

The Role of Alternate Hosts in the Epidemiology of *Ascochyta* Blight of Chickpea in Reduced Tillage Cropping Systems in the Pacific Northwest

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PROJECT OBJECTIVES:

Objective 1. To quantify *Ascochyta rabiei* infection of wheat and other alternate hosts grown in rotation with chickpea in rotational, minimum tillage or direct seeding cropping systems.

Objective 2. To determine the ability of *A. rabiei* to reproduce on wheat in rotational, minimum tillage and direct seeding cropping systems.

Objective 3. To develop a specific PCR primer to detect and quantify *Ascochyta rabiei* infection of wheat and other hosts.

KEYWORDS: alternate host, host specificity, inoculum, rotation

Statement of Problem: In US Pacific Northwest (PNW) cropping systems, wheat is typically grown in rotation with barley and/or legumes. Pathogens that cause disease on grasses do not generally cause disease on legumes and this specificity forms the basis for rotations as a disease control strategy. If pathogens have a wide host range and are able to colonize all hosts in the rotation, inoculum will build up resulting in high levels of disease and economic losses. The wheat-chickpea rotations used in the PNW are thought to reduce initial inoculum of the *Ascochyta* blight pathogen of chickpea. However, artificial inoculation studies indicate that the fungus may have a much wider host range than previously thought including wheat. If the pathogen can colonize and/or reproduce on wheat, this may provide a potential mechanism for the buildup of initial inoculum and a means for the pathogen to "bridge" across all crops in the rotation.

ZONE OF INTEREST: high rainfall zone - annual cropping systems

ABSTRACT OF RESEARCH FINDINGS:

Several greenhouse inoculation experiments have been performed that were designed to test the hypothesis that *A. rabiei* isolates originally isolated from chickpea as well as from several other presumed non-hosts are able to colonize plants other than chickpea. Results from both experiments clearly demonstrate that *A. rabiei* can survive on wheat, pea, alfalfa, and lentil without causing obvious disease symptoms. We have established a transformation system for *A. rabiei* and this procedure has been used to develop green and red fluorescent isolates of *Ascochyta*, which in turn has provided us with a powerful tool to observe the infection process of wheat and legumes *in planta*. The transformants have been validated as normal by observation of culture characteristics and morphology as well as ability to cause disease on chickpea hosts. In non-host infection studies, we have demonstrated that *A. rabiei* spores are capable of attaching to

non-host plant surfaces and subsequently germinating. Development beyond initial germ tube production on intact green non-host plants has not been demonstrated. However, when detached green wheat leaves were inoculated with *A. rabiei*, colonization ensued with leaf senescence. Significantly, *A. rabiei* developed asexual fruiting bodies (pycnidia) 12 days after inoculation. In a further experiment we inoculated the senescent leaf tips of an intact wheat plant and were able to demonstrate colonization by *A. rabiei*. These results suggest that *A. rabiei* is capable of colonizing and asexually reproducing on senescent non-host tissue, which may serve as an alternate source of inoculum. The remaining challenge will be to demonstrate this process of non-host colonization and fruiting body production under field conditions, and to investigate further the overwintering capability, saprophytic fitness and sexual stage formation on non-host tissue. The tools that we have developed as part of this STEEP project, such as the *A. rabiei* transformants and the real-time PCR assay will facilitate further studies into the role of alternative hosts in the epidemiology of *A. rabiei* in the Pacific Northwest.

RESULTS AND INTERPRETATION:

Inoculation and isolation experiments

In a series of replicated greenhouse experiments we have inoculated chickpea [cvs. Dwelley (resistant) and Spanish White (susceptible)], wheat (winter wheat cv. Madsen), lentil (cv. Crimson), field pea (cv. Columbian), and other potential hosts with *A. rabiei*. The *A. rabiei* isolates used represented the genetic diversity of known strains in the PNW according to molecular markers and virulence testing, as well as historical isolates obtained by Dr. W.J. Kaiser from a number of putative non-host plant species from *A. rabiei*-infected chickpea fields. California isolate CAB-00-12 was highly virulent on chickpea, and was used as a positive control for pathogenicity assays. Successful colonization of plants was confirmed by re-isolation from plant tissue at 4 wk post-inoculation after intensive ethanol/bleach surface-sterilization.

