PHOMA MEDICAGINIS: A MODEL PATHOSYSTEM

FOR *MEDICAGO TRUNCATULA*

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CHAPTER I

INTRODUCTION

Phoma medicaginis Malbr. & Roum. is a filamentous ascomycete causing spring black stem and leaf spot disease of the perennial alfalfa, *Medicago sativa* L. and annual medic species such as *M. truncatula* Gaertner. The existence of this disease was first reported in the US in 1908 (17). Spring black stem and leaf spot is characterized by necrosis and chlorosis of stem, petiole, and leaves, resulting in yield losses (8). *P. medicaginis* reproduces asexually (mitosporically) as conidia, parasitizes its host necrotrophically and produces the toxin brefeldin A. It is reported to be the dominant inhabitant of the leaves and stems of *Medicago* species and the brefeldin A it produces can exclude other fungi from infected host tissues, creating a niche for itself (19).

Medicago species belong to the legume family, Fabaceae. Members of this family provide high-quality protein and oil to humans and livestock and improve soil fertility by fixing atmospheric nitrogen in the soil (5) through the formation of root nodules with rhizobial bacteria such as *Sinorhizobium meliloti* (Dangeard) De Lajudie. Extensive research has been conducted on the mutualistic symbioses between these bacteria and the model legume *M. truncatula*, furthermore many symbiosis-defective mutants have been generated (3, 18). Also, the interactions between *Medicago* roots and arbuscular endomycorrhizal fungi such as *Glomus intraradices* (Schenck & Smith) have

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been investigated (3). These mycorrhizal fungi supply phosphorous and other minerals from soil to the host and get photosynthates in exchange (15, 16).

The foliar ascomycetes, *Ascochyta rabiei* (Pass.) Labr. and *Colletotrichum trifolii* Bain, have been studied to better understand their pathogenicity on legumes (9, 21). *A. rabiei* infects chickpea (*Cicer arietinum* L.) resulting in Ascochyta blight, while *C. trifolii* causes anthracnose of alfalfa. Research carried out on *A. rabiei* has been focused on its interaction with chickpea, a relatively intractable host. *Leptosphaeria maculans* (Desm.) Ces. & De Not., *Cladosporium fulvum* Cooke, and *Magnaporthe grisea* (Hebert) Barr have been proposed as hemibiotrophic fungal pathosystems for the genomically tractable model hosts, *Arabidopsis thaliana* (L.) Heynh., tomato (*Lycopersicon esculentum* Miller) and rice (*Oryza sativa* L.), respectively. So far, there are no well-studied pathosystems available for the model legumes, such as *M. truncatula* or *Lotus japonicus* (Regel) Larsen.

To design successful disease control strategies it is important to define a pathogen's interactions with its host, the virulence factors it employs, and the different genes controlling these interactions. Ideally, both the pathogen and the host should be tractable to molecular manipulation. One of the most direct approaches to understanding a pathogen's interaction with its host is to the generate mutants of the pathogen using various insertional mutagenesis methods, such as random mutagenesis, targeted gene disruption, and gene silencing. Changes in disease progression can then be analyzed by microscopy (7, 12).

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P. medicaginis is a suitable pathogen for such studies because it is: 1) ease to culture and maintain in the laboratory, 2) has a short generation time, 3) contains monokaryotic (uninucleate) hyphal cells and conidia, and 4) its amenability to transformation. *P. medicaginis* can grow on most common fungal media, (e.g. potato dextrose agar, oatmeal agar, malt extract agar, etc.) and is very stable under laboratory conditions even after repeated subculturing. Generally, conidia germinate within few hours in media and abundant pycnidia exuding spores are produced in one week. Extensive research has been conducted on the closely related fungus, *L. maculans* (anamorph: *Phoma lingam* (Tode) Desm.), and the sequencing of its genome is ongoing ((13); http://www.genoscope.cns.fr/). *L. maculans* causes black leg disease of canola, *Brassica napus* L., and studies with the model host *A. thaliana* have been initiated (1, 2).

In general, fungi can be mutagenized using chemical and physical mutagens or by insertional transgenic methods, such as restriction enzyme mediated integration (REMI) and *Agrobacterium tumefaciens*-mediated transformation (AMT) (4, 10, 14, 20). *A. tumefaciens* has the ability to transform fungal protoplasts, spores and hyphae (6). *A. tumefaciens* inserts T-DNA into its host's genome by heterologous recombination, potentially disrupting or perturbing the host's genes, and therefore acting as a mutagen. This method has advantages over chemical mutagenesis methods because the T-DNA "tags" the disrupted gene, aiding its identification. The high proportion of single T-DNA insertions in transformants simplifies gene identification over that of REMI, which often results in multiple insertions (10). Numerous fungal pathosystems of model plants have

begun to be examined at the cellular level using *Agrobacterium*-mediated transformation (11).

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CHAPTER II

LITERATURE REVIEW

Medicago species and their Diseases

Alfalfa, _Medicago sativa_ L.

Alfalfa (_Medicago sativa_ L.), also known as lucerne, is a perennial medic and a native of Iran and Central Asia (13, 50). It is currently cultivated in Asia, Europe, Australia, and the Americas. It was introduced into the United States in 1736 by colonists and again in the mid-1850s. Alfalfa is considered to be the most important forage crop in the United States (9). As with all legumes, alfalfa's mutualistic symbiosis with a soil bacterium, _Sinorhizobium meliloti_ (Dangeard) allows it to fix atmospheric nitrogen(9), resulting in a high-protein, high quality forage that is vital to the dairy industry. Another mutualistic symbiont of alfalfa roots is the arbuscular mycorrhizal fungus, _Glomus intraradices_, which supplies phosphorous and other minerals from soil to the host and gets photosynthates in exchange. Other valuable attributes of alfalfa include its relatively low input costs, its ability to improve soil fertility, and its use as the predominant source of nectar for honey production. In 2005, total production of alfalfa (hay) in the United States was about 76 million metric tons (NASS-USDA, 2005), valued at $7.3 billion. In Oklahoma, total production of alfalfa harvested from about 120,000

hectares in 2005 was 1.3 million metric tons, valued at $127 million (NASS-USDA, 2005).

The genus *Medicago* also includes annual medics such as *M. truncatula* (barrel medic), *M. polymorpha* (burr medic) and *M. scutellata* (snail medic). Annual medics are utilized as cover crops, short season forage crops, and weed-suppressing smother crops (35). Among the annual medics, *M. truncatula* has been developed as a model legume along with another legume, *Lotus japonicus*. *M. truncatula* shares many important characteristics with alfalfa, such as its mutualistic symbiotic associations with rhizobia and mycorrhizal fungi, and its high forage quality. Generally, most *Medicago* spp. are diploids or tetraploids with a basic haploid complement of 8 chromosomes (n = 8) (50). Cultivated alfalfa, for example, is a cross-pollinated (self-incompatible) tetraploid (59).

Medicago truncatula as a model legume

M. truncatula is a self-compatible, diploid (2n=16), with a small genome of about 500 Mbp. This combined with a fast generation time (~60 d) and robust seed production have made it the preferred medic for genetic research over alfalfa, which is a self-incompatible tetraploid (20, 90). As a legume, *M. truncatula* forms mutualistic symbioses with nitrogen-fixing rhizobacteria such as *Sinorhizobium meliloti* and arbuscular mycorrhizal fungi, such as *Glomus* spp. These symbiotic interactions are not formed by *Arabiodopis thaliana*, a major model plant system.

Numerous *M. truncatula* mutants in these symbioses have been generated using different mutagenesis methods such as exposing seed to the chemical, ethylmethane sulfonate (EMS) and ionizing radiation, like γ-rays, X-rays and fast neutrons. Mutants

with altered nodulation like *dmi* (3 loci, d̲oesn't m̲ake i̲nfection), *sunn* (s̲upern̲umerary n̲odulation mutant) and *skl* (s̲ic̲k̲le) were obtained from EMS-treated seed (17, 77). Several mutants affected in nodulation, nitrogen fixation and symbiosis with *Sinorhizobium*, were also generated using γ-irradiation (74). Thus, numerous *M. truncatula* mutants affected in mutualistic symbioses are available for studying interactions with pathogens, such as the subject of this proposal, *Phoma medicaginis* (87). Other mutagenesis strategies such as T-DNA tagging and transposon-tagging are also used to generate mutants of *M. truncatula* (24, 78). However, T-DNA-tagging is not the preferred method because it is too inefficient to saturate the genome. Tnt1 is a retrotransposon derived from tobacco, is the currently preferred method of insertional mutagenesis for *M. truncatula*. Tnt1 integrates into non-repetitive regions of the genome and has no site specificity (24, 85).

The genome of *M. truncatula* has almost been completely sequenced and a large number of EST libraries have also been sequenced and utilized for genome annotation and to construct microarrays (86). EST libraries have been prepared from different tissues of *M. truncatula* during numerous developmental stages, symbiotic interactions with *S. meliloti* and *G. intraradices*, and pathogenic interactions with *P. medicaginis*, *Phytophthora medicaginis* Hansen & Maxwell, *Meloidogyne incognita* Kofoid & White, and *Colletotrichum trifolii* (www.medicago.org). Another EST library was enriched for transcripts from root hairs of *M. truncatula* (22, 46). Proteomes from six different tissues like stem, leaf, seed pods, roots, flowers and cell suspensions of *M. truncatula* were

analyzed and 304 significant proteins were found (90). All these resources make *M. truncatula* an attractive model system.

Foliar diseases of alfalfa and annual medics

Alfalfa is susceptible to numerous plant pathogens such as bacteria, fungi, viruses, and nematodes. Among these pathogens, fungi cause most of the identified diseases of alfalfa. Fungal foliar diseases can blight and defoliate plants thereby reducing overall forage yield and quality (23, 52). In addition to causing yield losses, foliar diseases can also reduce forage quality through stimulating the production of phytoestrogen compounds like coumestrol, which is toxic to livestock and affects the rate of ovulation in sheep (6, 7, 68). For example, spring black stem disease increases the coumestrol levels in the annual medic, *M. polymorpha* var. *brevispina* (8). The production of coumestrol in *Medicago* spp. is also influenced by soil type and growth stage (7, 79).

The important foliar diseases of *Medicago* spp. include spring black stem and leaf spot (*P. medicaginis*), Lepto leaf spot (*Leptosphaerulina trifolii* (Rostr.) Petr.), anthracnose (*Colletotrichum trifolii*.), common leaf spot (*Pseudopeziza medicaginis* (Lib.) Sacc.) and summer black stem and leaf spot (*Cercospora medicaginis* Ellis & Everh.) (58). Most of these fungi are thought to be necrotrophic pathogens of *Medicago* spp., however little is known about the mechanisms utilized by these fungi to infect their host. By exploiting the genetic resources available in *M. truncatula* and new insertional mutagenesis techniques available in the fungi, the molecular nature of these plant-microbe interactions can be efficiently investigated.

Spring Black Stem caused by *Phoma medicaginis* Malbr. & Roum.

Taxonomy of *Phoma medicaginis*

Taxonomically, *P. medicaginis* belongs to the phylum Ascomycota, class Dothideomycetes, and order Pleosporales (72). However, familial placement of this asexual, mitosporic ascomycete is uncertain as it displays phylogenetic affinity to members of both the Leptosphaeriaceae and the Pleosporaceae. The fungus has had numerous names: *P. herbarum* var. *medicaginis* Westend. ex. Rabenh., *P. medicaginis* Malbr. & Roum., *Diplodinia medicaginis* Oud., *Ascochyta imperfecta* Peck, and finally as the variety *medicaginis*, to distinguish it from the chlamydosporous variety *pinodella.* Since var. *pinodella* has been raised to the species level, it follows that var. *medicaginis* should also be consolidated back to the level of species sensu Malbranche & Roumeguère (58).

With *Phoma herbarum* Westend. as the type species, the genus *Phoma* Fr. has been divided into 9 different sections to facilitate identification up to species. These subdivisions or sections include *Phoma, Heterospora, Paraphoma, Peyronellaea, Phyllostictoides, Sclerophomela, Plenodomus, Macrospora,* and *Pilosa* (12). Different teleomorphs associated with the genus *Phoma* include *Didymella* Sacc., *Leptosphearia* Ces. & De Not., *Mycosphearella* Johanson, and *Pleospora* Rabenh. ex Ces. & Des Not. *Phoma* species are identified based on the shape, size, and septation of conidia and pycnidial wall structure (12). *P. medicaginis* var. *medicaginis* belongs to the section *Phyllostictoides*. The only teleomorph reported in this section was *Didymella*. Important characteristics of members of this section include thin-walled, pseudoparenchymatous,

ostiolate pycnidia and conidia that are usually unicellular *in vitro*, but exhibit septa *in vivo,* depending on environmental conditions. Chlamydospores are rare, but, when present are unicellular and swollen. Members of the section, *Phyllostictoides* typically cause leaf spots and necrosis of leaves.

Closely Related Fungi

P. medicaginis var. *macrospora* Boerema, Pieters & Hamers is also a major pathogen of alfalfa and causes spring black stem and leaf spot of alfalfa (12). As the name suggests, *P. medicaginis* var. *macrospora* produces larger conidia (one to three septate) at low temperature (6°C) when compared to variety *medicaginis*. However, at room temperature (20-22°C) varietal differences in conidial size are insignificant, making distinguishing the two varieties difficult. Another significant difference between these two pathogens is that *P. medicaginis* var. *macrospora* seems to be more virulent on alfalfa than *P. medicaginis* var. *medicaginis*. This has been attributed to cold adaptation of *P. medicaginis* var. *macrospora,* which is thought to have originated in the cold mountains of southwest Asia.

Morphologically, *P. pinodella* appears to be closely related to *P. medicaginis* and was originally named *P. medicaginis* var. *pinodella* L.K. Jones. However, *P. pinodella* primarily infects pea and red clover and only rarely infects *Medicago* spp. Other features distinguishing *P. pinodella* from *P. medicaginis* include its frequent sectoring in culture, formation of crystals on MEA, and production of chains of chlamydospores. Because of

these and differences in the pycnidial wall, var. *pinodella* was raised to the status of species and became *P. pinodella* (L.K. Jones) Morgan-Jones & Burch (48).

Distribution and host range

P. medicaginis has been reported from Australia, Canada, Europe, and the United States. In the United States, the disease was first reported in New York in 1908 (83). In Australia, spring black stem is considered an important disease of annual medics such as *M. truncatula*, resulting in extensive defoliation and premature death of plants (2, 4, 45). In the United States, *P. medicaginis* has been reported from Idaho, Washington, Kentucky, Colorado, Kansas, Minnesota, Nebraska, and Oklahoma (34).

P. medicaginis has been reported to have a wide host range that includes many legumes such as *Arachis hypogaea* L. and *Glycine max* L. Merr. in Brazil, *Cicer arietinum* L., *Dolichos uniflorus* Lam., and *Pongamia pinnata* (L.) Pierre in India, *Glycine ussuriensis* Regel & Maack., (Zambia), *Medicago* spp. (worldwide), *Melilotus* spp. (China and U.S.) and *Trifolium* spp. (China, U.K. and Australia) (34). It has also been reported to be a pathogen of such non legumes as *Brassica* spp. (Canada), *Capsicum annum* L. (Brazil) and *Pennisetum clandestinum* Hochst. ex Chiov. (New Zealand). Recently, however, the host range of *P. medicaginis* has been reported to be limited to *Medicago* spp. (32). Clearly, the host range of this fungus needs to be re-assessed using well-characterized isolates inoculated on a comprehensive series of previously reported hosts.

Disease symptoms and pathogen life cycle

P. medicaginis can infect all parts of an alfalfa plant, including the roots, crown, stems, petioles, leaves and seeds, thereby extensively reducing the yield and forage quality of susceptible cultivars (7, 40, 48, 54, 70). *P. medicaginis* is considered to be both a soilborne and seedborne pathogen with the primary inoculum surviving in the soil on plant debris and seeds as pycnidia, mycelia and chlamydospores. The severity of infection depends upon temperature and humidity, and during cool, rainy weather, disease develops and spreads rapidly (67). Seedborne mycelia directly infect seedlings, and conidia produced in pycnidia on debris are disseminated to new host tissues by rain. All parts of the plant, but particularly older leaves near the crown, are susceptible to attack.

