAPTER II

LITERATURE REVIEW

High number of studies were conducted around the World to characterize diversity of *Macrophomina phaseolina* populations due to its importance as the causal agent of charcoal rot disease on long list of crops that are cultivated for different purposes such as nutrition, oil and biofuel industries. In the second half of XX century, main aim of researches was phenotypic characterization and application of obtained information in breeding and disease management programs.

Dhingra and Sinclair (1973) revealed variation among *M. phaseolina* population collected from Arkansas, Illinois and Missouri based on virulence and growth rate under different temperature conditions. It was reported that isolates showing best growth at high temperatures are highly virulent.

Papavizas (1977) carried on research to reveal the surviving ability of microsclerotia under different conditions in soil. Effect of the factors such as fertilizer, temperature, moisture and fungicides were checked. It was reported that only a few fungicides had control on the microsclerotia population. Also, organic amendment produced from alfalfa exhibited restriction against microsclerotia, however, other N sources added to soil did not affect sclerotia. In addition, it was reported that the most important factors influencing survival of sclerotia were moisture and temperature of the soil, thus, high moisture at low temperature and low moisture at high temperature decreased survivability of sclerotia in soil.

Zazzerini and Tosi (1989) investigated variability of causal agent of charcoal rot on media containing chlorate and host specificity of isolates. Fungal material was isolated from different hosts and colony morphology of latter was checked on chlorate containing media. Isolates have shown different growth patterns defined as restricted, feathery, and dense. Relationship among host plant, regions and chlorate sensitivity was not found. Also, pathogenicity tests were performed using sunflower and results have shown that there is no host specialization.

Lodha (1995) have reported the effectiveness of soil solarization against *Fusarium oxysporum* f.sp. *cumini*, *M. phaseolina* and weed growth wherein soil solarization was effective in reduction of disease incidence for 2 consecutive years. There was significant difference in population and disease incidence between solarized and control fields. In addition, difference between dry and moist solarization was reported. Moist solarization was more effective against *M. phaseolina* population than dry solarization. Manure enriched with nitrogen had effect on success of solarization as well. Researchers concluded that moist solarization with nitrogen rich amendments were effective way for management of soil borne plant pathogens.

Pratt et al. (1998) have evaluated symptom development after inoculation of different parts of mature plants of alfalfa and white clover with *M. phaseolina*. Stems, leaves, roots and stolons of plants were inoculated using toothpick or agar plug methods. Causal agent induced brown-black lesions on vascular tissue that progressed until collapse of pith and epidermis of stolons. Taproot and crown of plants inoculated with pathogen suffered from discoloration, invasion and death of vascular tissue and tissues under the inoculation point. Leaves of plants inoculated with mycelium were rapidly colonized by the pathogen with latter formation of sclerotia. Different advancement rates on leaves of different genotypes set the basement for hypothesis of existence of resistance to some extent against disease agent.

In new millennia, PCR based marker systems started to gain importance due to its applicability in different organisms. High reproducibility and reliability of DNA based analysis is advantage when compared to those of conventional methods of plant pathology practices however it still conserves its importance. Combination of conventional and molecular studies became a trend recently. Although marker systems to be applied should be chosen carefully, as value of information is directly correlated with executed method (Shamim et al., 2017).

Su et al. (2001) aimed to check the existence of relationship between chlorate resistance and origin host, and to analyze genetic diversity using RAPD and rDNA-RFLP and to define the host specialization using standard pathogenicity assay. Results suggested impossibility of host differentiation based only on chlorate sensitivity, however, analysis performed based on RAPD polymorphism using neighbor joining method suggests existence of host specialization to some extent.

Cross inoculation studies were performed, and it was concluded that there is adaptation to host rather than host specialization.

Almeida et al. (2003) reported high genetic diversity of Brazilian population of *M. phaseolina* collected from soybean. Genetic diversity of Brazilian population was conducted via RAPD and PCR-RFLP marker systems. Seventy-four unique loci were amplified using 22 RAPD primers. UPGMA analysis of bands polymorphism clustered all isolates into 3 groups with 99%, 92% and 88% average similarity. In some cases, different samples isolated from same plant exhibited different haplotype pattern. Samples collected from fields with crop rotation systems exhibited more similarity than those collected from fields with single crop. PCR-RFLP of isolates was unsuccessful in differentiation of isolates. Researchers concluded that single crop cultivation induces divergence of population through years.

Jana et al. (2005a) carried on molecular analysis using Universal Rice Primers – PCR (URP-PCR) on *M. phaseolina* isolated from chickpea, cotton, and soybean. Forty isolates from 3 different hosts were used during study and only 5 URP primers were useful for obtaining amplification products for all isolates. DNA fingerprint analysis were performed using UPGMA method and 2 main clusters were formed. Isolates from soybean and cotton clustered in different groups, however chickpea isolates did not cluster in any group and separated from each other. It was concluded that *M. phaseolina* genome shares some repetitive DNA with rice and can be used for fingerprint analysis.

Jana et al. (2005b) performed SSR fingerprint analysis of populations of *M. phaseolina* isolated from soybean and cotton grown in USA and India. Cluster analysis based on 3 SSR fingerprint patterns was executed, and all isolates clustered in 3 groups. Cotton and soybean isolates have clustered in different groups and only 3 isolates did not belong to any of them. They have suggested the SSR based fingerprinting analysis as a useful tool for estimation of genetic variability in recent microorganism.

Genetic and pathogenic variation of *M. phaseolina* causing charcoal rot disease on common bean were studied by Reyes-Franco et al. (2006). Ninety-six isolates representing different geographical locations were used in the study. Pathogenic variation was studied using different common bean cultivars. Cluster analysis based

on obtained data was performed and all isolates clustered into high and low virulence groups. AFLP analysis of all isolates was carried on for revealing genetic diversity of different populations. Ninety isolates have gathered in one group and genetic dissimilarity in first group ranged between 3-15%. High genetic diversity was attributed to diverse origin of isolates.

Thilagavathi et al. (2007) examined effectiveness of 3 different biocontrol agents against *M. phaseolina* isolated from green gram. Individual and combinatory effect of 3 different biocontrol agents, *Trichoderma viride*, *Pseudomonas fluorescens* and *Bacillus subtilis* were studied. Based on the procured data it was assumed that individual and combinatory formulations of biocontrol agents had a significant effect on suppression of disease and enhancement of plant parameters such as root and shot length. Utilization of biocontrol agents instead of fungicidal applications was suggested as it is ecofriendly and economically more appropriate treatment for management of recent disease.

Omar et al. (2007), investigated chlorate resistance, genetic diversity and pathogenicity of *M. phaseolina* isolated from cotton. Relationship between pathogenicity on 3 cultivars and chlorate phenotype was not observed. All isolates of *M. phaseolina* were pathogenic on cotton, although difference in cultivar-isolate interaction was found when statistical analysis executed. Band polymorphisms obtained from amplification of 4 RAPD primers were subjected to UPGMA analysis. Phenogram based on host-pathogens interaction and dendrogram of band polymorphisms did not match with each other, however, isolates from upper Egypt clustered together in both graphics and RAPD profile correlated with chlorate phenotype.

Mengistu et al. (2007) have aimed to develop reproducible and precise method for characterization of reaction of different soybean varieties against *M. phaseolina*. Twenty-four soybean genotypes with high agricultural income value were chosen. Five different disease assessment methods were evaluated. The most reproducible and reliable one was CFUI (Colony forming unit index), but also time consuming. RSS (Root stem severity) was more rapid, but less accurate. Selection of suitable method depending on breeding program was suggested by researchers.

Babu et al. (2007) have successfully developed species specific primer and prob combination for fast and robust identification of *M. phaseolina*. Isolates collected from different regions and different hosts were used during the study. ITS (Internal Transcribed Spacer) region was amplified and sequenced. Primer and probe were designed based on conserved regions of ITS region sequence. The utility of primers was checked via PCR executed on genomic DNA of *M. phaseolina* and other fungi, and only *M. phaseolina* isolates gave an amplicon of size approximately 350 bp. They have assumed that MpKFI and MpKRI primer pair is useful tool for fast molecular identification of *M. phaseolina*.

Hernandez-Delgado et al. (2009) have investigated molecular markers associated with resistance to *M. phaseolina* in common bean. Study was carried on with F_2 generation obtained from cross of BAT 477 and UI-114. Chi-square analysis were applied to the data of pathogenicity assay. Results of analysis suggests that there are 2 dominant genes responsible for resistance to *M. phaseolina*. AFLP marker system were used for QTL mapping. Twenty most polymorphic AFLP primer combinations out of 81 were used for map construction. Total of 294 amplification products were observed in F2 and parental lines. Only 130 amplified loci were useful during map construction due to 50% level of distortion. In the results of this study 2 genes responsible for resistance to charcoal rot were reported however they have double epistasis and only 1 AFLP marker were associated with resistance gene.

Baird et al. (2010) analyzed genetic diversity of US *M. phaseolina* population using SSR marker system. Twelve SSR loci out of 46 were highly polymorphic and were used for to resume the analysis. Those loci were used for investigation of genetic diversity of *M. phaseolina* population and its transferability across different genera. Genetic diversity of analyzed population was high when investigated with SSR, however, ITS based analysis showed opposite results. SSRs used during this study were proven to be cross transferable across various genera from Botryosphaeriaceae family.

Mengistu et al. (2011) investigated reaction of 149 soybean genotypes to *M. phaseolina* in field. For 3 years CFUI and environmental conditions were registered. They have reported that CFUI data is useful and reliable method for analysis of cultivar reaction to disease in the case when research is performed for more than 1

year. They have reported 6 moderately resistant cultivars that can be used by breeders for further studies.

First case of charcoal rot of sunflower caused by *M. phaseolina* was reported by Mahmoud and Budak (2011) from Turkey . High number of fields with disease incidence between 10-50% were surveyed. After symptomatic evaluation and observing microsclerotia isolated agent were subjected to molecular and pathogenicity analysis. Causal agent was identified as *M. phaseolina* and genetic diversity of obtained isolates were investigated using SRAP marker system. SRAP were proven to be highly informative and high level of genetic diversity were reported.

Islam et al. (2012) performed genome sequence analysis of *M. phaseolina* using whole genome shotgun method and assume that sequence data will be useful in further studies. Approximately 49 Mb was sequenced that comprise 92.83% of genome. Number of open reading frames predicted was 14,249 and 9,934 was validated by transcriptome analysis. Abundance of secreted oxidases, peroxidases and hydrolytic enzymes was reported and significant number of P450s, MFS type membrane transporters, glycosidases and transposases were detected. Genome sequence analysis detected wide range of genes responsible for pathogen-host interaction and explains wide host range of current pathogen.

Twizeyimana et al. (2012) aimed to develop precise and fast inoculation method for investigation of variety reactions of soybean and common bean against *M. phaseolina*. They developed cut-stem inoculation method and applied it on different soybean and common bean genotypes. They suggested that developed method is fast and reliable method for successful differentiation of aggressiveness and host specialization of *M. phaseolina*.