Chickpeas began showing lesions within one week of inoculation. Spanish White, a susceptible chickpea variety, showed moderate to severe lesions and disease response to all tested *A. rabiei* strains. Dwelley, a resistant variety, showed a range of response from mild to severe depending on the *A. rabiei* isolate, confirming findings of Dr. W.J. Chen, USDA-ARS. Other potential host plants generally appeared healthy and developed only small disease spots at a slower pace. These spots may be due to hypersensitive response of the host to the pathogen. All tested *A. rabiei* strains recently isolated in the PNW were re-isolated from chickpea, wheat, and pea, and most were re-isolated from lentil, and/or alfalfa (Table 1). All tested *A. rabiei* strains isolated from plants other than chickpea were re-isolated from chickpea and pea, and most were re-isolated from wheat and/or lentil (Table 2). Therefore, it appears that *A. rabiei* can infect several plant species in addition to chickpea without causing any obvious disease symptoms, and viable *A. rabiei* is maintained in these alternate host tissues. Perhaps *A. rabiei* is capable of escaping the surface sterilization treatment by directly penetrating one of a few cells, colonization of hypersensitive response tissue (possible hypersensitive reaction was noted during the wheat inoculations) or by entering stomata. Fifteen cultures of *A. rabiei* inoculated to and re-isolated from wheat, pea, lentil, or alfalfa were subsequently inoculated onto Dwelley chickpea. Dwelley again developed lesions and disease ratings were similar to infection with initial isolate (data not shown). This demonstrates that *A. rabiei* retains its pathogenicity to chickpea after colonizing “non-host” plants. Although these non-hosts remain generally healthy and productive they may

serve as a significant reservoir of infective *A. rabiei*. This gives support to the hypothesis that reduced tillage may increase Ascochyta blight by maintaining residues of chickpea, wheat, and other crops that all may serve as disease reservoir.

Table 1. Summary of greenhouse trials, addressing the potential of plants other than chickpea to support *A. rabiei*. Note: wheat plants demonstrated small white lesions, most likely due to hypersensitive response of the plant to *A. rabiei*. Disease rating 1=healthy, 10=dead; *A. rabiei* re-isolated *=yes.

Isolate	Source	Sp. White Chickpea	Wheat	Lentil	Pea	Alfalfa
C-12	California	7 *	3 *	1 *	1 *	1
EV-22	Evans Farm, Genesee, ID	6 *	3 *	1 *	1 *	1 *
EV-3	Evans Farm, Genesee, ID	3 *	3 *	1 *	1 *	1 *
SC-20	Schaefer Farm, WA	3 *	3 *	1 *	1 *	1
SC-22	Schaefer Farm, WA	4 *	3 *	1 *	1 *	1 *
SF-12	Silflow Farm, Genesee, ID	4 *	3 *	1	1 *	1 *
SP-41	Spillman Farm, Pullman, WA	8 *	3 *	1 *	1 *	1 *
SP-42	Spillman Farm, Pullman, WA	7 *	3 *	1 *	1 *	1 *
SP-55	Spillman Farm, Pullman, WA	4 *	3 *	2	1 *	1
water		2	2	1	1	1

Table 2. Summary of greenhouse trials, addressing the potential of *A. rabiei* isolated from plants other than chickpea to infect chickpea or other common rotation crops. Disease rating 1=healthy, 10=dead; *A. rabiei* re-isolated *=yes. Chickpea cultivars “Dwelley” and “Spanish White” (Sp. White) are resistant and susceptible to PNW isolates of *A. rabiei*, respectively.