Germination of conidia starts within hours after inoculation and the emerging germ tubes grow rapidly. Inoculum between 10^5 to 10^7 spores/ml produce severe disease, but higher spore concentrations inhibit the germination of spores (69). The pycnidial ooze (mucilage or slime) surrounding conidial cirri possibly suppress germination until dilution in water. Chromatographic analysis of the pycnidial ooze indicated the presence of short chained polysaccharides, which give rise to glucose and fructose after hydrolysis. Germination of conidia and disease severity is also reduced when spores are washed and stored in water, possibly due to the production of another germination inhibitor in water . Host tissues and/or nutrients (organic carbon and nitrogen) overcome this inhibition and rapidly induce germ tube formation.

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The germ tubes of *P. medicaginis* directly penetrate and infect the host's epidermal cells. Germ tubes can also penetrate through the stomata and infect the underlying mesophyll cells. The death of these initially infected cells corresponds with the first visible symptoms (67). The initial disease symptoms begin as necrotic brown to black spots on leaves, stem, and petioles. As the disease progresses, the leaf spots coalesce, leading to chlorosis and ultimately the abscission of leaflets from the petioles. Symptom development is favored by high humidity between 24-48 h after inoculation, temperatures between 18-24°C and slight wounding of leaves. Younger leaves are more susceptible to the pathogen though the disease begins in lower canopy (69). Heavy defoliation within the canopy decreases the overall yield and forage quality (leaf loss reduces crude protein) of infected alfalfa. Water soaked lesions can form on young stems, with heavily infected stems turning dark brown and becoming girdled. Leaf spots increase in size during cool humid periods, resulting in chlorosis. Pycnidia develop on necrotic, senescing leaves, petioles and stems, and exude conidia under wet conditions, serving as a source of secondary inoculum throughout the spring and early summer. Under less favorable/dry conditions, pycnidia on plant debris can act as resting structures and serve as a source of primary inoculum the following season (67). Additionally, the fungus can become seed borne, reducing seedling germination and causing damping off of heavily infected seedlings (3). Symptoms of spring black stem and leaf spot can resemble those of common leaf spot caused by *Pseudopeziza medicaginis* and Leptosphaerulina leaf spot caused by *L. trifolii* (21).

The seedborne nature of the pathogen has been investigated several times. In surveys of seed lots of alfalfa and annual medic cultivars, up to 40% of seeds were infested with *P. medicaginis* and the pathogen remained viable on seeds up to three years and in field debris up to five years. Up to 83% of the seed samples from Canada had some level of infestation and the infested seeds showed reduced germination rates and more root rot. The pathogen was more common in soils where alfalfa was grown continuously (3, 5, 11, 21).

Biology of *Phoma medicaginis*

A. Growth in culture:

Colony morphology has been studied on several different types of media, such as oat meal agar, malt extract agar and potato dextrose agar (PDA) (18), on which colonies form wavy, concentric, irregular margins. On malt agar (MA), *P. medicaginis* forms aerial mycelium and with age, produces dendritic crystals in the agar, which contain brefeldin A (62, 64). On PDA, young colonies are white in the beginning, and later turn olive-green in color, with pale buff margins. As the cultures become mature, the aerial hyphae collapse and occasionally sector. Pycnidia are produced abundantly in concentric circles, with some formed inside the agar or on aerial mycelia. Maximum growth of the pathogen is observed between 21-24°C. Pycnidia are produced most profusely between 21-27°C with exposure to light (58). On PDA plates, growth is slow at 9°C, increases rapidly until 21°C and ceases at 33°C. Abundant production of pycnidia is seen between 21°C to 27°C. Pycnidial production is favored at 27°C and enlarged pycnidia are

observed at 21°C (67). Inorganic compounds such as nitrate promote the formation of pycnidia, when compared to ammonia in the medium (19).

B. Reproduction of *Phoma medicaginis*

In nature, the pathogen is mitosporic, forming only asexual pycnidiospores in ostiolate pycnidia. Although the pseudothecia of *Pleospora* spp., *Leptosphaeria* spp. and *Ophiobolus* spp., have been found overwintering on alfalfa stems also infected with spring black stem, no ascospores from these fungi produced cultures of *P. medicaginis* (21, 43). As with most anamorphic ascomycetes, this fungus is haploid, reproducing only by mitosis (asexual reproduction). Conidiogenesis occurs inside pycnidia. Pycnidia of *P. medicaginis* are globose, ostiolate, and glabrous. The pycnidiospores are cylindrical, hyaline and single-celled (occasionally septate) which ooze from pycnidia in wet, pink-colored masses. Unicellular chlamydospores are produced only in older cultures (43, 75).

C. Host-pathogen interactions

Even though spring black stem was first reported in the early 1900s, not many mechanistic studies have been made on the disease. When seeds were inoculated with blended mycelia, seedling emergence was reduced due to damping off that caused the hypocotyls to turn brown to black (75). When whole plants were inoculated with blended mycelium, lesions appeared on stems and leaves. Hyphae of the pathogen penetrated either directly or through stomata. Appressoria were not observed. Lesion development started slowly, but it was rapid once the fungus was established inside the host. The fungal hyphae grew intercellularly and intracellularly in living cells in alfalfa stems

initially but later grew intracellularly in dead leaves. The vascular bundles in older stems were free of fungus and fungal growth was restricted to the cortical tissue. Young stems were more susceptible and were often girdled. Pycnidial development occurred beneath the epidermis of dead tissue.

Secondary metabolites produced by *Phoma* spp.

Few secondary metabolites specifically produced by *P. medicaginis* have been studied. However, numerous compounds have been isolated from other species of *Phoma* produced *in vivo* as well as *in vitro*. Among these, the macrocyclic lactone, brefeldin A is secreted by several species of *Phoma*, including *P. medicaginis*, as well fungi from other genera (62). This toxin inhibits the Golgi apparatus of plants and animals preventing Golgi-mediated extracellular protein secretion. According to Driouich (30), electron microscope studies showed that brefeldin A caused swelling of the endoplasmic reticulum, induced stacking of cisternae, and increased the number of vesicles in cells of sycamore maple (*Acer pseudoplatanus* L.). Biochemical and immunocytochemical studies suggest that xyloglucan (XG) accumulated in the cisternae of plant cells after brefeldin A treatment. The effects of the toxin are reversible and disappear two hours after its removal (30, 61). Typically, alfalfa plant tissues are completely colonized by *P. medicaginis* nine days after the death of the tissue. Brefeldin A has been isolated from the dead tissue and could inhibit spore germination of several epiphytic fungi co-inhabiting the same phyllosphere, implying brefeldin A is used by *P. medicaginis* to exclude competing fungi (91). Brefeldin A is produced by other fungi like *Alternaria* spp and *Penicillium*.spp. The toxin was first isolated from *Penicillium*

decumbens Thom and was later isolated from other species of *Penicillium*, including *Eupenicillium brefeldianum* (Dodge) Stolk & Scott, from which it gets its name, and other genera of fungi (10, 80).

Other than brefeldin, numerous other secondary metabolites are produced by *Phoma* species (Table 1)**.** *Phoma betae* Frank, a pathogen of beet (*Beta vulgaris* L.), produces betaenone, a toxin causing leaf spot and chlorosis (63). *Phoma lingam* (teleomorph: *L. maculans*), which causes black leg and leaf spot on members of Brassicaceae, produces several phytotoxins, including, phomalairdenone , phomalide and sirodesmin (37, 38, 66, 81).

Table 1. Toxins secreted by *Phoma* spp. and closely related Pleosporalean fungi

Compound	Pathogen	Class	Activity (site of action)	Ref.
Brefeldin A	*P. medicaginis*	Macrocyclic lactone	Antifungal, Phytotoxic (golgi inhibitor)	(30, 33, 91)
Sirodesmin	*L. maculans*	Epipolythiodioxo-piperazine (ETP)	Antibacterial, Antiviral, Phytotoxic (unknown)	(37)
AM-Toxin	*Alternaria alternata* (Fr.) Keissl.	Cyclic peptide	Phytotoxin (chloroplast, cell wall-membrane)	(44)
T-Toxin	*Cochliobolus heterostrophus* (Drechsler) Drechsler	Polyketide	Phytotoxin (mitochondria)	(1)
Ptr ToxA	*Pyrenophora tritici-repentis* (Died.) Drechsler	Peptide	Phytotoxin (chloroplasts)	(53)

Management of spring black stem and leaf spot

Spring black stem and leaf spot can be managed using multiple practices, such as seed treatments, cutting herbage, planting resistant cultivars of alfalfa, and spraying

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fungicides. Disease severity depends heavily upon the susceptibility of cultivars planted, the conduciveness of environmental conditions to the disease (e.g. cool, humid weather) and the number of years a field has been cultivated continuously in alfalfa (3, 4). The annual medic cultivars, *M. truncatula* cvs. Jemalong and Cyprus were more susceptible to the disease when compared to the cultivars of *M. polymorpha* L. var. *brevispina* such as 'Circle Valley' and 'Serena'. The age of the plant plays an important role on the resulting disease severity. Generally, a 10-week old plant is more susceptible compared to 1-4 week old plants (5). Inch et al. also reported that disease was more severe on older shoots (42).

Application of fungicides to control the disease was thoroughly researched by several groups. In early 1950s, research conducted in Minnesota suggested that contact fungicides such as ziram, zineb, nabam and ferbam were not effective against spring black stem. Rather, this study recommended growing resistant cultivars followed by rotation to a non-host crop to control the disease (58). The application of a fungicide such as, benomyl, carbendazim, flutriafol, propicanazole or triadimefton, effectively controlled spring black stem and leaf spot when combined with the planting of resistant cultivars (4, 5). However, fungicide applications to *Medicago* forage crops may not be economically feasible and may negatively affect livestock.

Transformation of Fungi

Transformation is the introduction of genetic material into the genome of a recipient organism and is an important tool for the genetic manipulation of filamentous

fungi and investigating their host-pathogen interactions. Transformation of fungi can result in either random insertions (heterologous or ectopic recombination) or gene replacement (homologous recombination). Both can be used to determine the function of a gene of interest through characterization of the resulting transformant's phenotype. With random insertions (insertional mutagenesis), genes are disrupted randomly with a selectable marker, resulting in an array of mutants defective in various genes. The inserted transgenes can then act as tags permitting the molecular identification of disrupted gene, more rapidly than conventional chemical or UV mutagenesis (56, 60). In gene replacement, the upstream and downstream genomic DNA sequences flanking a targeted gene are fused to a marker gene and used as the transgene. A double-crossover, homologous recombination of the transgene into the targeted endogenous sequence, results in a gene "knockout".

Transgenic DNA can be delivered into a recipient fungal cell's genome using numerous methods. Most protocols require the production of fungal protoplasts or spheroplasts using cell wall degrading enzymes in an isotonic solution. Then polyethylene glycol (PEG), restriction enzyme mediated integration (REMI), electroporation, particle bombardment (biolistics) or *Agrobacterium*-mediated transformation (AMT) are used to introduce and integrate the transgene into the fungal cell's nucleus (25, 26, 47, 56).

Electroporation has been used to transform several species of *Aspergillus* and has been a popular method because the generation of protoplasts is not necessarily required. However, this technique often results in multiple transgene integrations into the genome,

complicating analysis of transformants, thus precluding its use for random mutagenesis or site-directed mutagenesis since single integrations are preferred (15).

REMI has been successfully applied to transform the protoplasts of numerous fungi such as *M. grisea* and *C. heterostrophus* (51, 73, 84). This technique involves the addition of restriction enzymes to the transforming DNA to boost transformation efficiency. It is hypothesized that suitable restriction enzymes partially digest the fungal genome and permit similarly digested plasmid DNA to integrate at restriction sites possessing compatible sticky ends (47, 76, 84). This method has been effective for generating insertional mutants of various phytopathogenic fungi and has resulted in single-copy integrations of transgenes, though significant optimization was required (47). However, this method has its share of disadvantages too. Plasmid DNA can integrate into the same locus more than once and inaccurate DNA repair after restriction digestion of the genome and integration of the transgene (84). Due to DNA repair errors, transgenes can become unlinked from mutated genes making the subsequent rescue of the flanking DNA difficult (60).

Agrobacterium-mediated transformation (AMT) of filamentous fungi

Agrobacterium tumefaciens is the soil-inhabiting, gram-negative bacterium causing crown galls of plants. *A. tumefaciens* can transfer a piece of DNA (T-DNA) from its tumor inducing (Ti)-plasmid into its host plant cell's nucleus. In virulent *A. tumefaciens*, the T-DNA encodes genes producing phytohormones, causing the formation of galls in dicot plants, and opine amino acids only *Agrobacterium* can metabolize. The T-DNA

can be removed from the Ti plasmid, "disarming" it, and transferred to another plasmid to permit manipulations. The phytohormone and opine genes can then be replaced with transgenes of interest (e.g. selectable markers, reporter genes) and this second binary plasmid re-introduced into the disarmed *A. tumefaciens* strain. These transformed strains can then be used to transform plants with the engineered T-DNA. This method has lead to the generation of transgenic plants for research and commercial production (82).

Filamentous fungi, such as *Aspergillus awamori*, were first transformed using *Agrobacterium* in 1998 (25). Either fungal protoplasts, conidia, or hyphal cells were transformed equally well with AMT. The high transformation efficiency, ease of use and high frequency of single integrations has made *A. tumefaciens*-mediated transformation a powerful tool to transform a broad array of filamentous fungi including *A. niger, Neurospora crassa* Shear & Dodge*, Fusarium venenatum* Nirenberg*, Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.*, Trichoderma reesei* Simmons*,* and *Agaricus bisporus* (Lange) Pilat. Numerous plant pathogenic fungi such as *Fusarium oxysporum* Schltdl., *Exserohilum turcicum* (Pass.) Leonard & Suggs, *Colletotrichum trifolii*, and *Mycospharella graminicola* (Fuckel) J. Schröt. have also been transformed with *A. tumefaciens* (27, 41, 94). Prior to filamentous fungi, *Saccharomyces cerevisiae* Meyen ex. Hansen was also transformed using *A. tumefaciens* (16).

Table 2. Some plant pathogenic ascomycetes transformed using AMT

Fungus	Class	Purpose of transformation	Ref.
Magnaporthe grisea	Sordariomyctes	T-DNA tagging, gene replacement	(49)
Fusarium oxysporum	Sordariomyctes	T-DNA tagging	(49)
Verticillium dahliae	Sordariomyctes	Gene replacement	(28)
Colletotrichum lagenarium	Sordariomycetes	T-DNA tagging	(87)
C. trifolii	Sordariomycetes	T-DNA tagging	(41)
C. graminicola	Sordariomycetes	T-DNA tagging	(36)
Leptosphaeria maculans	Dothideomycetes	Gene replacement	(31, 37, 38)
M. graminicola	Dothideomycetes	Gene replacement	(55, 94)
Ascochyta rabiei	Dothideomycetes	T-DNA tagging	(57, 93)
Cochliobolus heterostrophus	Dothideomycetes	T-DNA tagging	(1)
Botrytis cinerea	Leotiomycetes	T-DNA tagging, gene replacement	(71, 88)

The mechanism of *Agrobacterium*-mediated transformation of plant systems is fairly well understood and the same mechanism is believed to occur when it is employed to transform a fungus (65). Briefly, *Agrobacterium* perceives inducing sugars and phenolics released by wounded plants and activates the virulence (*vir*) genes encoded on the Ti plasmid and the *chv* genes present in the chromosome (14). The *vir* genes facilitate the transfer of T-DNA and the *chv* genes are essential for attachment (29). Perception of sugars and phenolics results in the phosphorylation of the constitutively expressed VirA, which then phosphorylates the VirG transcription factor. VirG then activates the expression of the *vir* gene operons on the Ti-plasmid. The VirD proteins nick the Ti-plasmid and acts as a pilot to deliver T-DNA into the host. The VirE proteins bind to the single-stranded T-DNA forming a protective complex and have nuclear localization signals that direct it into the host's nucleus (39).