Multi locus phylogenetic analysis of large collection representing *M. phaseolina* populations from different continents was reported by Sarr et al. (2014). ITS, TEF (Translation Elongation Factor), ACT (Actin), CAL (Calmodulin) and TUB (β-Tubulin) gene regions were amplified and sequenced during the study. Phylogenetic tree was generated based on sequence data and new species namely *M. pseudophaseolina* was described. General correlation between host or geographical origin of isolate were not observed.

Hyperspectral imaging system were developed by Jones (2017) for precise assessment of disease development and for easy correlation of results with for QTL (Quantitative Trait Loci). It was stated that disease symptoms invisible to human eye can be observed with hyperspectral imaging system more precisely and minimize experimental error. QTL mapping of RILs identified 2 QTL of small effect on resistance to *M. phaseolina*.

Al-Ahmadi et al. (2018) aimed to develop method for determining effect of toxin on soybean seedlings using hyperspectral spectroscopy (HS). Soybean seedlings were placed into toxin containing solution and symptoms developed on leaves were checked with HS systems. They assume that HS at 1940 nm wavelength can be used as addition to conventional methods of disease assessment. HS measurement of leaves can detect wilting at the beginning of the disease.

Reznikov et al. (2018) investigated genetic and pathogenic profile of *M. phaseolina* population from north-western Argentina and compared them with US (United States) population. *M. phaseolina* was isolated from soybean and other hosts subjected to charcoal rot. AFLP and SSR fingerprinting were executed for genetic characterization of population. Fingerprinting analysis was ineffective for grouping isolates according to origin, host, and collection year.

Defense response of *Arabidopsis thaliana* against *M. phaseolina* were investigated by Saima and Wu (2019). Physiological and molecular responses were analyzed. Shoot length, root length, pigmentation and water content reduction and increase in sugar and proline contents of the leaves were main physiological responses. During molecular analysis using RT-PCR high increase in mitogen activated protein kinas and thaumatin proteins, and slight increase in chitinase and beta 1-3 glucanase were reported. Therefore, important role of defense related genes together with sugar and proline level were defined to be generated against *M. phaseolina*.

Effect of irrigation on disease severity and yield parameters of soybean plants subjected to charcoal rot were reported by Jordaan et al. (2019). Results suggest that irrigation is not preventive factor against charcoal rot disease, but it can reduce disease severity on plants. Necessity of drought conditions for higher disease severity rather that for infection was hypothesized. Researchers also mentioned that there is requirement for further studies of effect created by environmental factors on disease

development for development of successful management programs against *M. phaseolina.*

CHAPTER III

MATERIAL AND METHODS

The research was performed during 2018-2019 in the Gaziantep University, Biology Department, Mycology Laboratory.

3.1 Materials

3.1.1 Plant Material

Plant materials used throughout pathogenicity, inoculation and variety reaction studies were obtained from the Directorate of Trakya Agricultural Research Institute (Edirne) and Eastern Mediterranean Agricultural Research Institute (Adana). Susceptible varieties Yesilsoy and Deray were used during pathogenicity and inoculation method trials for soybean and sunflower, respectively.

Figure 3.1 Soybean and sunflower seeds; A: Soybean cultivar Yesilsoy; B: Sunflower cultivar Deray

3.1.2 Pathogen Material

Soybean, maize, and sunflower plant materials exhibiting charcoal rot symptoms were obtained from the experimental plots of the Directorate of Trakya Agricultural Research Institute (Edirne) and Eastern Mediterranean Agricultural Research Institute (Adana) during September-October of 2018 and 2019.

3.2 Methods

3.2.1 Pathogen Isolation

Isolation of *Macrophomina phaseolina* was performed from plant parts showing symptoms of charcoal rot. Plant material was checked for presence of microsclerotia in stem sections with binocular microscope.

Small parts of microsclerotia containing plant materials were taken with sterile scalpel, wrapped in cheese cloth, and placed into sterile 100 ml bottles. Fifty ml of 1.5% NaOCl was poured into each bottle and incubated for 3 minutes. Afterwards NaOCl was discharged and plant materials were incubated in sterile distilled water 3 times for 3 minutes. Later plant materials were left for drying on blotting paper in sterile Petri dishes using sterile forceps. Dry plant materials were placed into Petri dishes containing PDA and incubated at 30 ^{o}C for 3 days. Single hyphae isolates were obtained and stocked in cryotubes filled with 20% glycerol solution and conserved in deep freezer (Altinok and Can, 2010).

3.2.2 Morphological Characterization of Isolates

Species identifications were executed based on morphological characters. In planta microsclerotia size and colony morphology were main parameters noted during morphological identification (Mahmoud and Budak, 2011).

3.2.3 DNA Isolation

Agar plugs were transferred into 100 ml bottles containing 50 ml Potato Dextrose Broth (PDB) media and placed into shaker at 30 ^{o}C and in dark conditions. Melanized mycelial mass were filtered with cheese cloth, washed with sterile distilled water and wrapped into aluminum foil and then placed into liquid nitrogen

for fast freezing. Afterwards small part of isolated mycelial mass was blended to powder with mortar and liquid nitrogen for physical homogenization. Manual DNA extraction method was performed according to Peever et al. (1999) with some modifications.

1. 600 µl CTAB buffer (2% CTAB, 1.4 M NaCl, 0.1 Tris-base, 20 mM EDTA) was added to the micelles in the tube and incubated at 60 °C at 350 rpm for 30 minutes in the heater mixer.
2. After incubation, 600 µl of chloroform-isoamyl alcohol (24: 1) was added to the tube and centrifuged at 5000 rpm for 20 minutes.
3. 400 µl of supernatant were transferred to the new sterile 1.5 ml tube.
4. 400 µl of cold isopropyl alcohol was added into the tube with supernatant, mixed and incubated at cold for 10 minutes.
5. After incubation tubes were centrifuged for 20 minutes at 5000 rpm.
6. After centrifugation, supernatant was poured, 70% ethyl alcohol was added on pellet part, washed, and left to dry.
7. 50-100 µl TE buffer was added to the dry pellet.
8. Dissolved DNA was placed in deep freezer

3.2.4 Molecular Identification

Molecular identification of isolates were performed using species specific primer pair suggested by Babu et al., (2007). All isolates identified as *M. phaseolina* based on morphological features were subjected to molecular analysis with species specific primer combination MpKFI (5'-CCG CCA GAG GAC TAT CAA AC- 3') and MpKRI 5('-CGT CCG AAG CGA GGT GTA TT-3'). PCR reaction mixture containing 2 µl 10x Taq buffer, 0.2 mM dNTP, 1.25 mM $MgCl_2$, 5 pmol of each primer and 1 unit of Taq polymerase with final volume of 20 µl was used for amplification. PCR reaction was 1 minute of initial denaturation at 95 °C followed by 25 cycles of denaturation at 95 C for 30 seconds, 1 minute of annealing at 56 °C and extension for 2 minutes at 72 °C with final extension at 72 C for 10 minutes. PCR product was electrophoresed via EtBr stained agarose gel electrophoresis and visualized at UV monitoring system.

3.2.5 Inoculation Methods

3.2.5.1 Growth Chamber Conditions

Surface sterilized soybean, sunflower and maize seeds were sown into pots containing peat: perlite: sand (2:1:1) mixture and watered. Pots were placed into growth chamber at 12:12 photoperiod with 26 °C and 60% relative humidity conditions. Plants were watered if necessary. Different inoculation methods were executed on 3 weeks old seedlings. Trials were performed 2 times with 4 repeats containing 4 pots (Zazzerini and Tosi, 1989; Twizeyimana et al., 2012).

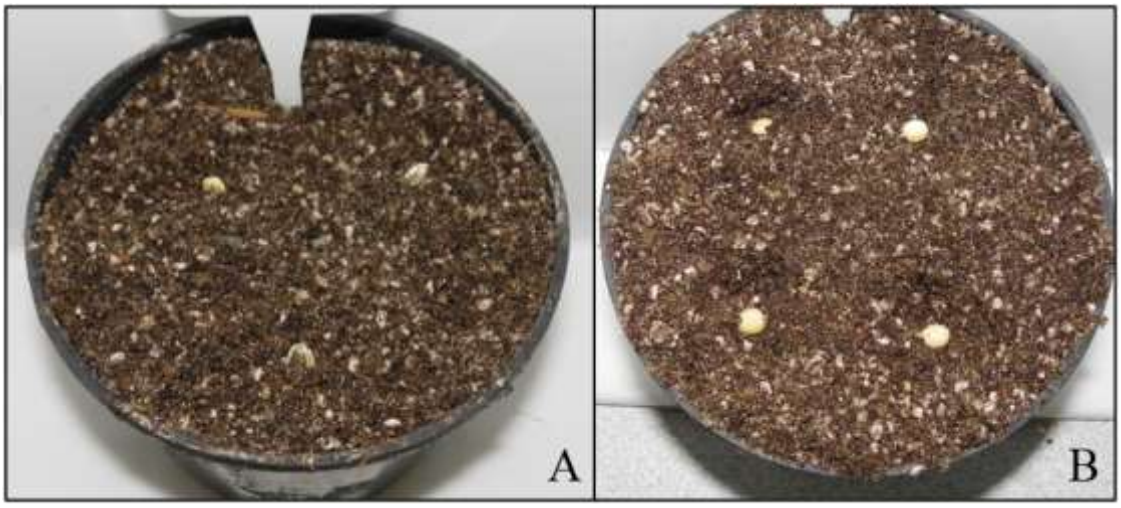

Figure 3.2 Seeds planted in peat: perlite: sand mixture containing pots; A. Sunflower seeds; B. Soybean seeds

3.2.5.2 Cut Stem Inoculation Method

This method was performed only for sunflower and soybean plants. 3 weeks old seedlings were cut 2 cm above the second trifoliate leaf with sterile scalpel. Ten days old and 3 days old cultures were taken with sterile 100 µl pipette tips and placed on the open wound of the plant. Pipette tips containing uninoculated PDA plugs were placed on control plants. Growth chamber conditions were setup to 14:10 photo period, 60% relative humidity and 30 °C. Pipette tips were removed from plants 3 days after inoculation (Twizeyimana et al., 2012).

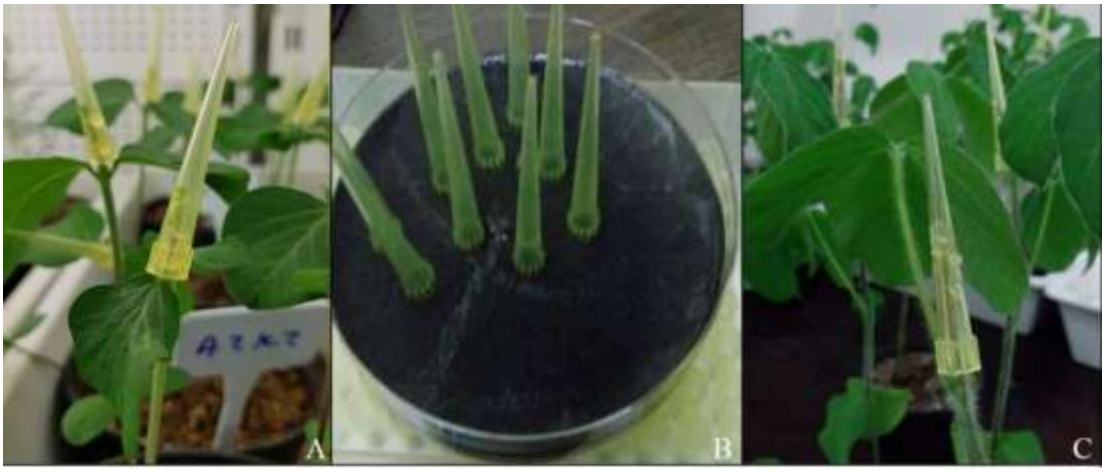

Figure 3.3 Cut stem inoculation method; A. Inoculated sunflower seedlings; B. Three days old inoculum; C. Inoculated soybean seedlings.