Isolate	Source and Location	Dwelley Chickpea	Sp. White Chickpea	Wheat	Lentil	Pea
AR240	Tumble pigweed, ID	2 *	6 *	3 *	1	1 *
AR265	Alfalfa, WA	2 *	8 *	3 *	1	2 *
AR28	Wheat, ID	3 *	9 *	2	1 *	1 *
AR639	Lentil, WA	6 *	6 *	2 *	1	1 *
AF40	fabia bean, Lebanon	2 *	8 *	3 *	1 *	1 *
water		2	1	2	1	1

*In addition, where possible the isolates of Trial 2 were also inoculated onto their original host plant, i.e. AR265 was also inoculated onto alfalfa, AF40 was also inoculated onto Faba bean.

Cytological studies of host and non-host colonization and transformation of *A. rabiei*

To examine the interaction between *A. rabiei* and non-host plants in more detail, we performed inoculations and examined the interaction microscopically with electron, fluorescent and light microscopy. For fluorescent microscopy, we developed a transformation procedure using polyethylene glycol and transformation cassettes carrying hygromycin B and geneticin resistance genes (*hph* and *nptII*) as selectable markers. *Ascochyta rabiei* strains were transformed with

either pTEFDsRed2 or pTEFEGFP reporter plasmids to express red or green fluorescence, respectively. To represent the two *A. rabiei* chickpea pathotype groups, two isolates of *A. rabiei* AR20 and AR628, which are weakly and highly pathogenic on resistant chickpea, respectively were transformed for infection studies. Protoplasts prepared from each fungal isolate were transformed or co-transformed with plasmids indicated in Table 4 and 5. Inoculated plants were observed using an Olympus BH-2 fluorescence microscope equipped with 490 nm and 545 nm excitation filters.

Table 4. Transformation frequency (Tf).

Plasmid	Selectable marker	Reporter gene	Total no. of transformants	Tf/ μg plasmid/ 10^7 protoplasts (%)
pSH75	<i>hph</i>	-	216	2.12
pII99	<i>nptII</i>	-	127	2.82
pHygEGFPex	<i>hph</i>	GFP	376	4.65
pTEFEGFP	-	GFP	-	-
pTEFDsRed2	-	DsRed2	-	-

Table 5. Co-transformation frequency (CTf).

Selection plasmid	Reporter plasmid	No. of co-transformants	Total no. of transformants	CTf (%)
pSH75	pTEFEGFP	80	103	77.7
pSH75	pTEFDsRed2	146	228	64.0
pII99	pTEFEGFP	18	29	62.1
pII99	pTEFDsRed2	16	28	57.1

The percentage of *A. rabiei* conidia germinating on chickpea was significantly higher than on non-hosts. Sporadic germination was detected on the non-hosts, faba bean and wheat (Fig. 1A-C). As the number of conidia that germinated on non-hosts was very low and the distribution of *A. rabiei* conidia was non-uniform it was difficult to quantify this phenomenon. After inoculation of *A. rabiei* conidia onto an intact wheat plant, the number of conidia to be found on the wheat leaf surface decreased with time. After initial germination of a few *A. rabiei* conidia on the intact wheat plant leaf surface, no further development of *A. rabiei* occurred. The number of *A. rabiei* conidia found after inoculation on an intact healthy wheat plant decreased with time, also after the initial germination of a few conidia and no further development was noted. In addition, eight days after inoculation onto intact wheat leaves the distribution of GFP within *A. rabiei* conidia was found to be abnormal with uneven distribution throughout the conidia, perhaps suggestive of a substance toxic to *A. rabiei* present on the wheat leaf surface or a signalling molecule produced by the wheat plant that affected the *A. rabiei* conidia. As noted with both fluorescent and electron microscopy some *A. rabiei* conidia on the faba bean leaf had apparently swollen and burst, perhaps suggestive of some pre-penetration response by the plant (Fig 1B – lower image). These phenomena warrant further investigation. The cytological findings contradict those of the inoculation and isolation experiments as we were not able to observe infection of the intact non-host plants. Perhaps we were not able to observe the possible few limited infection events using our sampling and observation techniques. We speculate that *A. rabiei* is capable of occasionally

infecting non-host plants, be it by directly penetrating cells, colonization of hypersensitive response tissue or by entering stomata to escape the surface sterilization procedure.