Engineered T-DNA vectors are of two types: 1) co-integration vectors and 2) binary vectors. Co-integration vectors are modified Ti plasmids. These vectors are more stable

in nature and do not require selection pressure for maintenance in *A. tumefaciens*. Binary vectors are capable of replicating in either *E. coli* or *A. tumefaciens* and contain only the right and left borders of the T-DNA (89). Binary vectors must be maintained under selection in *A. tumefaciens* strains carrying the Ti-plasmid in order to successfully transform a host cell.

Selectable marker genes for fungal transformation

Auxotrophic and drug resistance markers are the two main types of selectable markers used to transform fungi. Auxotrophic mutant strains are generated using antimetabolites preventing prototrophic growth. For example, 5-fluoro-orotic acid is toxic to prototrophs but mutants lacking *pyrG* (or at some other step in the pyrimidine biosynthetic pathway) are resistant this compound. Such auxotrophic mutants must be supplemented with a source of uracil in order to grow. These mutants can then be transformed with a transgene construct carrying the complementary *pyrG* (*ura3*) marker, permitting the positive selection of transformants on defined media lacking uracil. This marker is routinely used in the transformation of *S. cerevisiae*, *A. nidulans* and *N. crassa*. Another such selectable marker, acetamidase (*amdS*), permits wild type fungi to survive on acetamide, a poor nitrogen source, insufficient for growth without the *amdS* transgene. Antibiotic resistance genes, such as hygromycin phosphotransferase (*hph*), bleomycin-binding protein (*ble*), benomyl-resistant β-tubulin (*tub*) and phosphinothricin acetyltransferase (*bar*) are examples of negatively selectable markers and are used widely to transformed filamentous fungi (73, 92).

T-DNA-tagging of filamentous fungi

Agrobacterium-mediated transformation has become the preferred method over other forms of transformation because of its ease (protoplasts are not required), high efficiency, and high frequency of a single-copy of the transgene in each transformant. Mutants with single T-DNA insertions simplify rescue and complementation of disrupted genes in asexual fungi like *P. medicaginis*. It was reported that 85% of the T-DNA-tagged transformants of *C. lagenarium* and 75% T-DNA transformants of *C. trifolii* have single insertions (41, 87).

Research Objectives

The objectives of this thesis research project were the following: 1) transform *P. medicaginis* using *A. tumefaciens* to generate a library of ~1000 transformants and identify T-DNA-tagged mutants with altered morphology, 2) characterize the T-DNA insertions in the genomes of selected transformants of *P. medicaginis* and 3) assess the comparative virulences of the wild type strains and selected transformants on *M. truncatula* and *M. sativa* and use *P. medicaginis* transformants expressing green fluorescent protein to investigate the cytology of the infection process using fluorescent microscopy.

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CHAPTER III

AGROBACTERIUM-MEDIATED TRANSFORMATION OF *P. MEDICAGINIS*

Introduction

Transformation of plant pathogenic fungi helps to characterize the gene of interest and to understand the host-pathogen interactions. Transformation introduces a selectable marker into the fungus either through random integration (heterologous recombination) or disrupting a gene of interest (homologous). Random mutagenesis is commonly followed to generate an array of transformants by tagging them with a selectable marker such as hygromycin phosphotransferase (*hph*). Insertion of a selectable marker facilitates the identification of the disrupted gene, which is difficult with UV irradiation or chemical mutagenesis. Thus, random insertional mutagenesis can help identify genes important for the virulence and metabolism. Restriction enzyme mediated integration (REMI), electroporation, particle bombardment and *Agrobacterium* mediated transformation are the commonly used methods for transformation of fungi.

A. tumefaciens can transform the host by transferring a region (T-DNA) of its Ti-plasmid. The opine genes on the Ti-plasmids are replaced with selectable markers and reporter genes and maintained in *E. coli*. *Agrobacterium*-mediated transformation (AMT) is preferred over other methods because of its ease of use, high transformation efficiency and high frequency of single T-DNA inserts. Plant pathogenic fungi such as

C. trifolii, M. graminicola, E. turcicum and *F. oxysporum* are some examples that were transformed by *A. tumefaciens*.

Experimental Approach

Fungal cultures: Three single-spore isolates of *P. medicaginis* were collected from an alfalfa field at the Oklahoma State University Agronomy research farm in Stillwater, Oklahoma. These three isolates were designated as P1, P2, and P3, and they were maintained on YPS medium (0.1% yeast extract, 0.1% tryptone and 0.1% dextrose, and 1.8% agar). For transformation these three isolates were grown on 60 mm YPS plates incubated at 18°C under 20 W fluorescent lights with a 12 h light cycle. Spores were suspended from 7-10 d old plates, into ~2 ml of sterile milliQ water and spore number was determined using a hemocytometer. For transformation, spore concentrations were adjusted to 10^5 spores/ml in water (5).

Bacterial cultures: *A. tumefaciens* strains were streaked from 20% glycerol stocks stored at -80°C onto plates of Agrobacterium minimal media (AMM; 2.05g K_2HPO_4, 1.45g KH_2PO_4, 0.5 g NH_4NO_3, 1.5g NaCl, 0.01g $CaCl_2$, 0.25g $MgSO_4$, 2.5mg $FeSO_4$, 2g glucose, 20 μl trace elements stock solution (Vogel, 1964) and 18g agar in 1 liter of milliQ water (2) and incubated at 28°C for 2 d until single colonies were formed. All cultures of *A. tumefaciens* LBA 4404 were supplemented with streptomycin (200 μg ml^{-1}) to maintain the Ti plasmid, pLBA4404, and were additionally supplemented with kanamycin (100 μg ml^{-1}) to maintain the fungal transformation vectors, pPTGFPH and pBHt2 (Table 1; Fig. 1).

Table 3. Fungal isolates and bacterial strains used in this research

Species	Isolate/Strain	Binary vector	Bacterial selection	Fungal selection	Visual marker
A. tumefaciens	LBA4404	N/A[a]	Streptomycin	N/A	N/A
A. tumefaciens	LBA4404	pPTGFPH	Streptomycin, Kanamycin	Hyg^R (*hph*)[b] G418^R (*nptII*)[c]	GFP[d]
A. tumefaciens	LBA4404	pBHt2[e]	Streptomycin, Kanamycin	Hyg^R (*hph*)	N/A
P. medicaginis	P1	N/A	N/A	N/A	N/A
P. medicaginis	P2	N/A	N/A	N/A	N/A
P. medicaginis	P3	N/A	N/A	N/A	N/A

[a] N/A, not applicable

[b] *hph*, hygromycin phosphotransferase from *Escherichia coli* driven by the *trpC* promoter from *Aspergillus nidulans*

[c] *nptII*, neomycin phosphotransferase from *E. coli* driven by the nopaline synthase (*nos*) promoter and terminator from *A tumefaciens*.

[d] GFP, green fluorescent protein, sGFP (S65T) variant, driven by the *toxA* promoter from *Pyrenophora tritici-repentis* and the *nos* terminator.

[e] see Mullins et al. (2001)
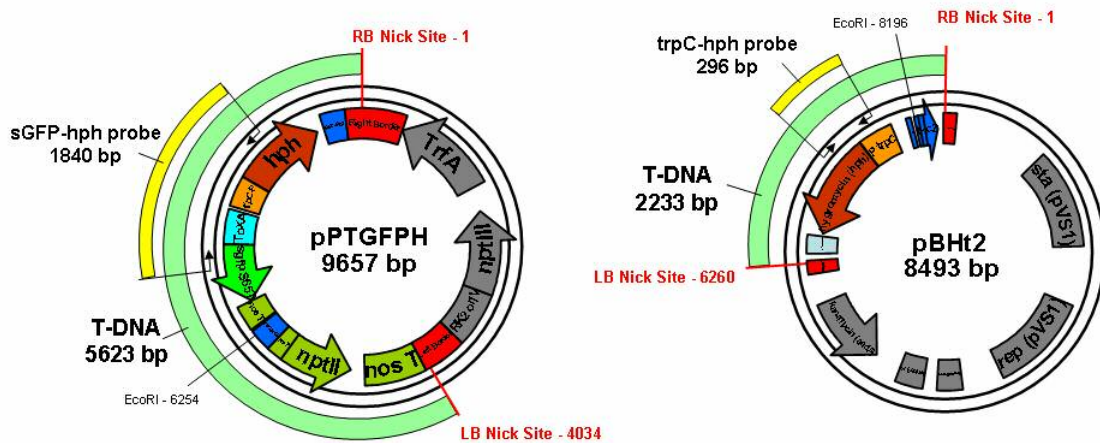
Figure 1. Binary vectors pPTGFPH and pBHt2 used for T-DNA tagging of *Phoma medicaginis*. The T-DNA regions (lime) between the red borders were integrated into the genome of *P. medicaginis* strains. Transgenes carried on the T-DNAs are described in the text. The PCR products used to confirm transformants are indicated (yellow). The sGFP-hph PCR product was used to probe Southern blots of *EcoR*I-digested genomic DNA. Gray-colored ORFs are required for maintenance of transformation vectors in bacterial hosts.

Fungal Transformation: Single colonies of LBA4404 (vector-less control), LBA4404 (pPTGFPH), and LBA4404 (pBHt2), were used to inoculate 5 ml of 523 broth (1% sucrose, 0.8% tryptone, 0.4% yeast extract, 0.3% K_2HPO_4, and 0.03% $MgSO_4·7H_2O$) supplemented with the appropriate antibiotics (Table 3) in gamma-irradiated, sterile 15 ml falcon tubes, and incubated in an angled rack overnight on a shaker at 28°C. Optical densities (OD) of the cultures were measured using a spectrophotometer (UV-265, UV-Visible spectrophotometer, Shimadzu, Kyoto, Japan) at 600 nm. Bacterial cells were pelleted by centrifugation at 2,000× *g* for 1 min and diluted to an OD 0.2 in virulence-inducing minimal medium broth (IMM; 2.05 g K_2HPO_4, 1.45g KH_2PO_4, 0.5g NH_4NO_3, 0.15g NaCl, 0.0025g $FeSO_4$, 0.01g $CaCl_2$, 0.25g $MgSO_4$, 0.9g glucose, 5.33g MES, 5 ml glycerol, 20 µl Vogel trace elements solution in 1 liter of milliQ water and supplemented

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with 200 μM acetosyringone (200 μl per liter from 1 M DMSO stock) and appropriate antibiotics) and incubated shaking (250 rpm) overnight at 28°C as 5 ml IMM broth cultures in 15 ml Falcon tubes. The OD's of cultures were measured and again diluted to OD 0.2 with IMM broth and used as the working stock suspensions for subsequent transformations. These virulent induced cultures could be stored at 4°C and used for 7 d.

For transformation, 500 μl induced *Agrobacterium* culture and 500 μl *P. medicaginis* spore suspension (10^5 conidia ml^{-1}) were mixed and 200 μl aliquots of the mixture were each spread onto 47 mm nitrocellulose membranes (Fisher Scientific, Pittsburgh, PA) overlaid on a 60 mm IMM plates supplemented with the appropriate antibiotics. Mixed culture plates were co-incubated at 20°C in the dark for 3 d. As a transformation control, conidia were co-incubated with induced vector-less LBA4404. After co-incubation, nitrocellulose membranes with fungal and bacterial cells were transferred to 60 mm plates containing selection medium (YPS supplemented with 50 μg ml^{-1} hygromycin, to select fungal transformants, and 200 μg ml^{-1} timentin and 50 μg ml^{-1} cefotaxime, to eliminate *Agrobacterium*) and incubated at room temperature.

After 7-14 d, hygromycin-resistant colonies of *P. medicaginis* were observed and transferred to 24-well plates containing selection medium. No fungal colonies developed on vector-less LBA4404 control plates. Unless stated otherwise, the fungal transformants were selected and maintained on YPS selection medium containing hygromycin and timentin.

Purification of transformants: Transformants were each purified to stable homokaryons by streaking hygromycin-resistant colonies from the wells of 24-well plates each onto 60 mm plates of selection medium. After ~3 d, hyphal tips from a single colony of each transformant were transferred to the wells of new 24-well plates containing selection medium. This procedure was repeated three times for each transformants and the stability of transformants was tested by subculturing on YPS plates without selection followed by transfer back to YPS selection medium proving that the integrated transgene was not lost. The resulting transformants were considered to be homokaryons with stably integrated T-DNA. Transformants were stored by streaking on filter paper placed on the selection medium and incubated until sufficient growth had colonized the paper. The colonized paper was peeled off, desiccated, and kept inside sterile, plastic WhirlPak pouches. The transformant library was stored at 4°C in a sealed plastic box with desiccant.

Analysis of transformants: Sixty transformants (10 transformants for each isolate-vector combination) were selected to analyze T-DNA integration. Briefly, mycelia of wild type strains and transformants were cultured in 15 ml falcon tubes containing 5 ml of PDYP broth (2.4% potato dextrose broth, 0.1% tryptone and 0.1% yeast extract) supplemented with or without the appropriate antibiotics and incubated at room temperature in the dark on a tissue culture rotator (35-40 rpm) for 5 d. Preventing exposure of the cultures to light suppressed melanization of transformants, thereby increasing DNA yields. After 5d, mycelial cultures were centrifuged at 3,500 rpm for 5 min, the supernatants discarded, and the pelleted mycelia washed with 1x PBS and then lyophilized for 2 d.

DNA was isolated from lyophilized mycelia using DNeasy plant mini kit (Qiagen Inc., Valencia, CA), and quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The presence of T-DNA in each transformant was determined by PCR with primers (IDT techologies, Coralville, IA) "sGFP-reverse" (5'-AAGTCGTGCTGCTTCATGTG-3') and "hph-reverse-Leclerque" (5'-CCGTCAACCAAGCTCTGATAG-3') for transformants derived from pPTGFPH and primers "hph-reverse" (5'-GCCGATGCAAAGCCGATAAACA-3') and "trpC-forward" (5'-GCTGCTTGGTGCACGATAA-3') for pBHt2 transformants. Eleven of the 60 transformants were selected for further analysis of T-DNA copy number by Southern blot hybridization (Table 4)**.** Since many of the selected transformants were morphologically abnormal, we amplified and sequenced the ribosomal internal transcribed spacer (ITS) region of each of the 11 transformants, using primers ITS4 and ITS5 (6), to confirm the identity of these transformants as *P. medicaginis.*
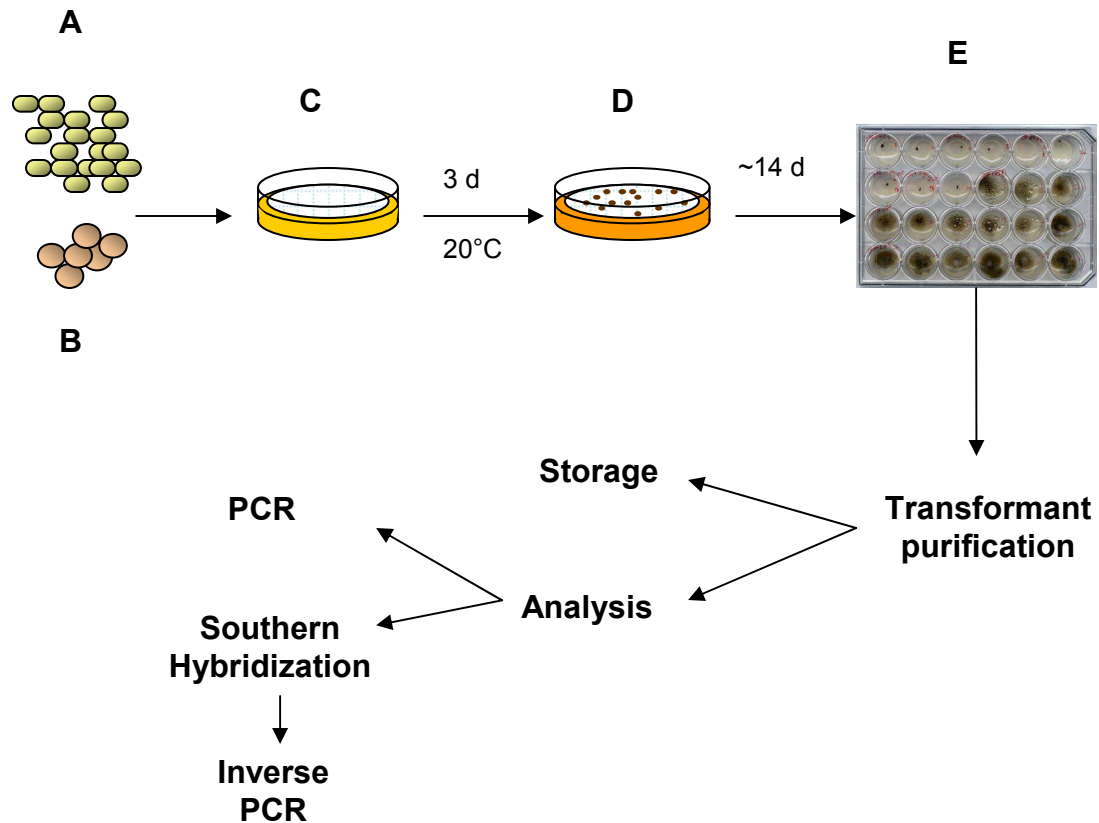
Figure 2. Flowchart of *Agrobacterium*-mediated transformation of *P. medicaginis* (see text for details)**.** A) induced *Agrobacterium* cells and B) conidia of *P. medicaginis* are mixed and the mixture spread on C) a membrane-covered IMM plate. After co-incubation, the membrane is transferred to D) a selection medium on which *Agrobacterium* is eliminated and colonies of fungal transformants are recovered and transferred to E) a multiwell selection plate. T-DNA tagged transformants are then further processed and characterized.