3.2.5.3 Toothpick Inoculation Method

Toothpicks were soaked into deionized water, boiled for 1 hour and left for drying on blotting paper. Dry toothpicks were then placed into jar and autoclaved for 15 minutes at 121 °C and at 1.5 atm. Later sterile toothpicks were placed into Petri dishes containing 3 days old *M. phaseolina* culture and incubated for 7 days. Toothpicks colonized with pathogen were transferred into empty petri dishes and left to dry.

Figure 3.4 Toothpick inoculation method; A. Inoculated soybean seedlings; B. Toothpick inoculum; C. Inoculated sunflower seedlings.

Toothpicks colonized with agent were used as inoculum and unused toothpicks were conserved at +4 C. Soybean and sunflower plants were inoculated with toothpick 2 cm below first node and controls were treated with sterile toothpicks. Toothpicks were removed 3 days after inoculation. Growth chamber conditions were setup to 14:10 photo period, 60% relative humidity and 30 °C (Pratt et al., 1998).

3.2.5.4 Stem-Tape Inoculation Method

Three weeks old sunflower and soybean seedlings were wounded 2 cm under first node with sterile scalpel. Agar plugs with 10 and/or 3-day old cultures were placed on the wound and wrapped with parafilm. Control group were treated with uninoculated agar plugs. Three days after inoculation parafilm was removed (Zazzerini and Tosi, 1989). Growth chamber conditions were setup to 14:10 photoperiod, 60% relative humidity and 30 °C

Figure 3.5 Stem-tape inoculation method; A. Inoculated soybean seedlings; B. Inoculated sunflower seedlings; C. Three days old inoculum

3.2.5.5 Scoring

Scoring of cut stem inoculated soybean plants were performed according to Viteri and Linares, (2017) and based on disease progression across the plant starting from the top (Table 3.1).

Table 3.3 0-8 scaling system according to Viteri and Linares (2017)

Score	Symptom
0	No sign of infection
1	Infection local to wound
2	Infection did not reach first node
3	Infection reached first node
4	Infection passed first node
5	Infection did not reach second node
6	Infection reached second node
7	Infection passed second node
8	Infection reached or passed third node

All other groups were scored according to Pratt et al. (1998) and length of lesions were measured (Table 3.2).

Table 3.4 0-4 Scaling system according to Pratt et al. (1998)

Score	Lesion length
0	0-0.2 cm
1	0.21-0.5 cm
2	0.51-1 cm
3	1.01-1.5 cm
4	>1.5 cm

All plants were scored every 3 days until 21 DAI. Area Under Disease Progression Curve (AUDPC)was calculated (Kocalar et al., 2020).

Statistical analysis was performed using SPSS 23.0 (Statistical Package for Social Sciences). Independent sample t-test and One-Way ANOVA analysis was performed ($p<0.05$).

3.2.6 Pathogenicity and Variety Reactions

Pathogenicity and variety reactions were performed using stem tape method for sunflower and maize plants and cut stem method were executed for soybean plants. Scores were taken at 3, 7 and 14 DAI.

Table 3.5 Soybean and sunflower varieties used in variety reactions

Soybean varieties	Sunflower Varieties
Lider	Palanci
Turksoy	Cetinbey
Ataem 7	Metinbey
Bravo	Sems Or
Mersoy	Orfey
Mono	17 Imi Tr
	Kayra
	Ahmetbey
	11 TR 077

Pathogenicity studies of collected isolates were performed on their host of origin (table 3.4).

Table 3.6 List of isolates collected from soybean, sunflower and maize plants

Soybean isolates	**Sunflower isolates**	**Maize isolates**
SMP 7	AMP 3	CMP 1
SMP 3	AMP 1	CMP 5
SMP 6	AMP 4	CMP 6
SMP 9	TAE 7	CMP 4
SMP 8	AMP 2	

3.2.7 Molecular Analysis of Pathogenic Isolates.

Complete ITS (Internal Transcribed Spacer) and part of β-Tubulin genes were amplified via PCR using ITS 5 (5’ TCC GTA GGT GAA CCT GCG G 3’) and ITS 4 (5’ GGA AGT AAA AGT CGT AAC AAG G 3’) (White et al., 1990), and Bt1a (5′ TTC CCC CGT CTC CAC TTC TTC ATG 3′) and Bt1b (5′ GAC GAG ATC GTT CAT GTT GAA CTC 3′) (Glass and Donaldson, 1995) primer pairs respectively. PCR reaction mixture containing 2.5 µl 10x Taq buffer, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 4 pmol of each primer and 1 unit of Taq polymerase with final volume of 25

µl was used for amplification. PCR reaction was 4 minute of initial denaturation at 94 °C followed by 35 cycles of denaturation at 94 C for 2 minutes, 1 minute of annealing at 56 °C for β-Tubulin and 53 °C for ITS and extension for 2 minutes at 72 °C with final extension at 72 °C for 10 minutes. PCR product was electrophoresed via EtBr stained 1.5% agarose gel electrophoresis and visualized at UV monitoring system (Altinok and Can, 2010).

3.2.8 Analysis of Sequence Data

Sequences were aligned using ClustalW alignment algorithm and suitable phylogenetic analysis model were chosen using MEGAX software (Kumar et al., 2018). The evolutionary history of both regions was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method and Tamura 3-parameter method for ITS and β-tubulin, respectively (Tamura, 1992).

CHAPTER IV

RESULTS

4.1 Pathogen Isolation and Identification

Macrophomina phaseolina was successfully isolated from maize, soybean and sunflower plants exhibiting symptoms of charcoal rot disease (Figure 4.1). All of the isolated samples were prepared as stock cultures at -20 ^{o}C for further studies.

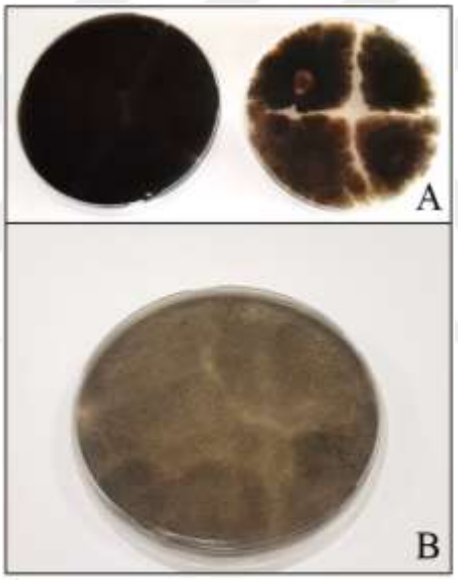

Figure 4.1 Colonies developed from symptom exhibiting plant parts on PDA media; A: Primary colonies developed from plant parts; B: Colony morphology of single hyphae isolate

4.2 Morphological Identification

All the symptom exhibiting plants were checked for presence of microsclerotia within the stem tissues and sizes of microsclerotia was measured (Table 4.1, Figure 4.3).

Table 4.7 Average length and width (**µm)** of microsclerotia

CROP	SUNFLOWER	SOYBEAN	MAIZE
LENGTH	67.31	63.83	61.6
WIDTH	68.37	67.55	60.2

Diseased soybean, maize and sunflower plants had microsclerotia embedded in the stem tissue of mature plants (Figure 4.3). Colony morphology on PDA was with aerial mycelia and in some cases having irregular shape. Slightly ellipsoid microsclerotia were observed in mature culture on PDA media (Figure 4.2).

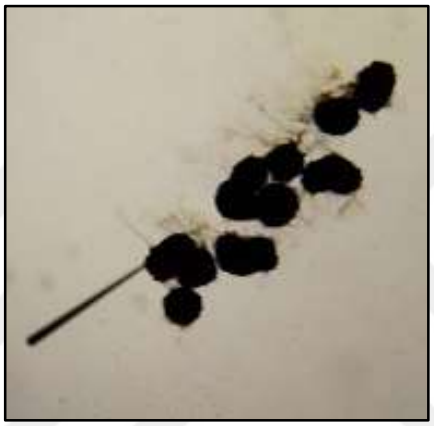

Figure 4.2 Microsclerotia developed on PDA media within 10 days at 400x magnification

Figure 4.3 Inner stem tissue of plants exhibiting charcoal rot symptoms viewed with stereo microscope; A: Sunflower inner stem tissue at 7x magnification; B: Sunflower inner stem tissue at 32x magnification; C: Inner stem tissue of soybean plants at 12x magnification; D:Inner stem tissue of soybean plant with microsclerotia 12x; E,F,G: inner stem tissue of maize plants subjected to charcoal rot at 7x, 7x and 40x magnifications respectively.

4.3 Molecular Identification

Species specific primers successfully amplified 350 bp region and all the isolates included in this study was identified as *M. phaseolina* based on molecular and phenotypic characters (Figure 4.4).

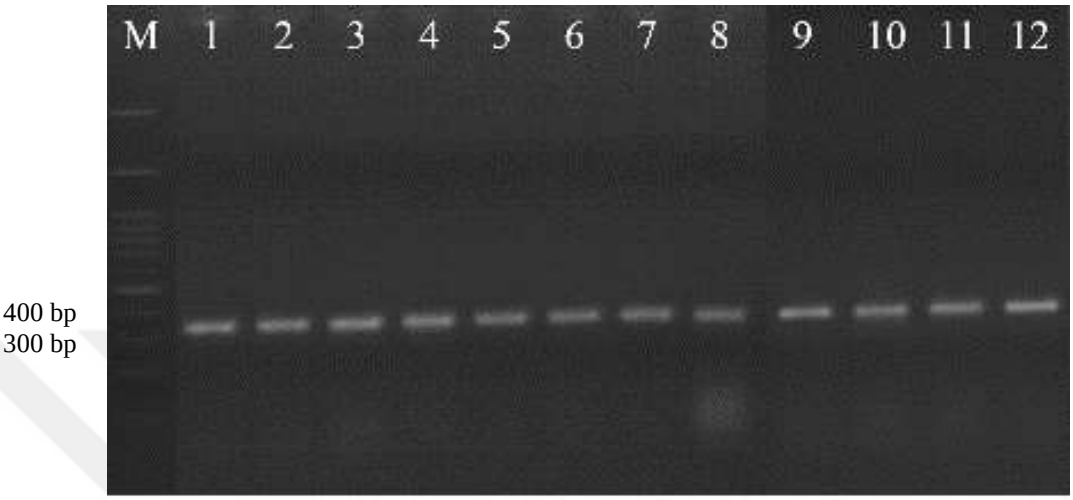

Figure 4.4 Amplicons obtained using species-specific primer pair MpKFI-MpKRI. M: 100 bp marker. 1-12 *Macrophomina phaseolina* isolates

4.4 Inoculation, Pathogenicity, and Variety Reaction Studies

Re-isolation studies were performed for plants inoculated using different inoculation methods and *M. phaseolina* were observed. Un-inoculated control plants did not exhibit any symptoms of disease and pathogen was not observed after Koch postulate application. *M. phaseolina* was successfully re-isolated from plants exhibiting disease symptoms. AUDPC % value was calculated based on disease severity % values on scoring days.