In contrast to the inoculations of healthy intact non-host plants, we were able to demonstrate that *A. rabiei* germinates on, penetrates and colonizes detached senescing wheat leaves (Figs 2A,B). The thickened and intercellular *A. rabiei* hyphae appeared to align with the wheat leaf epidermal cells (Fig. 2A). Significantly, pycnidia developed on detached wheat leaves 12 days after inoculation (Fig. 2B). Given this finding, we attempted to determine if *A. rabiei* was capable of colonizing the senescent leaves of intact non-host plants. The naturally senescing leaf tips of intact wheat plants were inoculated with an *A. rabiei* conidial suspension. Six days after inoculation *A. rabiei* was found to have colonized the senescing wheat leaf tip (Fig. 2C). In an attempt to determine if *A. rabiei* is capable of sexual development on non-host tissue, we inoculated senesced wheat stems (both autoclaved and non-autoclaved) and senesced chickpea stems (control) with a mixed isolate conidial suspension. The wheat and chickpea stems were incubated in a growth chamber under conditions suitable for *A. rabiei* sexual stage development. Pseudothecia and pycnidial bodies were observed on the chickpea stems, however the sterilized and non-sterilized wheat stems contained no obvious sign of colonization by *A. rabiei*. These results do not preclude the possibility of *A. rabiei* sexual stage development on non-host tissue, further conditions and non-host tissues need to be tested.

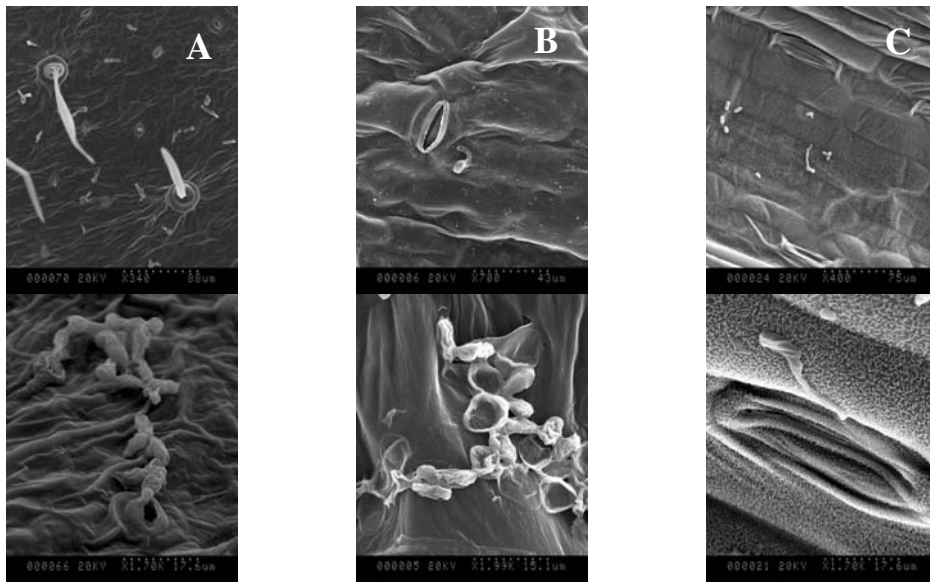


Fig. 1: Electron micrographs of (A) chickpea (*Cicer arietinum* L.), (B) faba bean (*Vicia faba* L.), and (C) wheat (*Triticum aestivum* L.) leaves inoculated with conidia from the chickpea pathogen *Ascochyta rabiei*, 3 days post-inoculation. The images demonstrate germination of conidia and germ tube growth of *A. rabiei* on host and non-host surfaces. Note: in the lower image of plate B, the swollen and burst appearance of *A. rabiei* conidia on the faba bean leaf.