Results and discussion

Nine hundred eighty-two transformants of *P. medicaginis* were obtained by AMT (Table 4). Most of the transformants were generated using the vector pBHt2 with AMT efficiency estimated to be about ~ 0.017 % (i.e. 18 transformants were generated per 10^5 conidia). The other vector used in this study, pPTGFPH, was not as efficient a vector for transformation producing only five transformants per 10^5 conidia, ~ 0.005%. Often times

pPTGFPH did not produce any viable transformants (data not shown). Two of the transformants generated using pPTGFPH lost the transgene, which was further confirmed by PCR.

Table 4. Summary of T-DNA tagged transformants generated in this study

Fungal Isolate	*Agrobacterium* Strain	Vector	# transformants	PCR confirmed[a]
P. medicaginis P1	LBA 4404	pPTGFPH	24	10/10
	LBA 4404	pBHt2	319	10/10
P. medicaginis P2	LBA 4404	pPTGFPH	10	8/10
	LBA 4404	pBHt2	245	10/10
P. medicaginis P3	LBA 4404	pPTGFPH	60	10/10
	LBA 4404	pBHt2	324	10/10
		Total	**982**	**58/60**

[a] T-DNA transgene was amplified with hph R and sGFP R primers for pPTGFPH and trpC R and hph R for pBHt2.

The transformation efficiency and the number of transformants of *P. medicaginis* generated using AMT was similar compared to that achieved in other closely related plant pathogenic fungi like *Ascochyta rabiei* (4, 5). In *A.rabiei*, about 11 transformants were generated per 10^5 conidia, using the *Agrobacterium* strain LBA1126. A similar strain LBA4404 was used for transformation in our study (see materials and methods). Transformation efficiency varies from one strain to another. For example, in *A. rabiei* the *Agrobacterium* strain AGL1 produced more transformants, about 16 transformants per 10^5 conidia.

In the closely related black leg fungus, *L. maculans*, AMT efficiency was lower than that in *A. rabiei* and *P. medicaginis*, with only 0.35 transformants obtained per 10^5 conidia (1). Factors determining AMT efficiency include the fungal species and strain,

co-cultivation period, and density of bacteria (7). In *Mycosphaerella graminicola*, 5-7 transformants were produced per 10^7 spores and the number of transformants generated depended upon the age of the conidia (3).

Results indicated that pBHt2 was a better binary vector for transformation of *P. medicaginis*, while pPTGFPH was not as efficient, because fewer transformants were generated, possibly due to the instability of the T-DNA insertion. The two copies of the *nos* terminator, oriented as a direct repeat might have been recognized as a transposon and excised by the fungus' genomic defense machinery (Fig. 1).

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CHAPTER IV

CHARACTERIZATION OF T-DNA TAGGED MUTANTS OF *P. MEDICAGINIS*

Introduction

Before gene identification can proceed, insertional mutants must be analyzed to determine the number of transgenes integrated into their genomes. Southern hybridization is frequently used to estimate the number of T-DNA inserts in a fungal genome after AMT and determine if any mutant phenotypes in virulence or morphology are a result of one or more T-DNA inserts. Since restriction fragment length polymorphisms are also assessed by Southern hybridization, relative genomic context of each T-DNA is determined for each transformant, indicating whether T-DNA integration into the *P. medicaginis* genome is random or not. T-DNA-tagged mutants containing single insertions can then be analyzed to identify the genes encoded on the genomic DNA flanking the T-DNA. Inverse PCR and TAIL-PCR are the commonly used methods to identify the flanking sequence (5, 10). Inverse PCR is reported to be more efficient than TAIL-PCR (10). Ten T-DNA tagged mutants and the three wild type strains were selected for Southern hybridization, and, one with a single insert and a readily scoreable phenotype, P1-A17, was selected for inverse PCR determination of flanking sequences.

The ten transformants were chosen from the transformant library based on their range of aberrant and wild type morphologies (Fig. 3; Table 5). Three transformants, P1-

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A17, P1-E90, and P1-A3, were derived from wild type P1. Transformants P1-A17 and P1-E90 were generated using the vector pBHt2 and P1-A3 was generated using pPTGFPH. Morphologically, the pycnidia and hyphae of P1-A17 appeared to lack melanin, though the pycnidia still produced conidia, which appeared pink en masse. P1-E90, another non-melanized mutant, produced only white aerial hyphae and did not form pycnidia and conidia. However, melanization was observed in older cultures of P1-E90 (>30 d). Transformant P1-A3 frequently produced aberrant pycnidia that were cracked, lacked distinct ostioles, and appeared more melanized compared to the wild type P1 (Fig. 3).

Four transformants from the wild type strain P2, P2-65, P2-70, P2-N234 and P2-P262 were selected for analysis. P2-65 and P2-70 were transformed using the vector pPTGFPH while P2-N234 and P2-P262 were transformed with pBHt2. P2-65 showed white, aerial hyphae and its pycnidial development was extremely delayed. The pycnidia of P2-70 were darkly melanized with reduced conidial production. P2-N234 appeared to be similar to wild type. P2-P262 had dark hyphae and conspicuously cracked pycnidia without spores.

Three transformants derived from the wild type strain P3, P3-A6, P3-Q278 and P3-T347 were selected. P3-A6 was generated using pPTGFPH and P3-Q278 and P3-T347 were obtained by using pBHt2. Fluorescent microscopy revealed that P3-A6 has very bright GFP expression in its hyphae and pycnidia, and thus, it was selected as the transformant used for the microscopy studies in Chapter V. P3-Q278 resembled the wild

type, P3, morphologically. The other transformant in this category P3-T347 possessed

highly melanized hyphae and lacked pycnidia or spores.

Table 5. Description of mutants selected for Southern hybridization analysis and number of T-DNA insertions in each

T-DNA-tagged mutant	Vector used	Phenotype: (pycnidia, conidia and other characteristics)	Southern Results	
			# inserts	Est Size (Kbp)
P1-A3	pPTGFPH	Cracked pycnidia, no ostioles Reduced conidiation	1	8.5
P1-A17	pBHt2	Non-melanization pycnidia Abundant conidia	1	9
P1-E90	pBHt2	Fluffy, white hyphae No pycnidia or conidia	1	10
P2-65	pPTGFPH	White, aerial hyphae Small or delayed pycnidia formation bright GFP in hyphae	1	8.5
P2-70	pPTGFPH	Darkly melanized pycnidia Reduced conidia	1	5
P2-N234	pBHt2	Apparently wild type	2	10, 9, 3.5
P2-P262	pBHt2	Dark colony Cracked pycnidia, no ostioles No conidia	2	10, 9
P3-A6	pPTGFPH	Apparently wild type bright GFP in hyphae	smear	4-9.7
P3-Q278	pBHt2	Apparently wild type	2	8.5, 3
P3-T347	pBHt2	Highly melanized hyphae No pycnidia or conidia	1	5

[a] N/A, not applicable
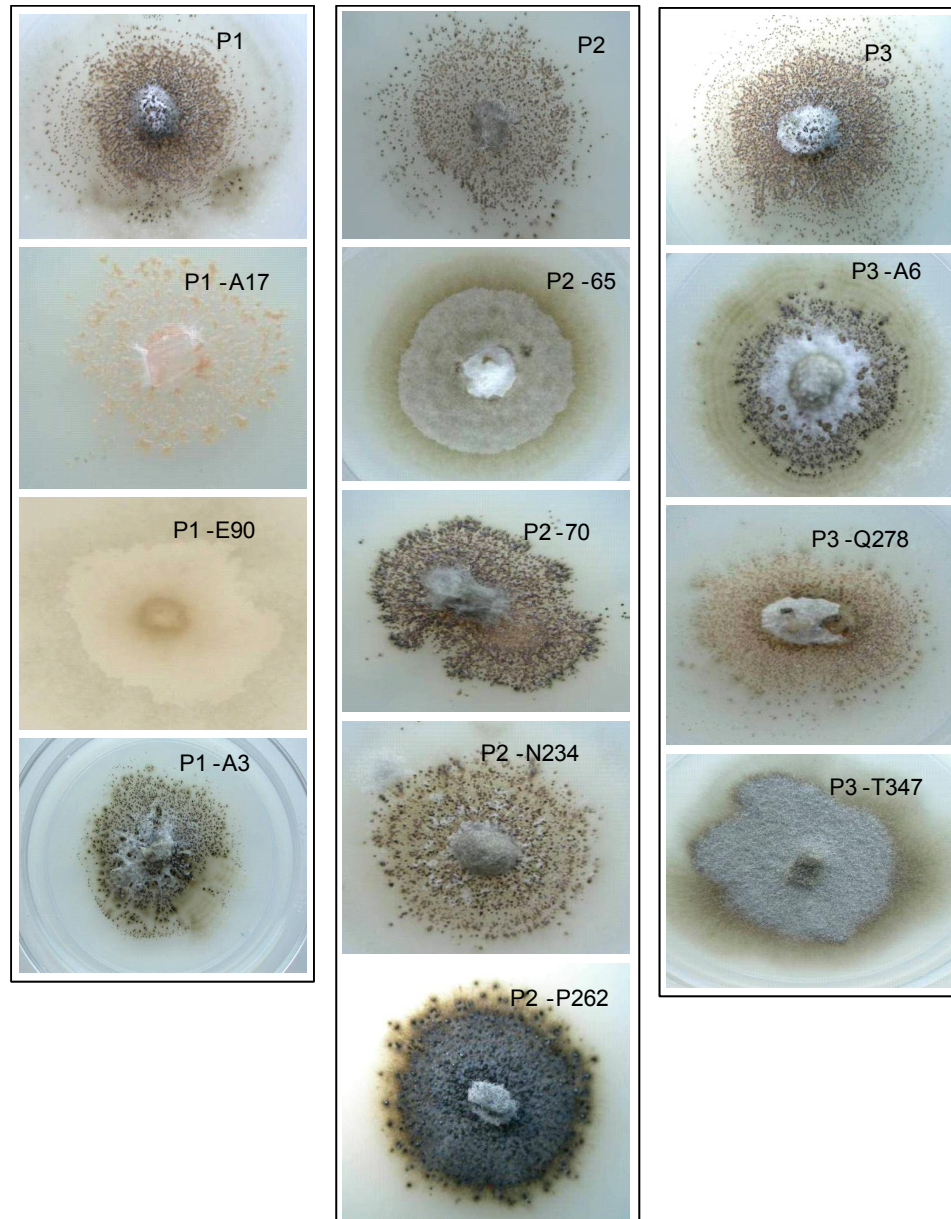
Figure 3. Cultural morphology of wild type strains and transformants of *P. medicaginis*. Top row, from left to right, the three wild type isolates, P1, P2, and P3. Column 1, mutants of P1, P1-A17 forming non-melanized ("albino") pycnidia, P1-E90 fails to form pycnidia or melanized hyphae and becomes hydrophilic (water-soaked) in the older part of the colony, and P1-A3, pycnidia fail to form ostioles and eventually crack open. Column 2, mutants of P2, P2-65, characterized by white aerial hyphae and delayed pycnida, P2-70, with darkly melanized pycnidia and reduced conidiation, P2-N234, morphologically wild type, and P2-P262, highly melanized colony with large pycnidia and no conidiation. Column 3, mutants of P3, P3-A6, similar to wild type with bright GFP expression (not shown), P3-Q278 morphologically wild type, and P3-T347, highly melanized hyphae with no pycnidia or conidia.

Experimental Approach

Southern blot hybridization: Southern blots were performed essentially according to Sambrook et. al. (6).   To obtain sufficient high molecular weight genomic DNA for Southern hybridization, wild types and 10 transformants were cultured as in objective 1. Genomic DNA was isolated according to Möller et al. (4) with the following modifications to improve the purity of resulting DNA: addition of 1.0% PVP and 1.0% PVPP to the extraction buffer. Addition of these compounds reduced co-precipitation of phenolics and polysaccharides with genomic DNA.   Commercial kits did not yield sufficient genomic DNA for a Southern blot, even though the genomic DNA was very clean.

Six micrograms of genomic DNA from each transformant were digested with *Eco*RI at 37°C overnight and separated on a 0.8% agarose-TBE gel at 50 V for 4 h. The gel was post-stained with 0.5 μg ml^{-1} ethidium bromide to check loading.   Separated DNA was blotted to a positively charged nylon membrane by upward capillary transfer, followed by UV cross-linking. The blot was probed with the sGFP-hph PCR product (~1.8 Kb) from the vector pPTGFPH (Fig. 1), which also hybridizes to the *trpC-hph* region of the T-DNA from pBHt2.   The purified PCR product was denatured and crosslinked with alkaline phosphatase using the Alkaphos Direct kit (Amersham Biosciences, Pittsburgh, PA) and hybridized with the blot overnight according to manufacturer's directions.   The hybridized probe was detected by chemiluminescence,

using CDP-Star as a substrate.   Southern hybridization revealed that six of the ten mutants had single insertions (Fig. 5).

Inverse PCR: Inverse PCR is used to obtain unknown sequence flanking known sequence. From among the mutants with single T-DNA insertions, P1-A17 was selected to identify the flanking sequence because of its colony morphology and relatively small left border-flanking sequence (~3 Kb of 5 Kb *EcoR*I genomic fragment based on Southern results; Figs. 4 and 5). Genomic DNA was isolated from mycelial cultures of P1-A17 and the wild type P1 as before for the Southern. Approximately 5 μg of genomic DNA was digested overnight at 37°C with *EcoR*I, then heat-inactivated at 70°C for 10 min. The digested sample was cleaned and precipitated using Geneclean Turbo kit (Qbiogene, Morgan Irvine, CA) and resolved on a 0.8% agarose gel prepared using 0.5× TBE gel. The gel was post-stained with ethidium bromide and the target DNA smear (about 4-6 Kb, Fig. 4) excised with a clean sharp razor blade and gel-purified with the Geneclean Turbo kit. Ligation reactions were set up in 20 μl volumes each containing 1× T4 ligation buffer (Promega, Madison, WI), 3 U T4 DNA ligase and 200 ng of digested DNA and milliQ water. The reaction mixture was incubated at 4°C overnight, then inactivated at 70°C for 5 min.