4.4.1 Sunflower Inoculation Studies

Disease symptoms were observed for all inoculation methods used in this study. After removal of toothpick, parafilm and pipette tips, disease development at the inoculation points and downwards was observed for all the methods (Figure 4.5). Despite that, they differed in disease progression and reproducibility. Independent sample t-test ($p<0.05$) was applied to AUDPC % data to check parameters such as repeatibility and effect of inoculum quality of applied methods.

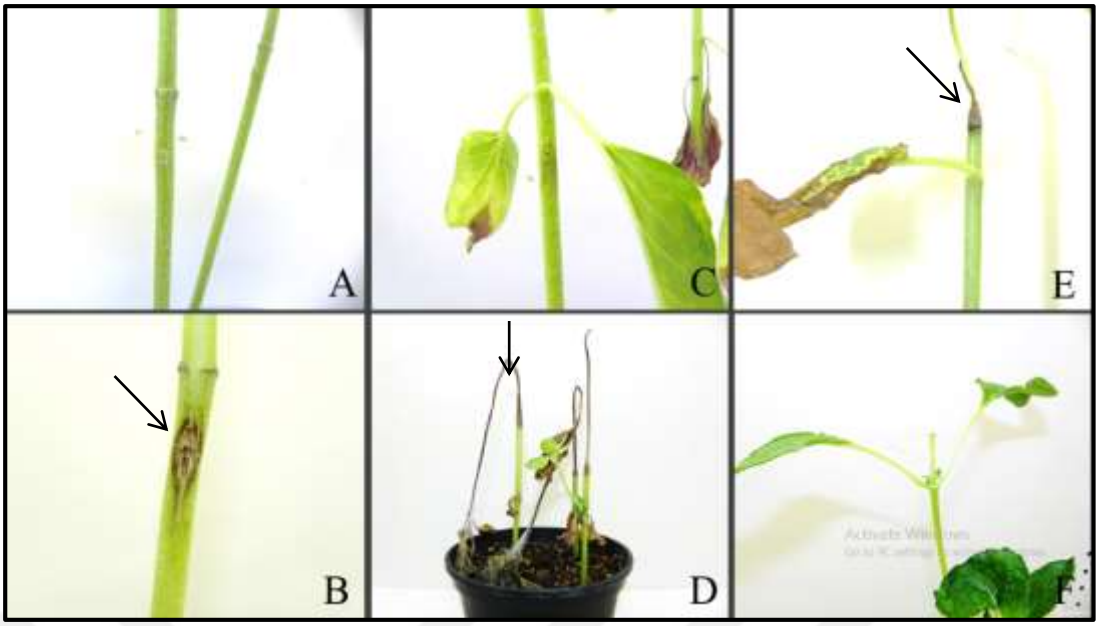

Figure 4.5 Symptoms developed on control and diseased sunflower plants inoculated using different inoculation methods. Toothpick method control (A) and inoculated (B); stem tape methods control (C) and inoculated (B); cut stem method inoculated (E) and control (F) plants. The symptoms developed *in vitro* were indicated with arrows.

4.4.1.1 Effect of Inoculum Maturity on Incubation Period of Disease on Sunflower

AUDPC % data was subjected to independent sample t-test ($p<0.05$) to determine the differences among the inoculum maturity types. AUDPC % data was significantly different between 3 days old inoculum and 10 days old inoculum for both repeats of stem tape inoculation method, however, effect of inoculum on disease progression of plants inoculated with cut stem method was significantly different only for second repeat (Table 4.2).

Table 4.8 Results of independent sample t-test applied on AUDPC % data to analyze significance of difference between inoculum maturity types ($p<0.05$)

Repeat	Method	t-test for Equality of Means		
		t	df	Sig. (2-tailed)
AUDPC% 1st repeat	CS3	1.177	14	0.259
	CS10	1.177	14	0.259
AUDPC% 2nd repeat	CS3	2.723	14	**0.016**
	CS10	2.723	7.373	**0.028**
AUDPC% 1st repeat	ST3	12.72	14	**0**
	ST10	12.72	13.52	**0**
AUDPC% 2nd repeat	ST3	10.94	14	**0**
	ST10	10.94	11.95	**0**

CS3: Cut-stem inoculation method with 3 days inoculum; CS10: Cut-stem inoculation method with 10 days old inoculum; ST3: Stem-tape inoculation method with 3 days old inoculum; ST10: Stem-tape inoculation method with 10 days old inoculum.

4.4.1.2 Disease Progression

Fastest progression was observed for stem-tape method and cut-stem method applied using 3 days old inoculum and was followed by same methods applied using 10 days old inoculum and toothpick method (Figure 4.6). Difference in disease progression of stem-tape highly depended on inoculum type, however the same situation was not observed for cut-stem inoculation method.

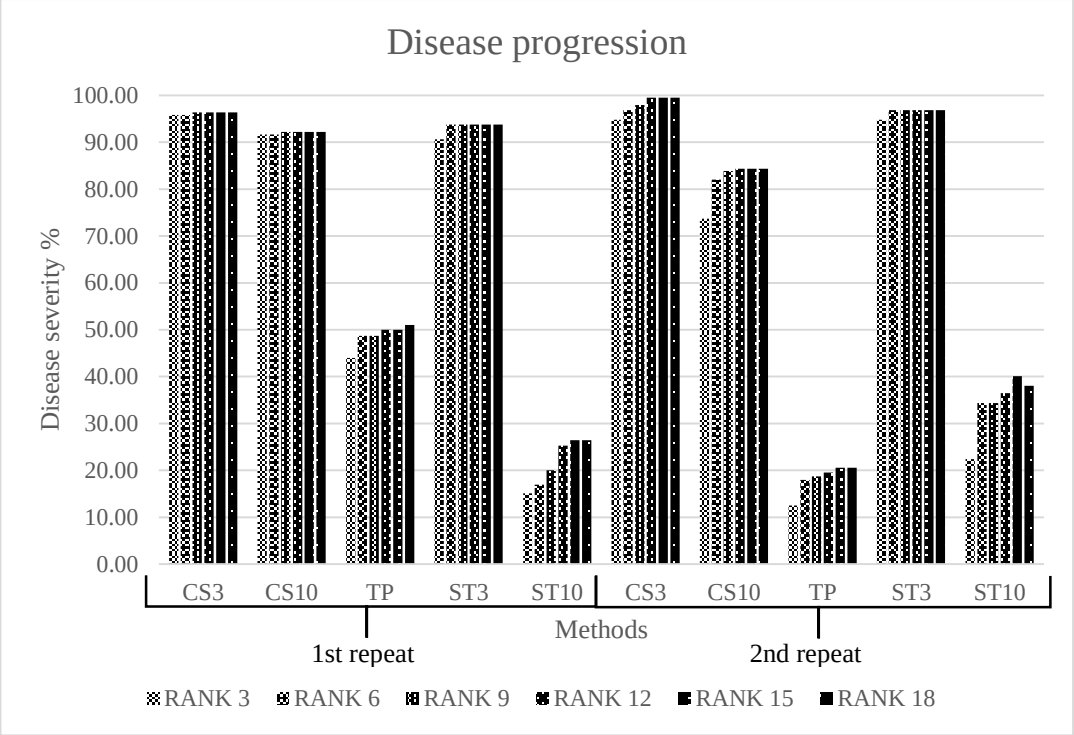

Figure 4.6 Disease severity% data of different methods on scoring days for sunflower; CS3: Cut-stem inoculation method with 3 days inoculum; CS10: Cut-stem inoculation method with 10 days old inoculum; ST3: Stem-tape inoculation method with 3 days old inoculum; ST10: Stem-tape inoculation method with 10 days old inoculum; TP: Toothpick inoculation method

One-Way ANOVA analysis and Tukey-HSD tests were applied on AUDPC % data to assess difference between the methods (stem tape, cut stem, toothpick). Stem-tape method applied using 10 days old inoculum and toothpick method were significantly different from 3 other inoculation methods for both repeats (p<0.05) (Table 4.3).

Table 4.9 Results of One-Way ANOVA applied on AUDPC % data according to methods applied on sunflower (Tukey-HSD; p< 0.05).

Methods*	1st Repeat**	2nd Repeat
ST10	21.27[a]	18.11[a]
TP	48.53[b]	33.94[a]
CS10	91.99[c]	81.91[b]
ST3	93.18[c]	96.49[b]
CS3	96.16[c]	97.86[b]

*ST10: Stem type, 10 days old inoculum; CS10: Cut stem, 10 days old inoculum; ST3: Stem type, 3 days old inoculum; CS3: Cut stem, 3 days old inoculum; TP: toothpick inoculation
**p<0.05 (Forms denoted by the same letter in the same column are not significantly different according
to TukeyHSD test (α= 0.05))

Independent sample t-test ($P < 0.05$) was applied on AUDPC % value to determine reproducibility of methods. Results of analysis suggests that 2 repeats were not significantly different for cut-stem and stem-tape inoculation methods for both inoculum types and repeats of toothpick inoculation method was significantly different from each other (Table 4.4).

Table 4.10 Results of independent sample t-test applied on AUDPC % data of methods according to repeats (p<0.05)

Methods*	t-test for Equality of Means		
	t	df	Sig. (2-tailed)
CS3	-0.633	14	**0.537**
	-0.633	8.922	**0.543**
CS10	1.603	14	**0.131**
	1.603	9.497	**0.142**
TP	3.517	14	0.003
	3.517	13.117	0.004
ST3	-0.698	14	**0.496**
	-0.698	13.692	**0.496**
ST10	-1.954	14	**0.071**
	-1.954	13.869	**0.071**

*ST10: Stem type, 10 days old inoculum; CS10: Cut stem, 10 days old inoculum; ST3: Stem type, 3 days old inoculum; CS3: Cut stem, 3 days old inoculum; TP: toothpick inoculation

4.4.1.3 Variety Reactions of Sunflower Cultivars

Stem-tape inoculation method with 3 days old inoculum was applied for variety reaction assay as this method exhibited minimum difference between repeats and fast

symptom development. Charcoal rot symptoms were observed on all the varieties and control plants did not developed any symptoms. No resistant variety was determined and of 9 varieties studied, all plants exhibited moderately susceptible or susceptible reaction to *M. phaseolina* (Figure 4.7). Some symptomatic differences between varieties were observed.