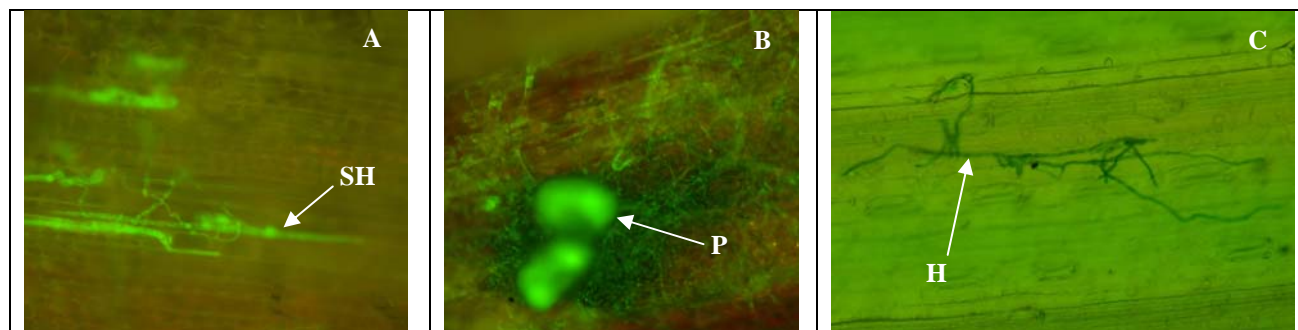


Fig. 2: (A) Fluorescent light micrograph of GFP-transformed *A. rabiei* on a detached wheat leaf 9-days after inoculation. SH indicates swollen *A. rabiei* hyphae. (B) Fluorescent light micrograph of GFP-transformed *A. rabiei* on a detached wheat leaf 12 days after inoculation. P indicates developing pycnidial bodies. (C) Light micrograph of *A. rabiei* 6 days post-inoculation on a naturally senescing wheat leaf tip of an intact healthy plant. The leaf was cleared with chloral hydrate and the *A. rabiei* hyphae (H) were stained with aniline blue.

According to our cytological observations, *A. rabiei* does not infect healthy non-host tissue. However, we have demonstrated that *A. rabiei* is capable of colonizing and producing asexual fruiting structures on detached senescing wheat leaf tissue. In addition, we have demonstrated the colonization of the naturally senescing leaf tips of an intact wheat plant. These findings together with those of the inoculation and isolation experiments suggest that alternate hosts may play a significant role in the epidemiology of *A. rabiei*. Further studies should attempt to quantify this potential inoculum source, the competitive saprophytic ability of *A. rabiei* and perhaps significantly the possibility of sexual stage development which is capable of producing long range aerially dispersed inoculum.

Development of specific PCR primer pair to detect and quantify *Ascochyta rabiei*

A real-time, fluorescent polymerase chain reaction (PCR) assay was developed to quantify *A. rabiei* in plant tissues. The nuclear ribosomal intergenic spacer (IGS) regions of target and non-target *Ascochyta* spp. were sequenced, aligned and used to design a specific primer pair to *A. rabiei*. SYBR green chemistry was used to detect the single *A. rabiei*-specific PCR amplicon. The real-time PCR primer pair was sensitive, reliably detecting down to 10 fg of genomic DNA, i.e. less than one *A. rabiei* genome (Fig. 3). The primer pair proved to be specific by screening against non-target DNA of closely related *Ascochyta* spp. and by BlastN searches against the GenBank database. Various sampling strategies, DNA extraction techniques, SYBR green master mixes and real-time PCR platforms have been tested. To date the assay has been successfully applied to seed, soil and chickpea stem debris. The development of this real-time PCR assay for the quantification of *A. rabiei* biomass in plant and environmental samples will facilitate future research and disease control projects. In addition, we hope to further develop the assay for the routine testing of chickpea seed. A rapid and reliable assay for the presence of *A. rabiei* on chickpea seed would prove to be a valuable tool for disease management decisions, such as whether or not to treat specific chickpea seed lots for *Ascochyta* infection, or identifying appropriate regions in which to plant specific chickpea seed lots based on the relative risks of *Ascochyta* blight associated with regional environmental conditions.