PCR reactions were set up in 20 μl volumes containing of 1 μl of ligated DNA, 1× reaction buffer, 1 U DNA polymerase (Mango Taq, Bioline, Randolph, MA), 200 μM dNTPs, 0.1 μM of each T-DNA specific primer ('dsRed-F', 5'-TCCGGCTCGTATGTTGTGTGGAAT-3' and 'pBHt2-LB1', 5'-GGGTTCCTATAGGGTTTCGCTCATG-3') and PCR carried out on a PTC-200 DNA

engine thermal cycler (MJ research, Waltham, MA) using the following program: 3 min at 95°C, 10 cycles of 30 s at 94°C, 1 min at 59°C, and 3 min at 72°C, another 25 cycles of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C and finally 7 min at 72°C. PCR products were confirmed by electrophoresis, purified (P1-A17 only, ~3 Kbp) using a PureLink PCR purification kit (Invitrogen, Carlsbad, CA) and submitted for sequencing, with the 'dsRed-F' and 'pBHt2-LB1' primers, on an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA) in the Recombinant DNA/Protein Resource Facility (Biochemistry & Molecular Biology Department, OSU). Nested primers were designed and based on the 3' sequences and the entire 3 Kb product sequence in both directions.   Sequences assembled into a single contig with ChromasPro software (Technelysium Pty Ltd, Tewantin, QLD, Australia) and similar sequences searched
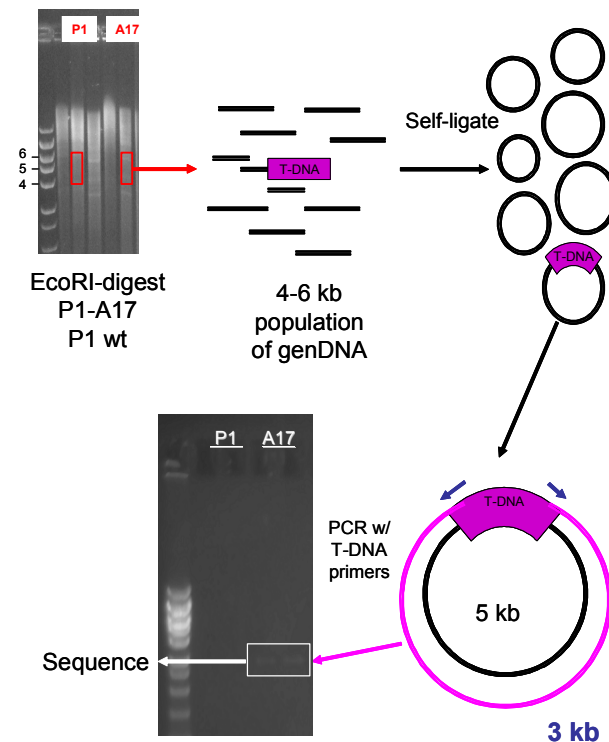
Figure 4. Schematic representation of inverse PCR amplification and sequencing of the genomic DNA flanking the left border of the T-DNA in P1-A17.

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Results and Discussion

Determination of T-DNA copy number

Six of the ten transformants analyzed by Southern hybridization were found to have single copies of the T-DNA inserted in their genome (Fig. 5). Transformants P1-A3, P1-A17, P1-E90, P2-65, P2-70, and P3-T347 each had single T-DNA insertions with *EcoR*I fragment sizes estimated to be 8.5, 5, 10, 8.5, 5, and 5 Kbp, respectively (Table 5). The remaining four transformants showed more than one insert. Transformant P2-N234 had at least 3 T-DNA insertions with estimated EcoRI fragment sizes of 10, 9, and 3.5 Kbp and may have had multiple copies of T-DNA with *EcoR*I fragment sizes of 9 Kbp (Fig. 5, lane 9). Transformant P2-P262 had two T-DNA insertions with EcoRI fragment sizes of 10 and 9 Kbp. Transformant P3-Q278 had at least two T-DNA insertions with *EcoR*I fragment sizes of 8.5 and 3 Kbp. Transformant P3-A6 had a smear (an undefined band) which spread between 4-9.7 Kb. No cross-hybridizing T-DNA regions were found in any of the control wild type strains.
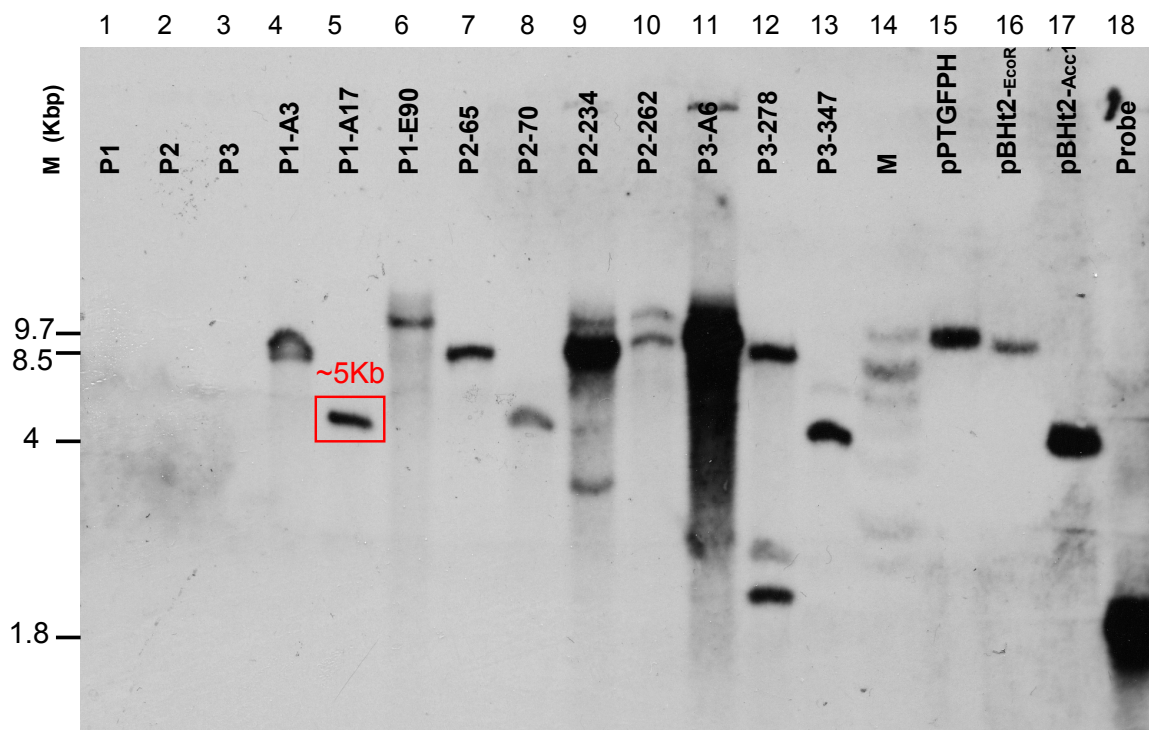
Figure. 5. Southern analysis of the T-DNA copy number in *P. medicaginis* transformants. Lanes 1-3, wild type strains P1, P2, and P3, lanes 4-13, transformants, lane 14, molecular weight markers, lane 15, pPTGFPH digested with *EcoR*I, lanes 17-18, pBHt2 digested with *EcoR*I or *Acc*I, respectively, and lane 18, hph-sGFP PCR product (unlabeled probe). Transformants P1-A3, P1-A17, P1-E90, P2-65, P2-70, and P3-T347 (lane 13) had single T-DNA inserts, while transformants P2-N234 (lane 9), P2-P262 (lane 10), P3-A6, and P3-Q278 (lane 12) had more than one T-DNA insert.

Southern hybridization indicated that ~60% of the selected *P. medicaginis* transformants had single T-DNA inserts. Other fungi transformed by AMT had similar frequencies of single T-DNA inserts. In *M. graminicola*, 85% of transformants had single T-DNA inserts (11). In the alfalfa anthracnose fungus, *C. trifolii*, 75% of AMT transformants had single copies of T-DNA (7), while in *F. oxysporum* 56% of transformants were found to have had single T-DNA inserts (5). Even with the resolving limitations of the blot preventing accurate *EcoR*I fragment size estimations, it appeared

that most T-DNA inserts were of different sizes, indicating that the T-DNA had inserted randomly in the genomes of the selected transformants.

The P3-A6 DNA smear (Fig. 5; lane 11) may have resulted due to the presence phenolics and polysaccharides contaminants in the genomic DNA preparation which would have inhibited *EcoR*I, resulting in the partial digestion of genomic DNA. Genomic DNA from *P. medicaginis* was isolated according to Möller et al. (4), since commercial kits did not yield sufficient DNA for Southern blots, and DNA enzyme inhibitors may have co-purified with the DNA. The presence of probe-hybridizing, high molecular weight DNA remaining in the well at the top of the gel blot supports this interpretation. Alternatively, DNA may have degraded during isolation, or multiple copies of T-DNA inserted at the same site resulting in an overloaded hybridization signal.

Mutant Phenotypes of Transformants

Some of the selected mutants P1-E90, P2-P262 and P3-T347 lost the ability to sporulate (Table 5). P2-P262 possessed visible pycnidia, but did not produce conidia, while P1-E90 and P3-347 lacked both pycnidia and spores. This could be due to the T-DNA disrupting a regulatory or coding regions of a gene either directly or pleiotrophically involved in the sporulation pathway. Also, secondary metabolism is often co-regulated with sporulation in fungi (2). According to Calvo et al. , sporulation is influenced by different factors such as metabolites that activate sporulation, pigments such as melanin and secondary metabolites such as toxins produced by colonies. In *Aspergillus* spp. (Eurotiomycetes), sporulation is induced by polyunsaturated fatty acids

such as linoleic acid (2).   In *Penicillium urticae*, calcium supplements induced conidiogenesis.

Only one mutant, P1-E90 possessed non-melanized, white hyphae among the three non-sporulating mutants. However, when P1-E90 was cultured in liquid media, hyphae melanized as cultures aged. Likewise, in the normally melanized *Cochliobolus heterostrophus*, a white colony morphology was observed in MAP kinase Chk1 deletion mutants, which also became melanized after prolonged growth (3).

P1-A17 had a single T-DNA insertion in its genome, which might have disrupted a gene upstream or downstream of melanin pathway. The pink color of P1-A17 may be due to accumulation of precursor of melanin synthesis pathway. A "*rosy*" mutant has been reported in *M. grisea*, where mutation of the scytalone dehydratase gene resulted in the accumulation of scytalone, a pink melanin precursor (8). In *C. heterostrophus,* an orange-pink colored colony was observed when the transcription factor *Cmr1* was deleted (3). Also in *C. heterostrophus*, mutations in the MAP kinases *Chk1* and *Mps1* affected the expression of transcription factor *Cmr1*, along with the genes of the melanin biosynthetic pathway, *scd1* (scytalone dehydratase), *brn1* and *brn2* (dihydroxynaphthalene reductase) and *pks1* (polyketide synthase). *Cmr1* transcription factors have also been identified in *C. lagenarium* and *M. grisea* (9).

Genomic DNA Flanking the Left Border of the T-DNA in P1-A17

Inverse PCR sequences of the genomic DNA flanking left border of the T-DNA in P1-A17 displayed 76% identity to a hypothetical serine/threonine protein kinase encoded by the SNOG_08128.1 locus of the genome sequence of *Stagonospora nodorum* (Berk.) Castell. & Germano available at the Broad Institute website (www.broad.mit.edu/annotation/genome/stagonospora_nodorum/). Using the blastx homology search (www.ncbi.nlm.nih.gov/BLAST/), the left border flanking sequence showed homology to the C-terminal end of this hypothetical protein in *S. nodorum*. The hypothetical serine threonine protein kinase shared homology to several hypothetical protein kinases from the other filamentous fungi, *Aspergillus nidulans*, *Botryotinia fuckeliana*, *Neurospora crassa*, and *Gibberella zeae*, and was similar to a functionally characterized Ran1-like protein kinase from budding yeast, *Saccharomyces cerevisiae*. The Ran1-like protein kinase in yeast plays an important role in suppressing the meiotic pathway during active growth in conditions when nutrients are not limiting (1). The yeast Ran1-like protein kinase is inactivated during starvation, which is thought to be an essential in the activation of the meiotic pathway. Other than in yeasts, this protein has not been characterized in other fungi.

A

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Score = 71.633 (174), Expect=1.07584E-12

Identities=32/42 (76%), Positives=34/42 (80%)