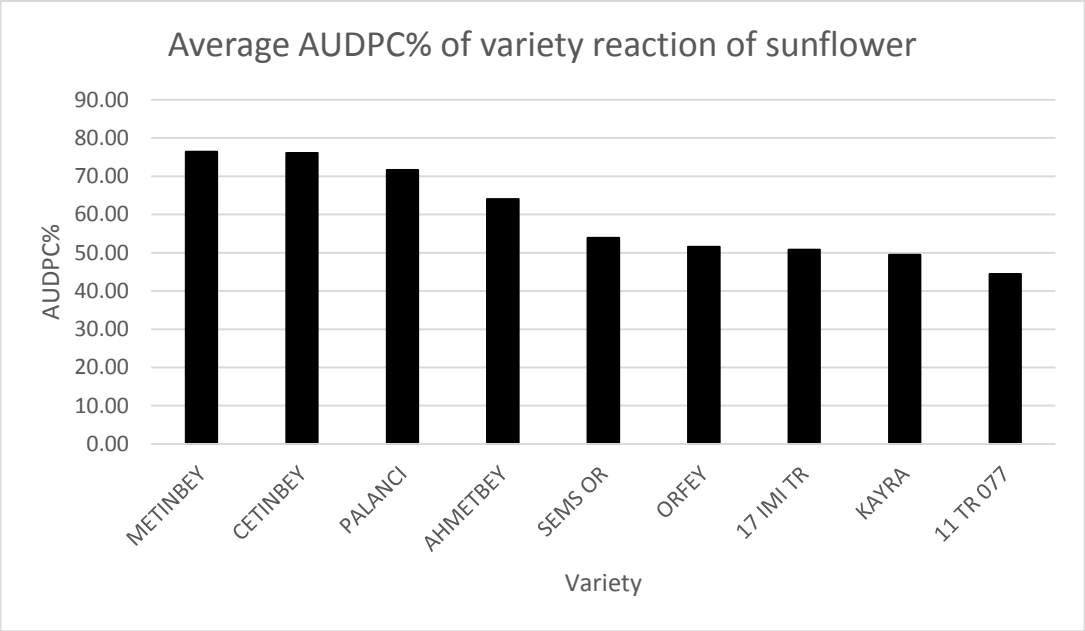

Figure 4.7 AUDPC % value of variety reactions of sunflower cultivars

Sems OR, 11 TR 077 and 17 TR Imi 003 varieties exhibited sub-epidermal lesions that cannot be seen without careful examination. Lesions that reached to node induced yellowing of the leaf and petiole. 11 TR 077 variety showed slow development of disease at the first week, but later disease progression gained speed. Lesions caused by *M. phaseolina* completely surrounded stem at the inoculation point for Palanci, Ahmetbey and Metinbey varieties, and after that disease progressed in both vertical directions. Lesions were observed on petioles of Kayra variety and on 17 IMI TR 003 variety lesions advanced as 2-3 thin lines. Lesions on Orfey and Sems OR varieties were local to inoculation point (Figure 4.8).

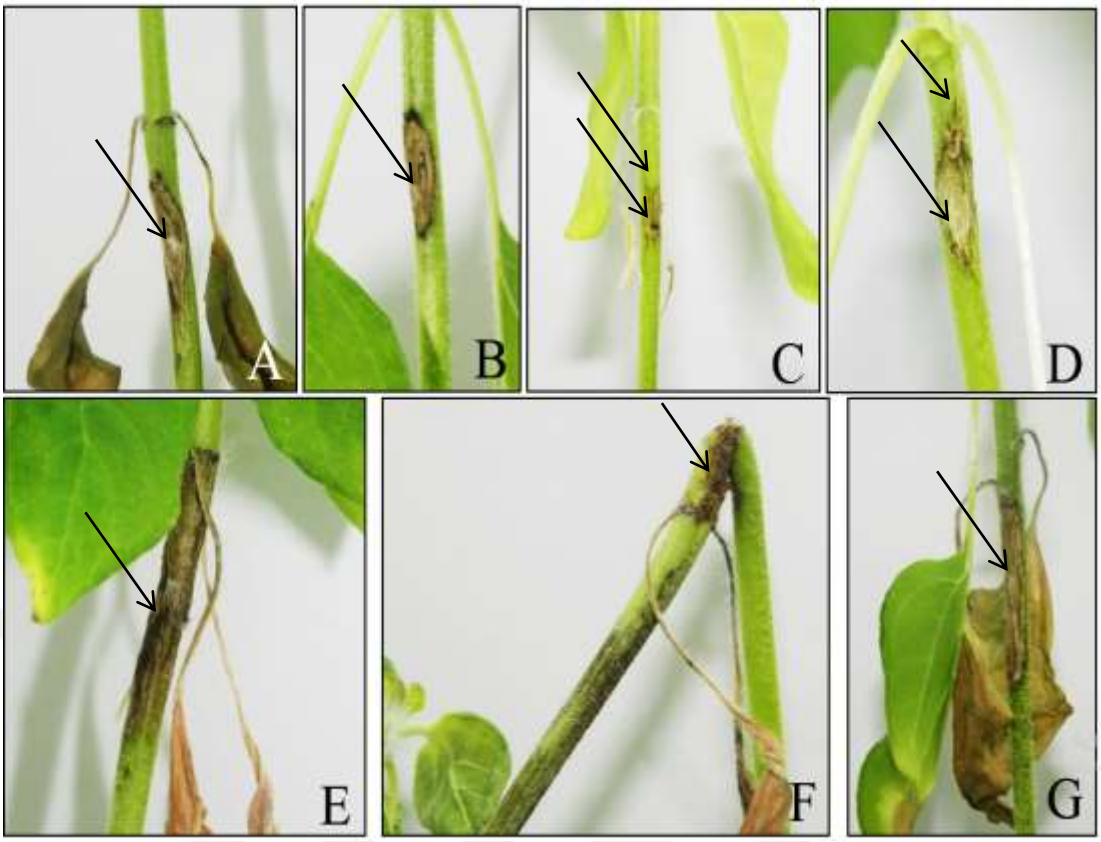

Figure 4.8 Symptoms developed on different sunflower varieties; A: Kayra; B: 17 TR IMI 003; C: Sems OR; D: 11 TR 077; E: Palanci; F: Metinbey; G: Deray. The lesions are indicated with arrows.

Independent sample t-test ($p<0.05$) was applied to AUPDC% value of both repeats to define if reactions of varieties were statistically different. Results showed no difference between repeats (Table 4.6).

Table 4.5 Results of independent sample t-test analysis applied to AUDPC% data of variety reactions data of both repeats ($p<0.05$)

Variable	t-test for Equality of Means		
	t	df	Sig. (2-tailed)
AUDPC %	.835	16	.416
	.835	16.000	.416

All parameters of methods are summarized in table 4.7. Results of applied statistical analysis suggest that inoculum maturity is important element for successful application of stem-tape inoculation method and not as important for cut-stem

inoculation method. In addition, cut-stem and stem-tape inoculation methods did not have significant difference between repeats, and difference between repeats of toothpick inoculation method was significant. Disease progression is fast for cut-stem inoculation method and stem-tape inoculation method applied using immature inoculum.

Table 4.6 Summarized results of all inoculation methods performed on sunflower

Parameters	**Methods***		
	CS	TP	ST
Inoculum	Not important	-	Important
Reproducibility	High	Low	High
Disease progression	Fast	Slow	Depends on inoculum maturity

* CS: Cut stem, TP: Toothpick, ST: Stem tape;

4.4.2 Soybean Inoculation Studies

Disease symptoms were observed for all inoculation methods used in this study. After removal of toothpick, parafilm and pipette tips, disease development was observed for all applied methods (Figure 4.9) and disease severity was scored every 3 days. Methods differed in disease progression and repeatability. Independent sample t-test ($p<0.05$) was applied on AUDPC% data to check parameters such as reproducibility and effect of inoculum maturity on applied methods.

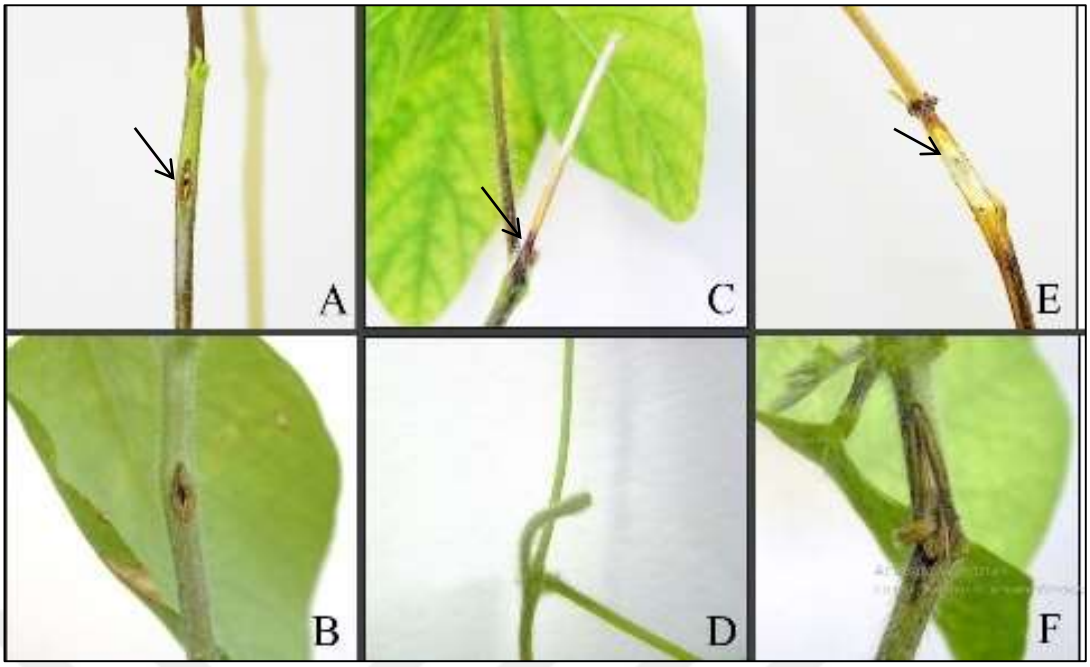

Figure 4.9 Symptoms developed on control and diseased sunflower plants inoculated using different inoculation methods. Toothpick method inoculated (A) and control (B) plants; cut stem inoculated (C) and control (D) plants; Stem tape inoculated (E) and control (F) plants. The lesions are indicated with arrows.

4.4.2.1 Effect of Inoculum Maturity on Disease Development on Soybean Plants

AUDPC% values were calculated and used for statistical analysis to determine significance of the effect of inoculum maturity on disease development. AUDPC % values of 2 different inoculum qualities and inoculation methods was subjected to independent sample t-test ($p<0.05$). AUDPC% values of 2 inoculum types were found significantly different for both repeats of stem tape inoculation method and significantly different only for 1 repeat of cut-stem inoculation method (Table 4.8).