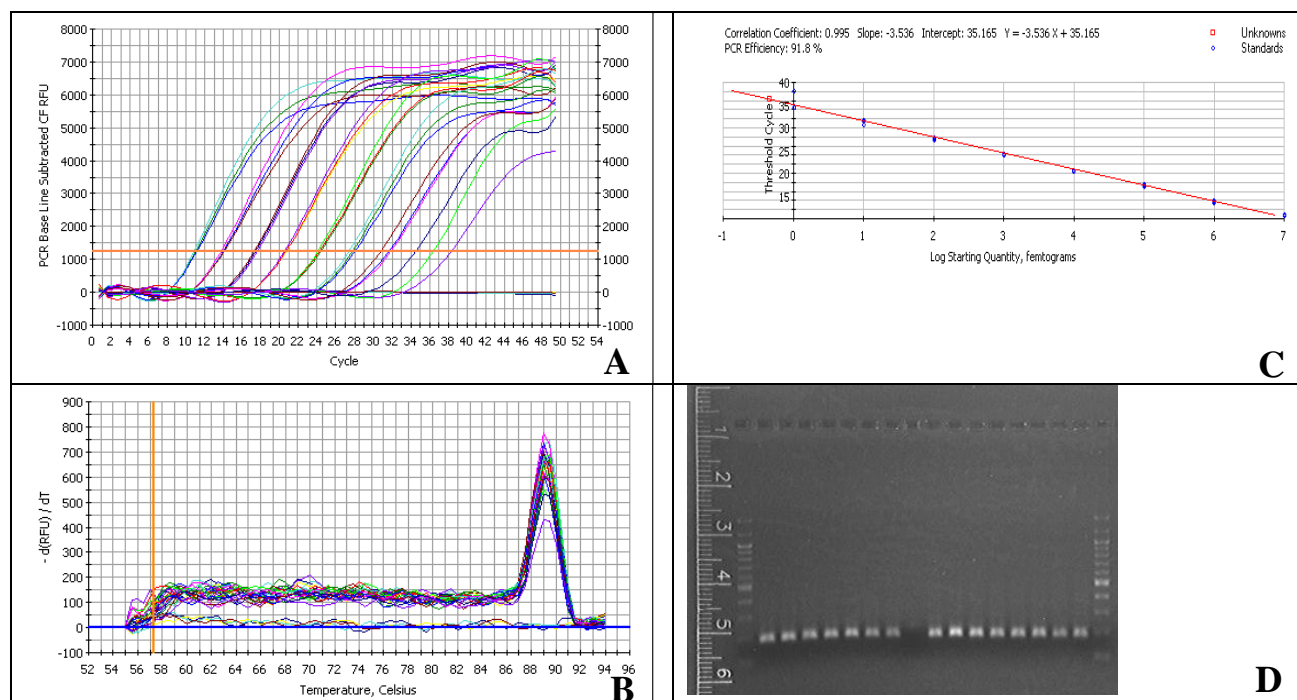


Fig. 3: Real-time PCR assay for *Ascochyta rabiei*, as detected with SYBR green chemistry. (A) Real-time PCR amplification graph, dilution series of standards from 10 ng to 1 fg (curves left to right) of DNA in triplicate. (B) Plot of standard curve for the interpolation of *A. rabiei* DNA concentrations of samples (C) Melt peak graph; a single PCR product is demonstrated by single peaks. (D) Agarose gel confirming the melt peak assay of single PCR products.

INTERACTION WITH OTHER SCIENTISTS CONDUCTING RELATED ACTIVITY:

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PUBLICATIONS AND PRESENTATIONS:

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