Pm P1-A17   QQQYVSPPPPTNNWFQPFIHAANLVKHVSFQPSMMAAPVHVH

            Q   YVSP P  N WFQPFI AAN+VKHVSFQPSMMA PVHV+

Sn SN15     QPTYVSPQPAGNTWFQPFIQAANIVKHVSFQPSMMAQPVHVY
```
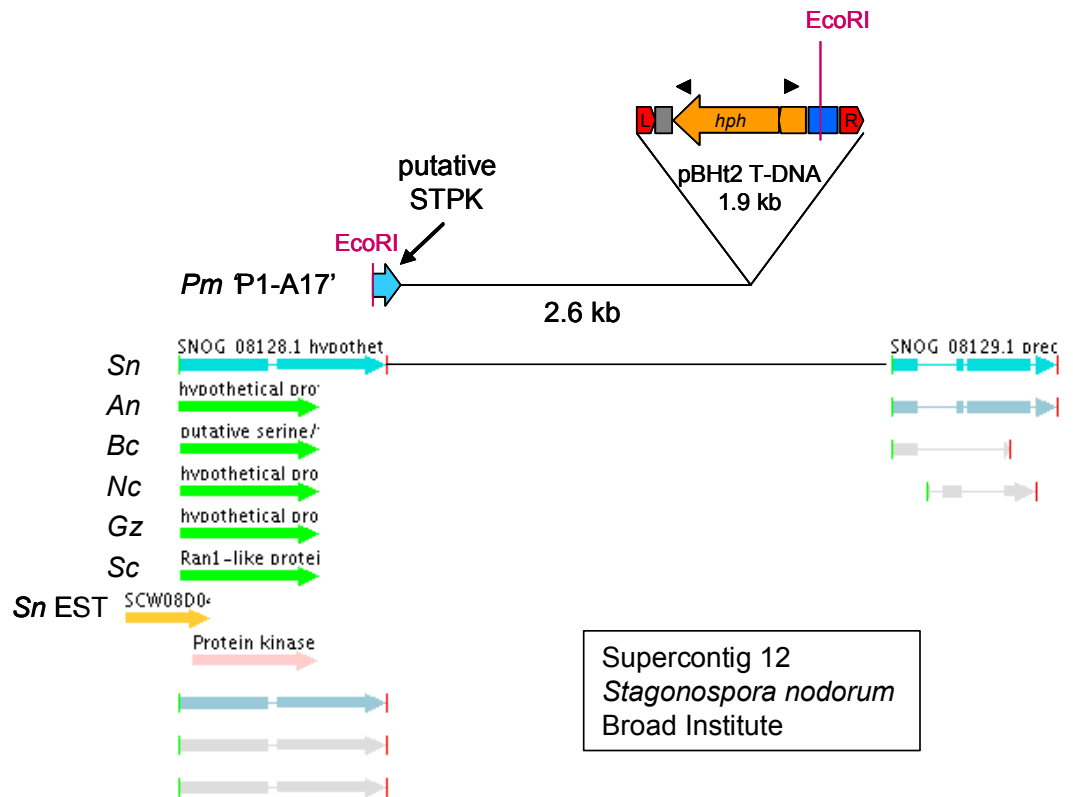
Figure 6. Similarity of the genomic DNA flanking the left border of the T-DNA in P1-A17 to hypothetical proteins encoded in the *S. nodorum* SN15 and other fungal genomes from the Broad Institute. A. BlastX alignment of the amino acid sequences of P1-A17 and *S. nodorum* SN15. B. Genome region alignment of the P1-A17 flanking DNA with hypothetical protein kinases identified in the genomes of various fungi, *Sn*, *S. nodorum*, *An*, *Aspergillus nidulans*, *Bc, Botrytis cinerea, Nc, Neurospora crassa, Gz, Gibberella zeae, Sc, Saccharomyces cerevisiae*, and *Sn* EST, EST genome annotation. Pink, blue and gray arrows indicate automated annotations of the *S. nodorum* genome. The approximate locations of the inverse PCR primers (outward facing arrow heads) on the pBHt2 T-DNA and the EcoRI cut sites are shown.

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CHAPTER V

ASSESSING THE VIRULENCE OF *P. MEDICAGINIS* STRAINS AND TRANSFORMANTS INFECTING *MEDICAGO* SPP.

Introduction

Macroscopic and microscopic analyses of host-pathogen interactions are essential to assess the virulence of wild type and mutant strains of the pathogen through detection of differences in disease progression. Previous studies have provided insights into spring black stem of alfalfa caused by *P. medicaginis* (3, 16). Fluorescent microscopy of transformants expressing green fluorescent protein (GFP) allows repeated observations of disease progression in inoculated tissues at the cellular level (4, 13, 19, 21). To assess the macroscopic disease progression and relative virulences, the three wild type and ten mutant strains of *P. medicaginis* (same strains as in Ch. IV) were assayed on three hosts, *M. truncatula* cv. Jemalong and *M. sativa* cvs. Baralfa 42 IQ and Vernal. For fluorescent microscopy, a bright GFP-expressing transformant, P3-A6, was used to inoculate *M. truncatula* and *M. sativa* cv. Baralfa 42 IQ.

Experimental Approach

Virulence Assays of Transformants

Detached Leaf Assay: Trifoliate leaves from 28-35 day old plants of the spring black

stem-susceptible cultivars Jemalong (*M. truncatula*) and Vernal (*M. sativa*) and the

resistant cultivar Baralfa 42 IQ (*M. sativa*, Barenbrug, Tangent, OR) were excised from

plants with a surface-sterilized razor blade. The leaves were surface-sterilized with a

solution containing 1% bleach (~0.05% NaOCl), 1% ethanol and 0.01% Tween 20 and

washed 3 times with distilled water. Three detached leaves were placed in 100mm

plastic petri dishes with two sterile, moistened paper filters. For each wild type strain and

transformant of *P. medicaginis*, four plates containing 3 leaves each were inoculated

using the droplet method (5). Briefly, two leaflets of each trifoliate leaf were each

inoculated with a 5 µl droplet of spore suspension (10^5 ml^{-1} in 0.01% Hi-Yield Spreader-

Sticker (Voluntary Purchasing Groups, Inc., Bonham, TX) or a 5 µl droplet mycelial

fragment suspension for the aconidial mutant transformants, P1-E90, P2-P262, and P3-

T347. The third leaflet of each leaf was mock-inoculated as a control with a 5 µl droplet

sterile 0.01% spreader sticker or sterile YPS agar suspension (prepared as with the

mycelial suspension), and the third leaflet mock-inoculated with 0.01% spreader-sticker

solution. The mycelial fragment suspensions were prepared by bead-beating (BioSpec

Products, Inc., Bartlesville, OK) six ~3 mm^3 agar plugs from 10 d old YPS cultures with

three 3 mm glass beads in 1 ml YPS broth. This suspension was incubated overnight at

room temperature, washed once with sterile distilled water and re-suspended in 1 ml of

sterile distilled water. Symptoms were scored 2, 4, 6 and 8 days post-inoculation (dpi)

using a 0 to 4 scale as follows: 0 healthy, no disease; 1 showing black spots (<1 mm diameter); 2 showing black spots, with partial chlorosis (< 50%); 3 showing black spots with extensive chlorosis (> 50%) and some water-soaking; and 4 complete tissue collapse with black spots, complete chlorosis and water-soaking, pycnidia, and leaflet abscission.

Statisical Analyses: Data from detached leaf assays were transformed by $\sqrt{X+0.5}$ (25) and analyzed by PROC ANOVA (SAS Institute, Inc., 2003). Multiple comparisons between treatment (isolate) means were conducted by the least significant difference (LSD) method at a significance level of 0.05. Untransformed data are presented by in the tables with F-values and their corresponding probabilities.

Cytology of infection of *Medicago* spp. by *P. medicaginis*

Plant culture and inoculation: Seedlings of Jemalong were grown in peat mix (Rediearth, Sungro, Bellevue, WA) and incubated in a growth chamber with a 16-8 h, light-dark cycle and 22°C (day) and 18°C (night) and 60% relative humidity. Fully expanded, 3-4 day-old cotyledons were excised with a sterile double edged razor blade and inoculated with the conidia from the transformant P3-A6. Conidia were suspended 10^5 ml^{-1} in 0.01% spreader-sticker and a 5 µl droplet of this spore suspension placed on each cotyledon (14). Control cotyledons were mock-inoculated with 5 µl 0.01% spreader-sticker solution. Also, 28-35 day-old detached trifoliate leaves and whole plants of Jemalong were inoculated with 10^5 ml^{-1} conidial suspension of P3-A6 using an hobby air brush (Testor Corp., Rockford, IL) and sprayed to run-off. Additionally, 28-35 day-old whole plants of Jemalong and Baralfa 42 IQ were spray inoculated as above. Two to

three leaves were selected and excised every 24 h and images of the disease symptoms recorded using a digital scanner (Epson Inc., Long Beach, CA) and cytology observed as follows.

Microscopy: Inoculated detached cotyledons and leaves were examined microscopically multiple times between 17-170 post-inoculation (hpi). Cotyledons were embedded in 3% agarose and hand-sectioned with a double-edged razor blade. Intact and cut cotyledons and whole leaflets were observed using a Nikon Eclipse E600 fluorescent microscope (Nikon Inc., Melville, NY) using a B2A longpass filter (ex 450-490 nm/dichroic 500 nm/em >515 nm) and images captured using a Magnafire CCD camera (Optronics, Goleta, CA). Additional images were captured using a Nikon Eclipse E800 fluorescent microscope with an Endow GFP longpass filter set (ex. 450-490nm/dichroic 495nm/em. >500nm) and a Retiga-2000R CCD camera (QImaging, Burnaby, Canada).

Results

Virulence Assays

Disease symptoms developed on detached leaves of *M. truncatula* and *M. sativa*, 4 days post inoculation (dpi). From 0-48 hours (to 2 dpi), no disease symptoms were observed on any of the inoculated detached leaves (Table 6). At 4 dpi, black spots without chlorosis appeared on the *M. sativa* cvs. (Table 8). Since leaves were inoculated with 5 µl droplets, black spots were not defined to single epidermal cells and instead formed aggregated clusters of black cells around the inoculation site. Chlorosis appeared

only occasionally on *M. sativa* cultivars with cv. Vernal showing higher disease ratings than Baralfa 42 IQ at 4 dpi (Table 8). However, most *M. truncatula* leaves at 4 dpi already showed about 25% chlorosis in addition to black spots (Table 8). At 6 dpi, the diameter of black leaf spots on *M. sativa* cvs. increased, due to merging of black spots (invasion of adjacent epidermal cells by the fungus), and leaves became chlorotic. At 6 dpi, *M. truncatula* leaves showed extensive chlorosis and water soaking and pycnidia appeared sporadically (Table 10). By day 8, leaves of *M. truncatula* were completely collapsed and pycnidia were observed. *M. sativa* leaves also showed widespread damage.

Leaves inoculated with mycelial fragments showed symptoms much more quickly than leaves inoculated with conidia. Brown spots appeared around the inoculation point on *M. truncatula* leaves by 2 dpi (Table 7). By 4 dpi, severe disease was widespread on *M. truncatula* leaves, with significant chlorosis and water-soaking (Table 9). By 6 dpi most *M. truncatula* leaves had completely collapsed. Disease also progressed more quickly on the mycelial fragment-inoculated leaves of *M. sativa* cvs. By 6 dpi, most mycelial-fragement inoculated *M. sativa* cvs. showed severe disease.

For the most part, none of the transformants appeared to differ significantly in virulence from the wild type strains. However, at 4 and 6 dpi, P2-65 showed reduced virulence on Vernal (Tables 8 and 10). P3-Q278 showed reduced virulence on *M. truncatula* comapared to the wild type. In contrast, at 8 dpi, P3-Q278 showed increased virulence along with P3-A6 on 42 IQ compared to P3 (Tables 10 and 12).

Table 6. Mean disease scores of three different *Medicago* host plants inoculated with conidia of three *P. medicaginis* isolates and their transformants after 2 dpi

Isolate	Day 2[a]		
	M. truncatula*	42 IQ	Vernal
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P1-A3	0.4 ± 0.2	0.1 ± 0.1	0.0 ± 0.0
P1-A17	0.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
P2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P2-65	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
P2-70	0.4 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
P2-N234	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
P3	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
P3-A6	0.3 ± 0.2	0.1 ± 0.1	0.0 ± 0.0
P3-Q278	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
$F_{(10, 33)}$	1.51	1.20	-
$Pr > F$[†]	0.1975	0.3277	-
$F_{(9, 30)}$	1.36	1.17	-
$Pr > F$[‡]	0.2507	0.3509	-

Table 7. Mean disease scores of three different *Medicago* host plants inoculated with mycelial fragments of three *P. medicaginis* isolates and their transformants after 2 dpi

Bead beat isolates**	Day 2[a]		
	M. truncatula*	42 IQ	Vernal
Agar control	$0.0 \pm 0.0d$	$0.0 \pm 0.0c$	0.0 ± 0.0
P1bb	$0.3 \pm 0.2cd$	$0.4 \pm 0.2ab$	0.2 ± 0.1
P1-E90	$0.5 \pm 0.1c$	$0.2 \pm 0.1bc$	0.2 ± 0.1
P2bb	$0.6 \pm 0.3bc$	$0.6 \pm 0.2a$	0.3 ± 0.2
P2-P262	$0.0 \pm 0.0d$	$0.0 \pm 0.0c$	0.0 ± 0.0
P3bb	$1.1 \pm 0.1a$	$0.5 \pm 0.0ab$	0.2 ± 0.1
P3-T347	$0.9 \pm 0.1ab$	$0.3 \pm 0.1abc$	0.3 ± 0.1
$F_{(6, 21)}$	8.81	3.29	1.84
$Pr > F$[†]	< 0.0001	0.0192	0.1404
$F_{(5, 18)}$	6.75	2.41	1.27
$Pr > F$[‡]	0.0010	0.0768	0.3185

* Based on 0 – 4 disease scale as described in Materials and Methods.
** Agar plugs colonized by strains of *P. medicaginis* were bead-beated with glass beads and suspended in spreader-sticker solution (see above)
[a] Means followed by the same lowercase letter in a column are not significantly different ($P < 0.05$).