Table 4.7 Results of independent sample t-test applied on AUDPC% data to analyze significance of difference between inoculum maturity types ($p<0.05$)

Repeats	Methods*	T-Test for Equality of		
		t	df	Sig. (2-Tailed)
1st Repeat	CS3	-	14	0.862
	CS10	-	13.43	0.862
1st Repeat	ST3	5.0	14	**.000**
	ST10	5.0	11.77	**.000**
2nd Repeat	CS3	2.2	14	**.043**
	CS10	2.2	12.244	**.046**
2nd Repeat	ST3	7.2	14	**.000**
	ST10	7.2	9.884	**.000**

*CS3: Cut-stem inoculation method with 3 days inoculum; CS10: Cut-stem inoculation method with 10 days old inoculum; ST3: Stem-tape inoculation method with 3 days old inoculum; ST10: Stem-tape inoculation method with 10 days old inoculum.

4.4.2.2 Disease Progression

Disease severity % values of scoring days is given in Figure 4.10. Disease severity % values was highest for stem-tape inoculation method applied using 3 days old inoculum and success of stem-tape inoculation method highly depends on inoculum maturity. However, the same statement is not appliable on cut-stem inoculation method (Figure 4.10).

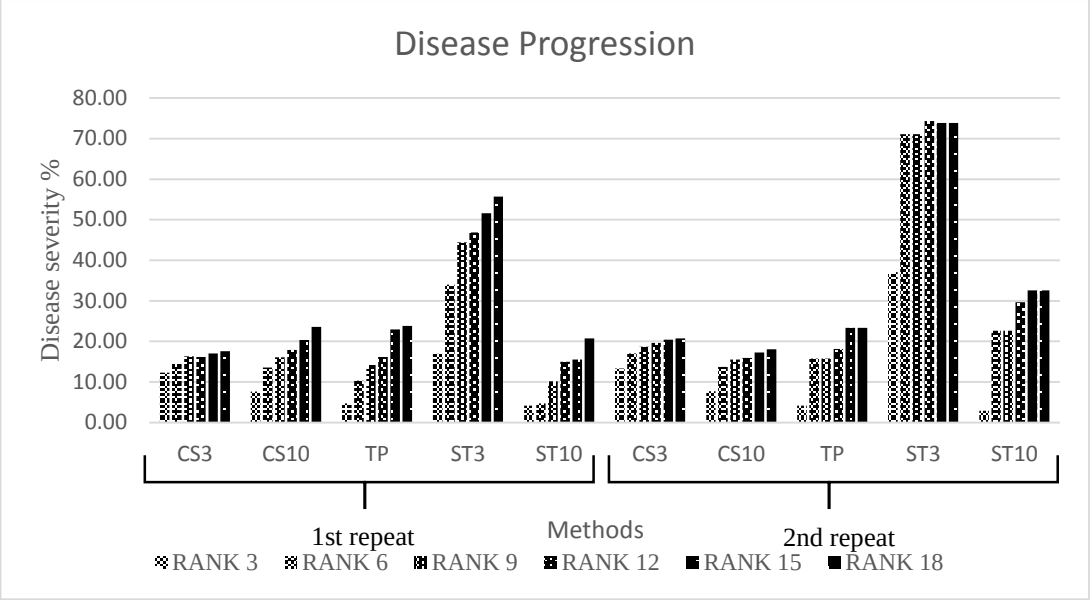

Figure 4.10 Disease severity% data of different methods on scoring days for soybean; CS3: Cut-stem inoculation method with 3 days inoculum; CS10: Cut-stem inoculation method with 10 days old inoculum; ST3: Stem-tape inoculation method with 3 days old inoculum; ST10: Stem-tape inoculation method with 10 days old inoculum. TP: toothpick inoculation method

One-Way ANOVA ($p<0.05$) analysis was applied together with Tukey-HSD to determine significance of difference between methods based on AUDPC% value. Results suggested that the stem-tape applied using 3 days old inoculum was significantly different from other 4 methods for both repeats ($p<0.05$) (Table 4.9).

Table 4.8 Results of One-Way ANOVA applied on AUDPC % data according to methods (Tukey-HSD; $p<0.05$).

Method	1st Repeat	2nd Repeat
CS3	10.87[a]	14.4 [a]
CS10	14.57 [a]	16.13 [a]
TP	15.47 [a]	18.08 [a]
ST3	15.86 [a]	23.03 [a]
ST10	40.29 [b]	66.25 [b]

$p<0.05$ (Forms denoted by the same letter in the same column are not significantly different according to TukeyHSD test ($\alpha= 0.05$))

*ST10: Stem type, 10 days old inoculum; CS10: Cut stem, 10 days old inoculum; ST3: Stem type, 3 days old inoculum; CS3: Cut stem, 3 days old inoculum; TP: toothpick inoculation

AUDPC% data was subjected to independent sample t-test to check significance of difference between 2 repeats of the same method ($p<0.05$). Results of applied analysis suggests that there is no difference between AUDPC% of both repeats for cut-stem and toothpick inoculation methods (Table 4.10).

Table 4.9 Results of independent sample t-test applied on AUDPC % data of methods according to repeats ($p<0.05$)

Methods*	**t-test for Equality of Means**		
	T	Df	Sig. (2-Tailed)
CS3	-1.19	14	**0.254**
	-1.19	13.382	**0.255**
CS10	0.875	14	**0.875**
	0.875	12.173	**0.875**
TP	-0.346	14	**0.735**
	-0.346	13.901	**0.735**
ST3	-3.559	14	0.003
	-3.559	13.889	0.003
ST10	-3.062	14	0.008
	-3.062	13.422	0.009

*CS3: Cut-stem inoculation method with 3 days inoculum; CS10: Cut-stem inoculation method with 10 days old inoculum; ST3: Stem-tape inoculation method with 3 days old inoculum; ST10: Stem-tape inoculation method with 10 days old inoculum; TP: Toothpick inoculation method

4.4.2.3 Variety Reactions of Soybean Cultivars

Cut-stem inoculation was applied using 3 days old inoculum and study was repeated 2 times as it was mentioned above this method have high reproducibility rate. Because of germination problems, variety reaction studies for Mersoy and Mono varieties was performed only once, and due to low number of groups, statistical analysis could not be applied (Figure 4.11).

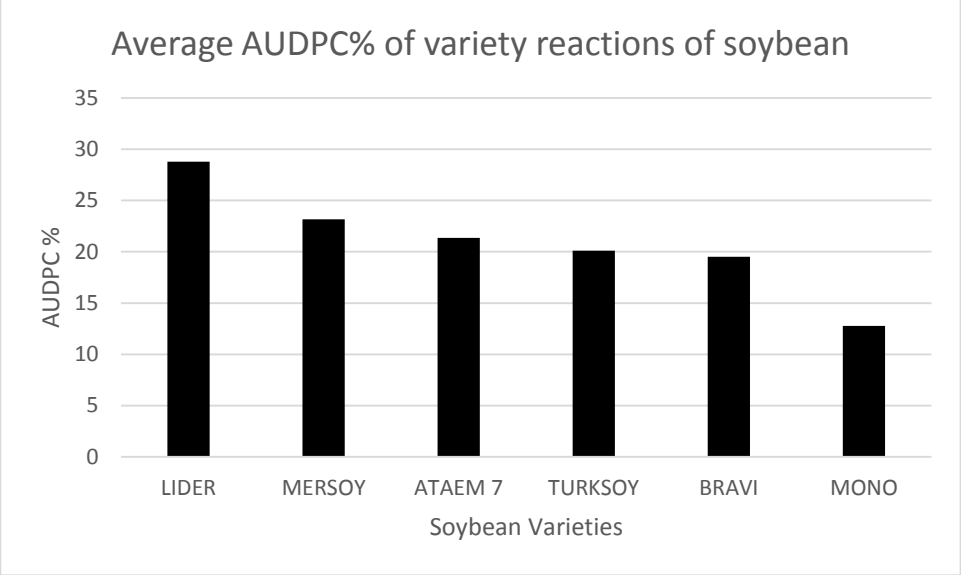

Figure 4.11 AUDPC % value of variety reactions of soybean cultivars

Difference in developed symptoms of varieties were as follows: local lesions on Ataem variety, yellowing of oldest leaves on Bravo variety, slight yellowing of all plant for Lider variety independent from lesion progress and yellow lesions on Mersoy variety (Figure 4.12).

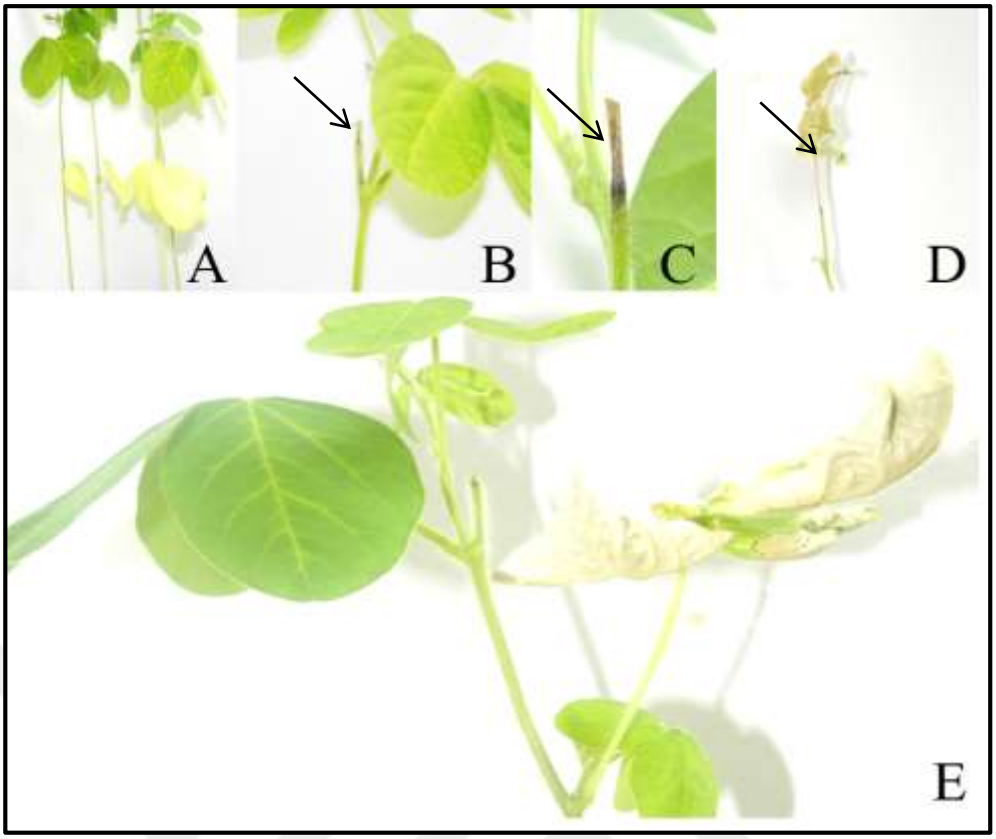

Figure 4.12 Symptoms developed on different soybean varieties; A: Bravo; B: Bravo; C: Ataem-7 ; D: Mersoy; E: Mersoy. The lesions were indicated with arrows.

Analyzed parameters are summarized in table 4.12. Inoculum maturity is important factor affecting disease development when stem-tape inoculation method is applied and have moderate importance for application of cut-stem inoculation method. Cut-stem and toothpick inoculation methods are repeatable methods, but disease development on plants inoculated using those methods is slow. Stem-tape inoculation method shows different between repeats and disease development speed highly depends on inoculum maturity, however this method shows difference between repeats.