[†] Significance level determined from ANOVA of square roots of all disease scores, including controls.
[‡] Significance level determined from ANOVA of square roots of all disease scores, excluding controls.

Table 8. Mean disease scores of three different *Medicago* host plants inoculated with conidia of three *P. medicaginis* isolates and their transformants after 4 dpi

Isolate	Day 4 [a]		
	*M. truncatula**	42 IQ	Vernal
Control	0.3 ± 0.3c	0.0 ± 0.0d	0.2 ± 0.2c
P1	1.6 ± 0.3ab	1.0 ± 0.2abc	1.2 ± 0.1b
P1-A3	2.3 ± 0.3ab	1.6 ± 0.4a	1.8 ± 0.2a
P1-A17	1.8 ± 0.4ab	1.1 ± 0.3abc	1.2 ± 0.2b
P2	2.3 ± 0.4ab	0.9 ± 0.1bc	1.7 ± 0.0a
P2-65	1.4 ± 0.3b	0.6 ± 0.2c	0.9 ± 0.1b
P2-70	2.3 ± 0.2ab	1.2 ± 0.2ab	1.1 ± 0.1b
P2-N234	1.6 ± 0.3ab	1.2 ± 0.1ab	1.1 ± 0.1b
P3	2.4 ± 0.4a	0.9 ± 0.3bc	1.2 ± 0.2b
P3-A6	1.8 ± 0.3ab	0.9 ± 0.1abc	1.2 ± 0.1b
P3-Q278	2.4 ± 0.1a	1.5 ± 0.2ab	1.1 ± 0.1b
$F_{(10, 33)}$	5.75	5.17	10.47
$Pr > F$[†]	< 0.0001	0.0002	< 0.0001
$F_{(9, 30)}$	1.61	4.64	1.74
$Pr > F$[‡]	0.5164	0.0007	0.1224

Table 9. Mean disease scores of three different *Medicago* host plants inoculated with mycelial fragments of three *P. medicaginis* isolates and their transformants after 4 dpi

Bead beat isolates	Day 4 [a]		
	*M. truncatula**	42 IQ	Vernal
Agar control	0.8 ± 0.5c	0.4 ± 0.2c	0.0 ± 0.0c
P1bb	2.4 ± 0.3ab	1.5 ± 0.2ab	1.4 ± 0.3b
P1-E90	3.0 ± 0.2ab	2.0 ± 0.4a	1.7 ± 0.4b
P2bb	1.9 ± 0.6b	0.9 ± 0.3bc	1.8 ± 0.3b
P2-P262	2.2 ± 0.4ab	1.6 ± 0.4ab	1.1 ± 0.1b
P3bb	3.3 ± 0.2a	1.9 ± 0.4a	1.5 ± 0.1b
P3-T347	3.2 ± 0.1a	2.2 ± 0.2a	2.7 ± 0.3a
$F_{(6, 21)}$	5.64	4.54	15.29
$Pr > F$[†]	0.0013	0.0042	< 0.0001
$F_{(5,18)}$	2.49	1.93	3.59
$Pr > F$[‡]	0.0703	0.1399	0.0199

* Based on 0 – 4 disease scale as described in Materials and Methods.
** Agar plugs colonized by strains of *P. medicaginis* were bead-beated with glass beads and suspended in spreader-sticker solution (see above)
[a] Means followed by the same lowercase letter in a column are not significantly different ($P < 0.05$).
[†] Significance level determined from ANOVA of square roots of all disease scores, including controls.
[‡] Significance level determined from ANOVA of square roots of all disease scores, excluding controls.

Table 10. Mean disease scores of three different *Medicago* host plants inoculated with conidia of three *P. medicaginis* isolates and their transformants after 6 dpi

Isolate	Day 6 [a]		
	*M. truncatula**	42 IQ	Vernal
Control	1.7 ± 0.5c	0.3 ± 0.1c	0.7 ± 0.3e
P1	4.0 ± 0.0a	3.3 ± 0.1a	2.3 ± 0.1bdc
P1-A3	3.8 ± 0.3a	3.4 ± 0.3a	3.2 ± 0.3a
P1-A17	4.0 ± 0.0a	2.6 ± 0.5ab	3.0 ± 0.2ab
P2	3.8 ± 0.2a	2.5 ± 0.2ab	2.8 ± 0.1abc
P2-65	3.6 ± 0.3a	2.3 ± 0.6ab	1.8 ± 0.3d
P2-70	4.0 ± 0.0a	2.8 ± 0.6ab	3.1 ± 0.3ab
P2-N234	3.3 ± 0.0ab	2.9 ± 0.5ab	2.7 ± 0.3abc
P3	4.0 ± 0.0a	2.0 ± 0.5b	2.1 ± 0.3dc
P3-A6	3.8 ± 0.2a	2.4 ± 0.5ab	2.5 ± 0.3abcd
P3-Q278	2.7 ± 0.3b	2.9 ± 0.2ab	2.7 ± 0.4abc
$F_{(10, 33)}$	7.56	5.48	7.17
Pr > F[†]	< 0.0001	< 0.0001	< 0.0001
$F_{(9, 30)}$	6.18	1.05	2.91
Pr > F[‡]	<0.001	0.4239	0.0134

Table 11. Mean disease scores of three different *Medicago* host plants inoculated with mycelial fragments of three *P. medicaginis* isolates and their transformants after 6 dpi

Bead beat isolates	Day 6 [a]		
	*M. truncatula**	42 IQ	Vernal
Agar control	1.8 ± 0.6b	1.0 ± 0.5b	0.1 ± 0.1b
P1bb	4.0 ± 0.0a	3.8 ± 0.2a	3.6 ± 0.1a
P1-E90	4.0 ± 0.0a	3.6 ± 0.1a	3.6 ± 0.3a
P2bb	4.0 ± 0.0a	2.6 ± 0.9a	2.9 ± 0.9a
P2-P262	3.8 ± 0.2a	2.6 ± 0.5a	2.7 ± 0.1a
P3bb	4.0 ± 0.0a	3.9 ± 0.1a	4.0 ± 0.0a
P3-T347	4.0 ± 0.0a	3.5 ± 0.2a	3.4 ± 0.1a
$F_{(6, 21)}$	6.48	4.81	15.88
Pr > F[†]	0.0006	0.0031	< 0.0001
$F_{(5, 18)}$	1.00	1.55	1.26
Pr > F[‡]	0.4457	0.2246	0.3224

* Based on 0 – 4 disease scale as described in Materials and Methods.
** Agar plugs colonized by strains of *P. medicaginis* were bead-beated with glass beads and suspended in spreader-sticker solution (see above)
[a] Means followed by the same lowercase letter in a column are not significantly different ($P < 0.05$).
[†] Significance level determined from ANOVA of square roots of all disease scores, including controls.
[‡] Significance level determined from ANOVA of square roots of all disease scores, excluding controls.

Table 12. Mean disease scores of three different *Medicago* host plants inoculated with conidia of three *P. medicaginis* isolates and their transformants after 8 dpi

Isolate	Day 8 [a]		
	*M. truncatula**	42 IQ	Vernal
Control	2.5 ± 0.3b	1.1 ± 0.3c	1.3 ± 0.5b
P1	4.0 ± 0.0a	3.8 ± 0.2a	3.9 ± 0.1a
P1-A3	4.0 ± 0.0a	4.0 ± 0.0a	3.9 ± 0.1a
P1-A17	4.0 ± 0.0a	3.5 ± 0.3ab	3.8 ± 0.2a
P2	4.0 ± 0.0a	3.6 ± 0.2a	4.0 ± 0.0a
P2-65	4.0 ± 0.0a	3.1 ± 0.4ab	3.9 ± 0.1a
P2-70	4.0 ± 0.0a	3.6 ± 0.4a	4.0 ± 0.0a
P2-N234	4.0 ± 0.0a	3.7 ± 0.2a	3.6 ± 0.2a
P3	4.0 ± 0.0a	2.7 ± 0.7b	4.0 ± 0.0a
P3-A6	4.0 ± 0.0a	3.9 ± 0.1a	3.7 ± 0.1a
P3-Q278	4.0 ± 0.0a	3.8 ± 0.2a	3.9 ± 0.1a
$F_{(10, 33)}$	17.87	7.98	13.36
$Pr > F$[†]	< 0.0001	< 0.0001	< 0.0001
$F_{(9, 30)}$	-	1.76	1.29
$Pr > F$[‡]	-	0.1194	0.2851

Table 13. Mean disease scores of three different *Medicago* host plants inoculated with mycelial fragments of three *P. medicaginis* isolates and their transformants after 8 dpi

Bead beat isolates	Day 8 [a]		
	*M. truncatula**	42 IQ	Vernal
Agar control	2.6 ± 0.9	1.7 ± 0.6b	0.2 ± 0.1b
P1bb	4.0 ± 0.0	4.0 ± 0.0a	4.0 ± 0.0a
P1-E90	4.0 ± 0.0	4.0 ± 0.0a	4.0 ± 0.0a
P2bb	4.0 ± 0.0	2.9 ± 1.0ab	4.0 ± 0.0a
P2-P262	4.0 ± 0.0	3.1 ± 0.4ab	3.6 ± 0.3a
P3bb	4.0 ± 0.0	3.9 ± 0.1a	4.0 ± 0.0a
P3-T347	4.0 ± 0.0	4.0 ± 0.0a	3.9 ± 0.1a
$F_{(10, 33)}$	2.13	3.09	170.62
$Pr > F$[†]	0.0920	0.0251	< 0.0001
$F_{(9, 30)}$	-	1.36	2.49
$Pr > F$[‡]	-	0.2859	0.0696

* Based on 0 – 4 disease scale as described in Materials and Methods.
** Agar plugs colonized by strains of *P. medicaginis* were bead-beated with glass beads and suspended in spreader-sticker solution (see above)
[a] Means followed by the same lowercase letter in a column are not significantly different ($P < 0.05$).
[†] Significance level determined from ANOVA of square roots of all disease scores, including controls.
[‡] Significance level determined from ANOVA of square roots of all disease scores, excluding controls.

Cytology of infection of *Medicago* spp. by *P. medicaginis*

Microscopic analysis of leaves of *Medicago* spp. inoculated with P3-A6 showed that conidia of *P. medicaginis* germinated within 24 hpi. Specialized penetration structures such as appressoria were not observed. Conidia also attached to the leaf hairs of *M. truncatula*. Spores which were spread more diffusely germinated more rapidly than spores in aggregates or clusters

During infection, *P. medicaginis* entered the host leaf by directly penetrating the epidermal cells and guard cells, and occasionally through the stomata (especially on cotyledons). Infected epidermal cells penetrated by the conidial germ tube and adjacent epidermal cells were frequently observed to be autofluorescent (Fig. 8.3) at the earlier stages of host cell infection, prior to the blackening of the invaded cell (see below). Between 48 to 72 hpi, thick primary hyphae were observed in individual or groups of epidermal cells. These hyphae appeared to be confined to only a few host cells. Hyphae were also observed directly infecting the guard cells (Fig. 8.5). In these initial 72 hpi (3 dpi), leaves remained asymptomatic and symptoms like black spot and chlorosis were not yet observed. The epidermal cells and other host cells harboring these primary hyphae eventually darkened after the initial autofluorescence (Fig. 8.4).

At 96 hpi (4 dpi), thin secondary hyphae spread to adjacent epidermal cells. Macroscopically, black spots and slight chlorosis became visible. These symptoms were frequently seen four to five dpi. On older leaves, pycnidial primordial were observed as early as 5 dpi on senescent tissues (Fig. 9.10).

After 6 dpi, black spots coalesced, leaflets became chlorotic, and also showed some degree of water soaking. Green islands were frequently observed surrounding the black spots in chlorotic areas. Hyphae were also observed in vascular tissue of the midrib petiole and petioles (Figs. 9.9 and 10.14).   On detached leaves the pathogen had completed its life cycle in 7-10 days, by forming pycnidia producing spores. Disease progressed more quickly on older leaves, which appeared to be more susceptible to the *P. medicaginis*.

On whole plants, symptoms appeared more slowly than on detached leaves. Young, apical leaves in the upper canopy appeared to be more resistant than older leaves occurring in the lower canopy. On the young upper leaves, germinating conidia were not observed until 4 dpi, 3 d after conidia had germinated on detached leaves. Secondary hyphae were not formed as rapidly and black spots appeared only intermittently on young leaves. In contrast, conidia germinated rapidly on the older, lower leaves and sometimes pycnidia appeared as early as 5 dpi (Fig. 9.12).

Inoculated cotyledons were hand sectioned at different stages of infection. Transverse sections of cotyledons showed the hyphae in epidermal cells (Fig. 8.6) and penetrating though stomata into the substomatal cavities and surrounding mesophyll (Fig. 9.7).
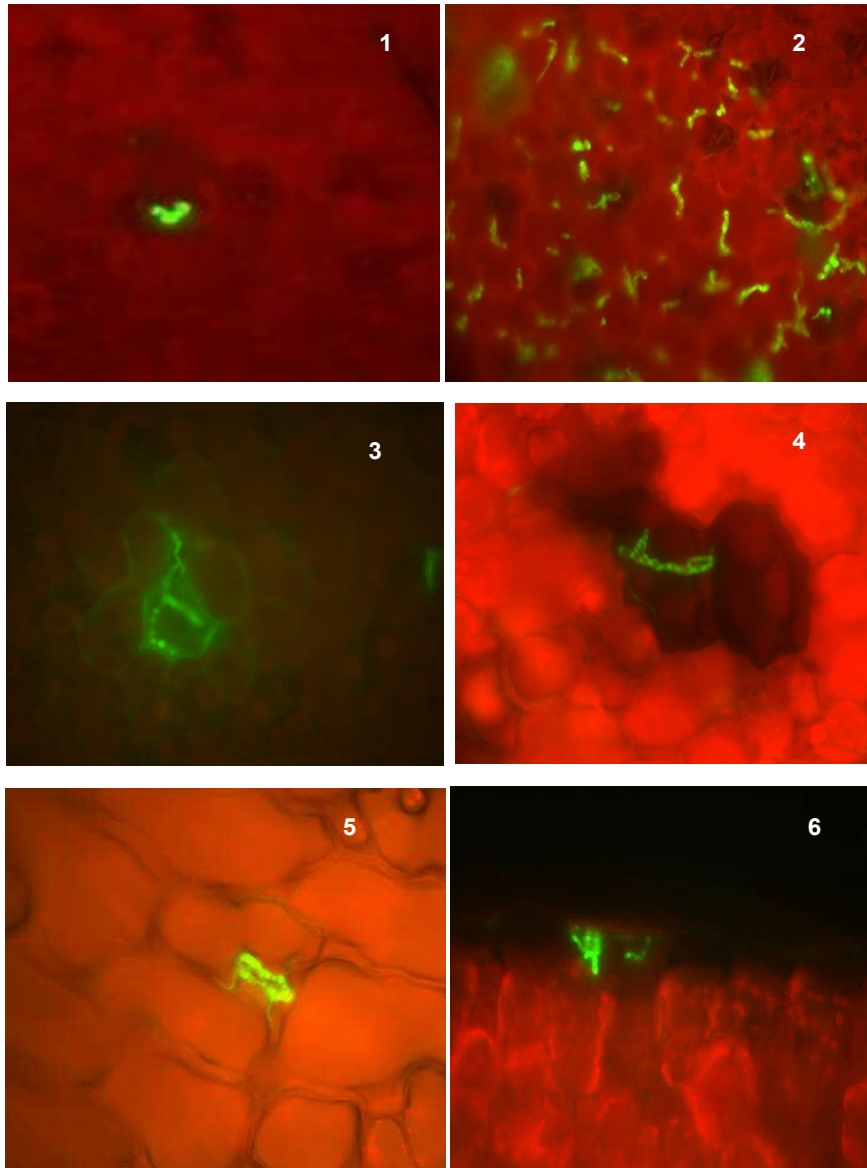
Figure 8. *M. truncatula* leaves and cotyledons inoculated with P3-A6 showing the initial stages of the disease. 1. Germinating conidium with 3-4 cells in an epidermal cell (60×). 2. Germ tubes of individual conidia that have penetrated epidermal cells and stomata (20×). 3. Multicellular primary hypha inside autofluorescent epidermal cells (40×). 4. Primary hypha in an epidermal cell, that turned dark after infection (40×). 5. Primary hyphae inside two guard cells surrounding a stoma of a cotyledon. 6. Transverse section of a cotyledon showing green hyphae inside and exiting an epidermal cell (40×).
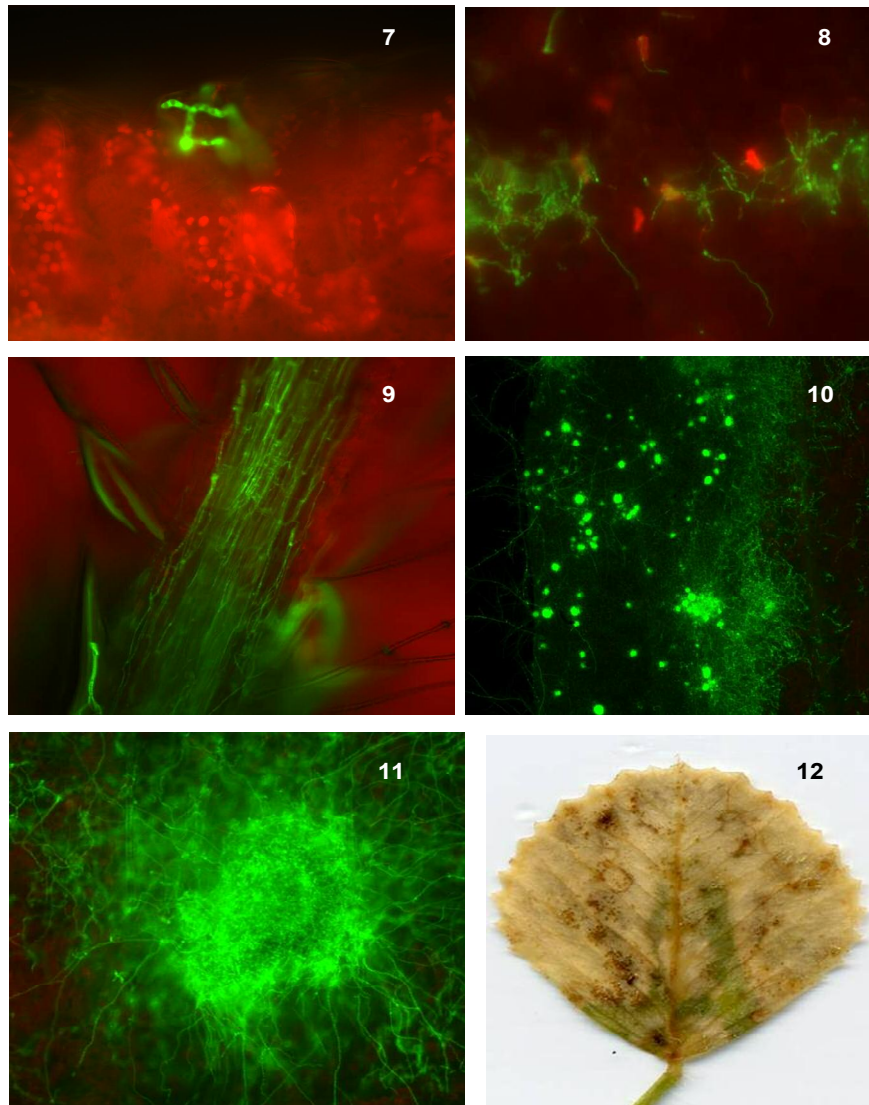
Figure 9. *M. truncatula* leaves and cotyledons inoculated with P3-A6. 7. Transverse section of a cotyledon showing hyphae inside the mesophyll tissue (40×). 8. Hyphae penetrating and colonizing epidermal cells. Red autofluorescent host cells correspond to the presence of infecting fungal hyphae, and likely represent a host response to the hyphae (20×). 9. Green hyphae inside the vascular tissue of the mid vein of a detached leaf (10×). 10. An old, attached *M. truncatula* leaf that has been colonized by P3-A6. Bright green spots are the developing pycnidia (4×). 11. Mature pycnidium from picture 10 (20×). 12. Scanned image of a senescing leaflet. Brownish-black spots are pycnidia.
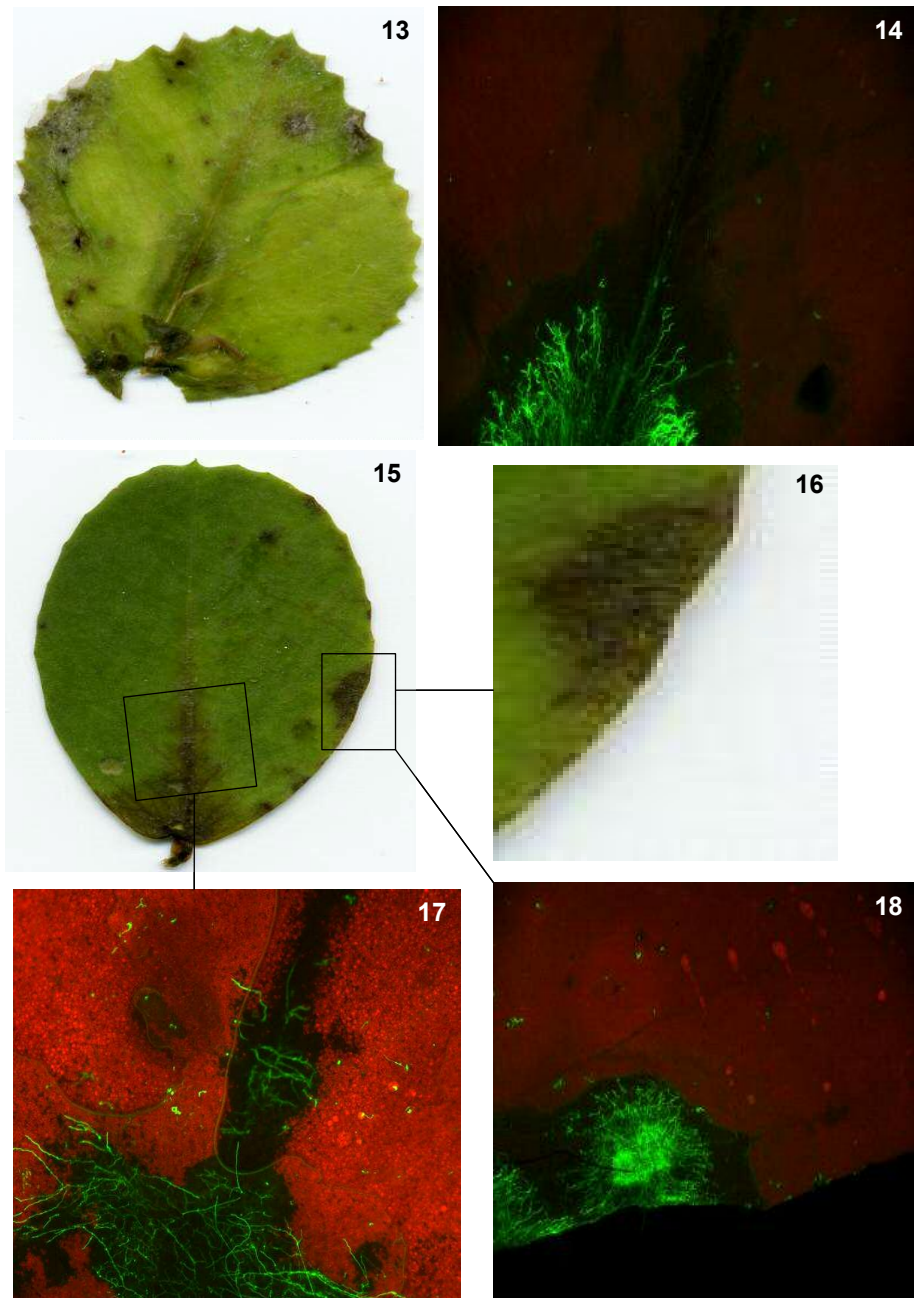
Figure 10. Symptoms on detached leaves. 13. Detached leaflet of *M. truncatula* scanned 7 dpi. Black spots, chlorosis and water soaking on either side of the mid vein were visible. 14. Hyphae growing towards the apex of the leaf inside the vascular tissue (4×). 15. Detached leaflet of *M. sativa* cv. 42 IQ 8 dpi. Necrosis can be observed at the leaf's margins of and spreading acropetally along the mid vein of the leaflet. 16. Cropped area of the leaflet in 15 to showing the necrotic area. 17. Hyphae inside the vascular tissue of the mid vein of 42 IQ leaflet pictured in 15 (4×). 18. Necrotic spot on the margin of 42 IQ leaflet pictured in 15, colonized by the mycelia of P3-A6 (4×).
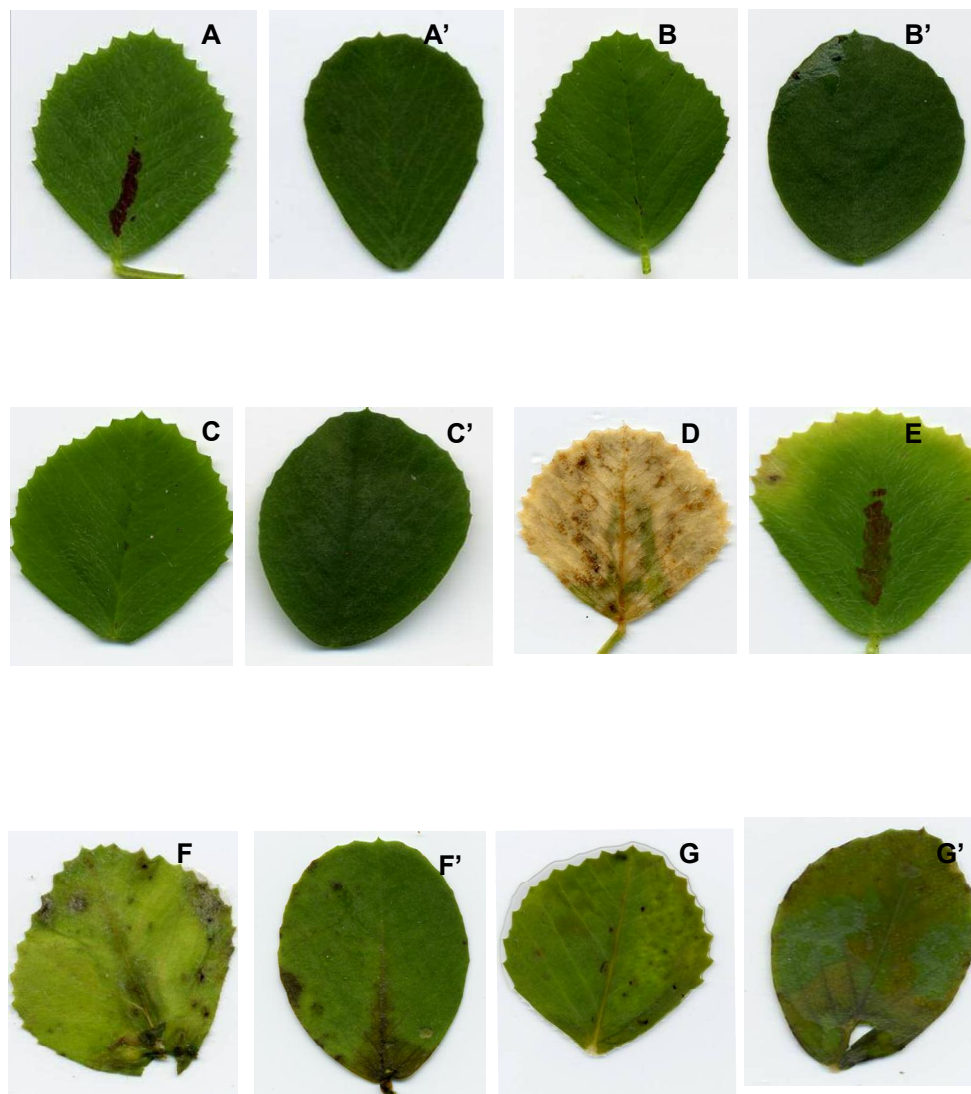
Figure 11. Scanned images of inoculated leaves of *M. truncatula* and *M. sativa* illustrating the disease progression on attached and detached leaves. A and A', Attached leaves of *M. truncatula* and *M. sativa*, respectively at 24 hpi (1 dpi). B and B', Attached leaves of *M. truncatula* and *M. sativa* at 3 dpi. C and C', Attached leaves of *M. truncatula* and *M. sativa* at 5 dpi. Disease symptoms were not observed on attached leaves at 1, 3 & 5 dpi. D and E, Attached leaves of *M. truncatula* at 8 dpi. D was a senescing leaf with black spots and green patches. F and F' Detached leaves of *M. truncatula* and *M. sativa* at 7 dpi showing disease. G and G' Detached leaves of *M. truncatula* and *M. sativa* at 8 dpi showing disease.

Discussion

Detached leaf Assay

Significant differences in virulence were not found among the transformants and wild type strains of *P. medicaginis*. Possible reasons for this may have been due to uneven spore load and/or the variability of the detached leaves used for the assay. Chung et al. (3) found that high conidial concentrations inhibited germination. The droplet inoculation method may have resulted conidial aggregations, which would have reduced germination and biased the findings. Since older leaves are more susceptible than younger leaves, then the age variability of the leaves used in these assays could have introduced sufficient experimental error to prohibit mean separations. The greater susceptiblity of older leaves may be due to the decreasing levels of phytoalexins, such as medicarpin, which decline with leaf age (8). The levels of medicarpin might be low in detached leaves, hence they are more susceptible to the pathogen.

Cytology of disease

Phoma medicaginis enters leaf tissues primarily by directly penetrating into the epidermal cells and only occasionally penetrating the through stomata. Stomata were more frequently penetrated by conidial germ tube inoculated on cotyledons. Similar findings were previously reported by Schenck et al. (18). Contrary to our findings, Castell-Miller (1) reported that *P. medicaginis* was never observed entering the host's leaf through stomatal openings. Other foliar pathogens, related to *P. medicaginis*, such as *Stagonospora nodorum*, *A. rabiei*, *L. maculans*, and *M. graminicola* also entered their

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hosts' leaves through the stomata. Conidia of *S. nodorum* germinated in three hours on detached wheat leaves and penetrated of host epidermal cells between 8-12 hrs (21).

Specialized penetration structures such as well-defined appressoria were not observed being produced by *P. medicagini*s in this study or previous studies (1). However, Hijano et al. (9), reported appresoria were seen at the tips of penetrating hyphae. Also, appressoria were not observed in *S. nodorum* (21). When compared to the conidia of *S. nodorum*, conidia of *P. medicaginis* germinated and penetrated host cells more slowly. *Medicago* epidermal cells exhibited autofluorescence when penetrated by the germ tubes of *P. medicaginis*. This penetration-associated autofluorescence of host cells also has been reported in the host cells of *L. maculans* (19) and *S. nodorum*. Yellow autofluorescence of plant cells is thought to be indicative of the accumulation of phenolics and/or the lignification of cell walls (7).

S. nodorum completes its life cycle on detached wheat leaves in 7 days, forming pycnidia in necrotic sectors of chlorotic leaves. On detached leaves, *P. medicaginis* completed its life cycle in 6-8 days.

M. graminicola, which causes leaf blotch of wheat, enters the host primarily through stomatal openings and germ tubes of spores oriented towards stomata (4). Unlike *M. graminicola*, the germ tubes of *L. maculans* did not exhibit preferential growth towards stomata (11). However, *L. maculans* germ tubes entered stomata during an incompatible interaction with Indian mustard, *Brassica juncea*, resulting in the necrosis

of the guard cells, deposition of callose, and accumulation of phenolics (2). The ascospores and pycnidiospores of *L. maculans* germinated at different rates with the former germinating as quickly as 4 hpi, while the latter germinated only after 24 hpi.

A. rabiei, a necrotrophic pathogen of chick pea, also penetrates its host both directly and through stomatal openings (10). During infection, the pathogen secretes toxins such as solanapyrones and cytochalasins. Solanapyrones are also present in the spore germination fluid, and may play a role in the initial stages of infection.

As shown in this study, infection by *P. medicaginis* causes chlorosis and necrosis of leaf tissue. Phytotoxins produced by *P. medicaginis* likely play an important role in the necrotizing development of the black spot phase. One such toxin produced by *P. medicaginis*, brefeldin A (BFA) has phytotoxic, mycotoxic and antibiotic activities *in vitro* (22). It inhibits Golgi-dependent secretion and vacuolar protein transport in plant cells (12). Even though BFA interferes with this crucial intracellular transport, high levels (20 μg) failed to affect alfalfa leaves, four days after application (24). But a derivative of the toxin, 7-dehydrobrefeldin A, disrupted Golgi stacks of sycamore maple cells and produced leaf spots with diameters greater than 3 mm. Though the toxin is hypothesized to be involved in the development of spots on infected leaves, BFA's role in the early stages of black stem leaf spots has not been thoroughly investigated. A scanning electron microscopy study rejected BFA's role in early development of leaf spots because they did not observe any Golgi disruptions in the epidermal cells where leaf spots are initiated. The matrix surrounding conidia and hyphae on the host may also

play a role in the development of spots in the early stages of the disease (3). Putative toxins may also play a role in the later stages of the disease when leaf chlorosis leads to leaflet abscission (6). Other species of *Phoma* are known to produce toxins which induce chlorosis (6) and the necrosis of plant tissues (23).

Results from this study indicated that older leaves were more susceptible to *P. medicaginis* than younger leaves. Levels of antimicrobial compounds, such as medicacarpin, a phytoalexin, decrease with age in leaves. Intermediates of the phytoalexin pathway accumulate when alfalfa plants are inoculated with *P. medicaginis* . The age-related decrease of phytoalexin levels in grape berries, results in the increased susceptibility to *Rhizopus stolonifer* (17).

Colletotrichum lindemuthianum (Sacc. & Magnus) Briosi & Cavara, a hemi-biotrophic pathogen of bean (*Phaselous vulgaris* L.) displayed distinct biotrophic and necrotrophic phases (15). It produced thick primary (biotrophic) hyphae in the initially infected epidermal cell followed by cell-to-cell invasion of adjacent cells by thin, narrow secondary (necrotrophic) hyphae. The thick primary hyphae have large diameters, which are thought to help reduce the total surface area in contact with the host. The thick wall is also thought to protect the pathogen from the cell wall-degrading enzymes of the host. The thin, narrow secondary hyphae then increases the surface area of the fungus, and likely helps it to absorb nutrients from the host and secrete phytotoxins and enzymes (15). A similar infection pattern was shown for *C. destructivum*, a pathogen of tobacco, *Nicotiana tabacum* (20).

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Objectives and Method of Study: Spring black stem of alfalfa (*Medicago sativa*) and other annual medics, such as *M. truncatula*, is caused by the necrotrophic, mitosporic, filamentous fungus, *Phoma medicaginis*.   The objectives of this research was to transform *P. medicaginis* using *Agrobacterium tumefaciens*-mediated transformation and generate a collection of T-DNA tagged transformants with mutations in genes involved in pathogenesis, to molecularly characterize some of *P. medicaginis* mutants generated, and to investigate the interactions of *P. medicaginis* with the hosts, *M. truncatula* and *M. sativa*, using detached leaf bioassays and fluorescent microscopy.

Findings and Conclusions: Nine hundred eighty-two T-DNA tagged transformants of *P. medicaginis* were generated using *Agrobacterium tumefaciens* mediated transformation. Several transformants showed altered morphology such as lack of melanin, defective pycnidia and absence of conidia. Sixty transformants were randomly checked by PCR to verify the presence of T-DNA in their genome. Further, ten transformants were selected for Southern blot hybridization and six were found to have single T-DNA inserts. Inverse PCR was performed on a mutant transformant, P1-A17, lacking melanin in hyphae and pycnidia, to identify the disrupted gene. The genomic DNA flanking the left border of the T-DNA of P1-A17 showed similarity to a serine/threonine protein kinase in the genome of the cereal glume blotch fungus *Stagonospora nodorum*. Detached leaves of *M. truncatula* ('Jemalong') and *M. sativa* ('Vernal' and 'Baralfa 42 IQ') were inoculated with wild type strains and ten transformants of *P. medicaginis* and disease progress followed over eight days. No consistent differences in the virulences of the transformants from the wild types strains were observed. A brightly fluorescent GFP-tagged transformant of *P. medicaginis*, P3-A6, was used to study the cytology of the host-pathogen interaction. Conidia germinated within 24 h and directly penetrated and infected epidermal and guard cells of leaves and penetrated through the stomata of cotyledons. This study provides insight into the virulence of the pathogen and the different stages of disease progression.

Advisor's signature: _____