Table 4.11 Summarization of all results of soybean inoculation methods

Parameters	Method		
	CS	TP	ST
Inoculum	Average	-	Important
Repeatability	High	High	Low
Disease progression	Slow	Slow	Depends on inoculum maturity

*ST: Stem type; CS: Cut stem; TP: Toothpick

4.5 Pathogenicity

All isolates collected from soybean, maize and sunflower plants were pathogenic on hosts they were isolated from (Figure 4.13).

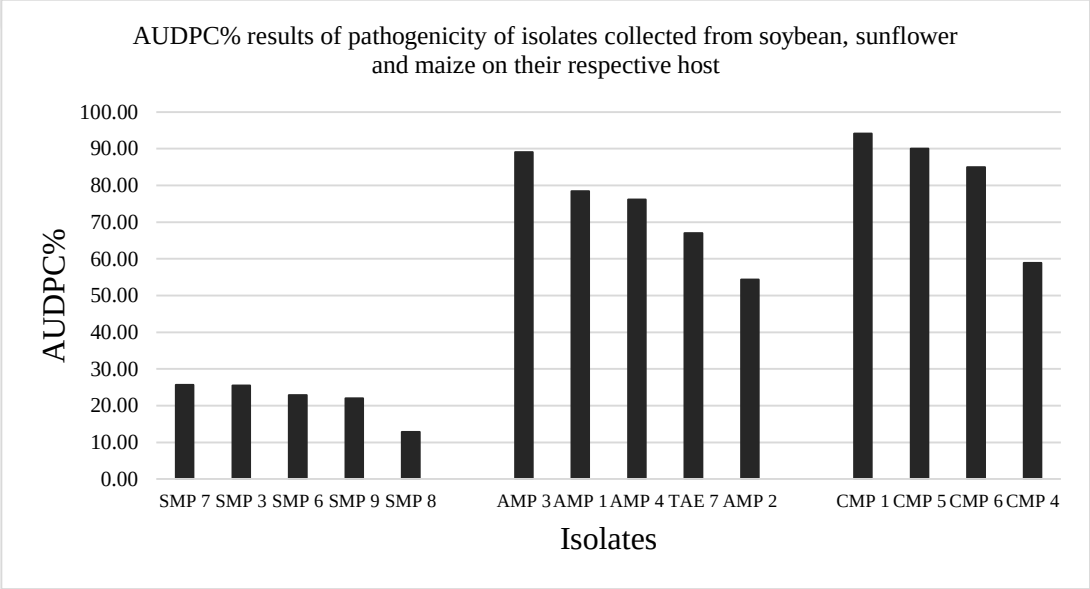

Figure 4.13 AUDPC % values of isolates collected from soybean, sunflower and maize

4.6 Phylogenetic Analysis

ITS and β-tubulin regions were amplified with ITS5-ITS4 and Bt1a-Bt1b primer pairs, respectively. Approximately 600-650 bp amplicon for ITS and 500 bp amplicon for β-tubulin were observed (Figure 4.14). BLASTn analysis was performed on sequence data for verification of species.

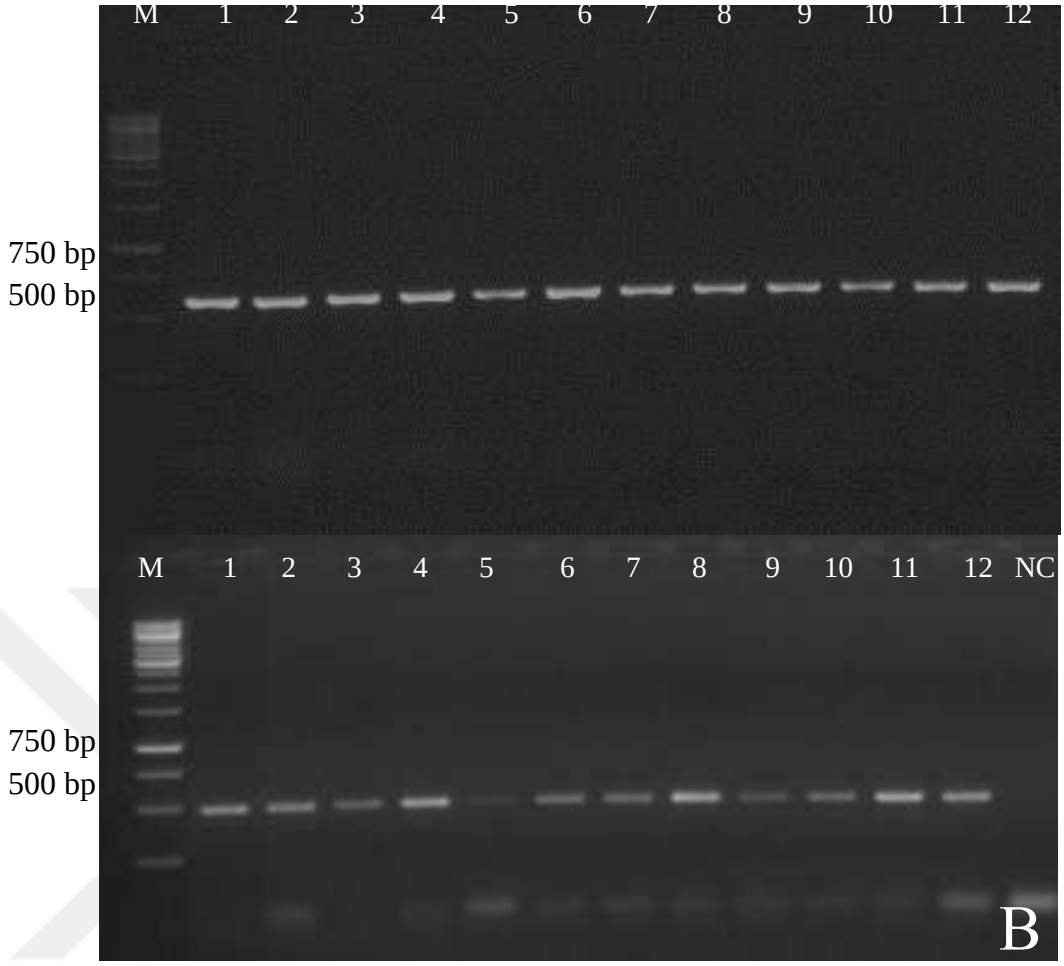

Figure 4.14 Agarose gel electrophoresis of PCR products; A: ITS region; B: β-tubulin region; M: 1 kb DNA ladder

Phylogenetic analyses involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. MN341226.1 and KU720484.1 sequences belong to *Lasiodiplodia theobromae* species and were used as outgroup sequences for ITS and β-tubulin, respectively. All phylogenetic analysis was supported with >50 bootstrap value.

Eleven isolates clustered in 3 different groups and SMP 3 isolate did not clustered in any of the groups based on ITS sequence data. Group 1 comprised of 4 sunflower isolates, 3 maize isolates gathered in group 2, and group 3 consists of 3 soybean and 1 maize isolates (Figure 4.15).

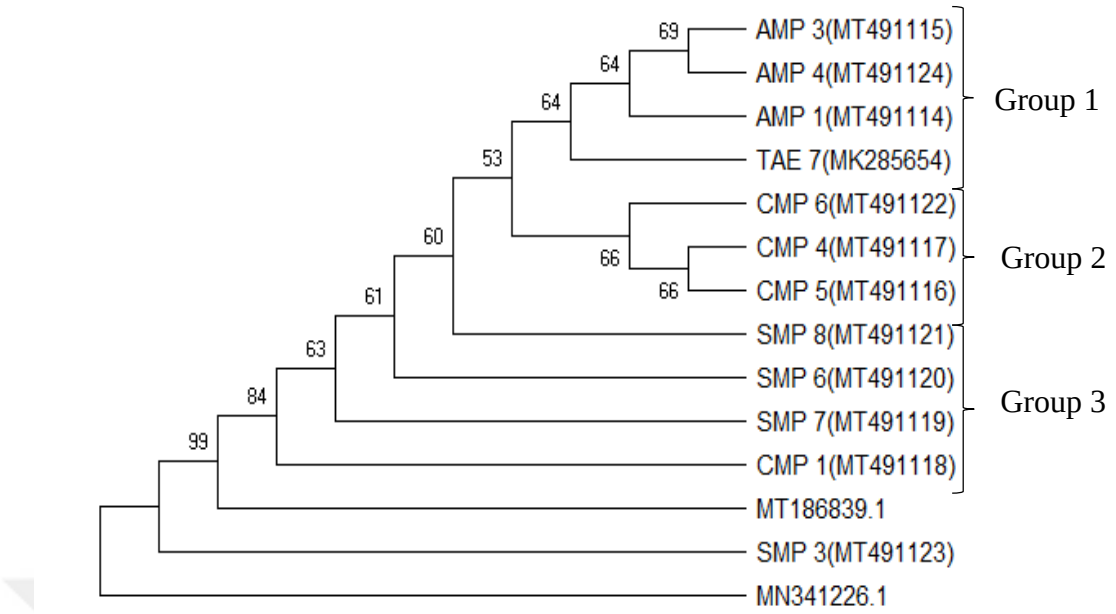

Figure 4.15 Phylogenetic tree of *M. phaseolina* isolates generated based on ITS sequence data

β-tubulin region failed to cluster any of the isolates into groups (Figure 4.16).

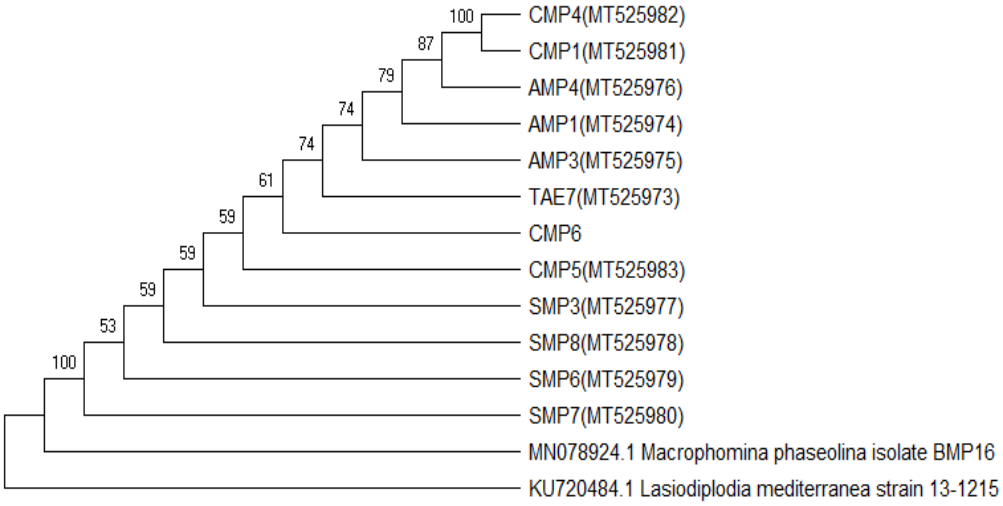

Figure 4.16 Phylogenetic tree of *M. phaseolina* isolates generated based on β-tubulin sequence data

CHAPTER V

DISCUSSION

Macrophomina phaseolina is fungal agent that causes charcoal rot disease on many economically important crop plants (Ijaz et al., 2013). Causal agent was reported from Turkey from maize, sunflower, soybean etc. (Karcılıoğlu et al., 1985; Tatlı and Sağır, 1992; Mahmoud and Budak, 2011) . There are a lot of diverse factors affecting disease development such as environmental conditions, inoculum load, exposure duration and plant age (Jordaan et al., 2019; Khamari et al., 2019; Chaturvedi, 2020). This study assessed different inoculation methods in combination with different inoculum maturity types were applied on 3 weeks old soybean and sunflower seedlings under growth chamber conditions to assess effect of inoculum maturity on disease development and applicability of chosen method for variety reaction studies. Phylogenetic analysis was also applied on *M. phaseolina* isolates collected from soybean, sunflower and maize plants showing charcoal rot disease symptoms.

Pathogen was successfully isolated from plant tissues with embedded microsclerotia. Average length and width of microsclerotia observed on sunflower, maize and soybean plants were higher than those of previously reported by Mahmoud and Budak (2011) and close to values reported by Bokor, (2007) on sunflower (Table 4.1). Since accurate and rapid identification of causal agent accelerates phytopathological studies, importance of species-specific primers grows as a valuable tool for species identification at molecular level. Primer pair suggested by Babu et al., (2007) was demonstrated as a useful tool for rapid molecular identification of current pathogen by many researchers. Current primer pair successfully amplified species-specific region (Figure 4.4) from *M. phaseolina* in our study.

Cut-stem, stem-tape and toothpick inoculation methods are among the most popular inoculation techniques used for pathological studies under field, greenhouse and growth chamber conditions.

(Zazzerini and Tosi, 1989; Pratt et al., 1998; Omar et al., 2007; Twizeyimana et al., 2012; Viteri and Linares, 2017). However, applicability of mentioned methods except cut-stem inoculation under growth chamber conditions remains unknown. Toothpick, stem tape and cut-stem inoculation methods were applied on 3 weeks old sunflower and soybean seedlings under fully controlled conditions in this study. Stem-tape and cut-stem inoculation methods applied using 3 days old inoculum under growth chamber conditions were found as the most suitable for application on sunflower and soybean, respectively.

According to Twizeyimana et al. (2012) equal distribution of inoculum amount among plants is important factor during phytopathological studies. In addition Khamari et al. (2019) indicated importance of inoculum load and exposure time on disease development. Quantification of inoculum amount on toothpicks is tedious process, and difference between toothpicks can cause differences not linked to inoculation conditions and germplasm characteristics. Chaturvedi (2020) highlighted importance of plant development stage on disease incidence. Their findings suggest that older plants become more susceptible to charcoal rot disease. Toothpick inoculation method were mainly used for pathogenicity studies on diverse array of hosts under greenhouse or field conditions on mature plants between 6-8 weeks old plants depending on crop species (Pratt et al., 1998; Avilés et al., 2008). Low repeatability on sunflower seedlings and slow disease development on both hosts inoculated with toothpicks possibly due to vegetative development stage of seedling and makes toothpick inoculation method inappropriate for fast screening studies on soybean and sunflower seedlings under growth chamber conditions (Figure 4.6; Figure 4.9; Table 4.4).

Effect of inoculum maturity on disease development was reported by Meyer et al. (1974). However, their study was performed under field conditions and effect of inoculum maturity on disease development *in vitro* was not previously reported. Hyphal and mature inoculum was applied on soybean and sunflower plants to study effect of inoculum on incubation period of disease. Results of independent sample t-test applied on AUDPC % data suggests that inoculum maturity is also important for *in vitro* studies and inoculation with fresh hyphae significantly accelerates disease development (Figure 4.6; Figure 4.9; Table 4.2; Table 4.8). Similarly Hemmati et al. (2018) performed histopathological studies and reported that susceptible and resistant

cultivar did not show difference in amount of microsclerotia attached to root and formation of appressoria and differences in disease development was observed after penetration of hyphae into inner plant tissues. As fresh inoculum consists mainly of hyphal structures rapid disease progression can be considered as normal due to skipping microsclerotia germination stage (Figure 4.6; Figure 4.9).

Role of jasmonate and auxin during necrotrophic pathogen attack was previously reviewed by Bari and Jones, (2009) and Pieterse et al. (2012). Auxin induces jasmonate biosynthesis and suppresses salicylic acid. Later jasmonate induces synthesis of defense related genes. Thus, auxin together with jasmonate are vital compounds for disease tolerance against necrotrophic fungi such as *M. phaseolina*. According to Gaige et al. (2010) and Mah et al. (2012) application of exogenous auxin and jasmonate decreased disease development rate during *M. phaseolina* infection. We have defined difference in symptom development on sunflower and soybean varieties and this could be explained by different disease resistance induced by auxins and jasmonate (Figure 4.7; Figure 4.10).

Importance of jasmonate and auxin during wound regeneration was reviewed by De Bruxelles and Roberts (2001). As wounding triggers jasmonate and auxin biosynthesis stem-tape and cut-stem inoculation methods applied plants enhances their resistance. We propose stem-tape inoculation technique for application on sunflower seedlings and cut-stem inoculation method for application on soybean seedlings under growth chamber conditions due to their wounding inoculation type that induces plant response. However, further studies are necessary to reveal molecular and physiological basis of disease response in soybean and sunflower to *M. phaseolina*, and variety reactions studies under growth chamber conditions should be analyzed not only symptomatically, but also physiologically.

As it is widely known, young leaves are main producers of auxin and seedling without young leaves cannot produce auxin, main inducer of jasmonate biosynthesis. Tuite (1969) mentioned that predisposition and susceptible host are among important factors affecting success of pathogenicity studies. Almost all young leaves were cut off on 3 weeks old sunflower seedlings at V4 development stage subjected to cut-stem inoculation method and new branches are not developed, and sunflower seedling becomes highly susceptible against necrotrophic pathogen attack. Therefore,

we propose cut-stem inoculation method on sunflower as fast pathogenicity screening method under fully controlled conditions, but it is not suitable for other studies such as variety reactions. Opposite situation is observed on soybean seedlings. Formation of new branches on lower nodes and development of new leaves was observed on soybean subjected to cut-stem inoculation method and this method could be used for fast germplasm and pathogen population screening assays under fully controlled conditions as suggested by Twizeyimana et al. (2012).

Mengistu et al. (2007) mentioned that AUDPC value is highly affected by environmental conditions when pathological studies are performed under field conditions, but results of our analysis suggest that AUDPC % value is highly informative when applied on disease severity % obtained during growth chamber studies when appropriate inoculation and scaling methods are applied (Table 4.4; Table 4.10). Disease development stages reported by Hemmati et al. (2018) suggests that time necessary for successful infection shows difference between susceptible and resistant cultivars. Since AUDPC % value is representing speed of disease progression over time we suggest AUDPC % value as a determining factor for characterization of resistance sources against charcoal rot disease together with disease severity and disease incidence parameters under fully controlled conditions and as repeatable quantitative value that could be used for phytopathological studies linked with charcoal rot disease.

Zazzerini and Tosi (1989) observed lesions at inoculation point within 48 hours after inoculation with 7 days old culture and reported that plants start to wilt 7 days after inoculation. However, during our study, death of plants was observed within 48 hours after inoculation on susceptible sunflower seedlings inoculated with hyphal inoculum (Table 4.6).

There is no resistance reported for cultivated sunflower, but potential resistance sources were reported in wild relatives. Among cultivated sunflower cultivars only moderately resistant and susceptible germplasms exist (Shehbaz et al., 2018). Results of variety reactions did not characterize any resistance among tested varieties and only 11 TR 077 variety can be considered as moderately resistant to charcoal rot (Table 4.5). Soybean cultivars with moderate resistance to charcoal rot were reported

by Mengistu et al. (2011). Our results suggest presence of resistance sources, but no complete resistance was observed (Table 4.11).

Su et al. (2001) reported high genetic similarity between *M. phaseolina* isolates collected from different hosts, but genetic variation according to host was observed based on RAPD and PCR-RFLP analysis. Jana et al. (2005b) reported that soybean originated isolates clustered in different cluster based on URP-PCR analysis. Arias et al. (2011) reported that SSR markers can differentiate *M. phaseolina* isolates according to hosts to some extent, however, Reznikov et al. (2018) did not observe any association between SSR and AFLP profile and host or geographic origin. In additions Sarr et al. (2014) and Machado et al. (2019) reported new speices that belongs to genus Macrophomina based on 5 gene regions analysis. ITS gene region is a useful tool for molecular barcoding of fungi according to Seifert (2009). Results of ITS sequence analysis of our study clearly differentiate sunflower isolates from other host originated ones. However, it can be linked also with geographical origin. Further studies with larger sample collection are necessary to generate profile of *M. phaseolina* population in Turkey. Cluster groups was not observed for β-tubulin gene region.

CHAPTER VI

CONCLUSION

This study aimed to apply widely used inoculation methods and test effect of inoculum maturity on disease development under fully controlled conditions, to check applicability of chosen methods during variety reactions studies on soybean and sunflower cultivars, and to define the phylogenetic analysis of *M. phaseolina* population. Results are as follows:

- Hyphal inoculum causes more rapid disease development and have short incubation period when compared to inoculum consisting of microsclerotia.
- On sunflower, the cut-stem inoculation method was found to be suitable only for pathogenicity studies.
- Stem-tape inoculation method applied using hyphal inoculum can be applied for variety reactions studies due to its high repeatability and symptomatic difference observed among cultivars during variety reactions.
- Cut-stem inoculation technique was proven to be repeatable method for pathogenicity and variety reactions studies on soybean.
- Toothpick inoculation method is not suggested for fast screening assays on soybean and sunflower, due to long incubation period and non-homogeneous distribution of inoculum.
- During variety reaction studies only 11 TR 077 variety was found moderately resistant among sunflower cultivars.
- No complete resistance was observed for soybean varieties tested.
- Phylogenetic analysis of ITS gene region formed 3 clusters with 1 cluster containing only sunflower originated isolates, 1 cluster containing only maize originated isolates and 1 cluster comprised of soybean and maize isolates. This data was supported with high bootstrap value >50.
- Phylogenetic analysis of β-tubulin did not form any cluster.

The data obtained through this study could enlarge our understanding in breeding studies against *M. phaseolina* of oilseed crop plants.

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APPENDIX

APPENDIX 1:

Mean AUDPC% of inoculation studies performed on soybean and sunflower

	Soybean İnoculation Studies AUDPC%		**SunflowerİnoculationStudies AUDPC%**	
Methods	First Repeat	Second Repeat	First Repeat	Second Repeat
CS3	15,47111742	18,08712121	96,16477273	97,86931818
CS10	15,86174242	14,39985795	91,99810606	81,91287879
TP	14,57149621	16,13399621	48,53219697	18,11079545
ST3	40,29356061	66,25236742	93,18181818	96,49621212
ST10	10,87831439	23,03503788	21,27130682	33,94886364