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Lifestyles of the wild grass-associated ascomycetes *Zymoseptoria pseudotritici* and *Zymoseptoria ardabiliae*

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1 Introduction

1.1 Interaction between fungi and plants

Fungi are important players in natural as well as in agro-ecosystems and many fungal species establish diverse and complex interactions with plants. Some species are important decomposers and recyclers of dead organic materials while others positively or negatively interact with plant roots in the rhizosphere or with aboveground plant components (Zeilinger et al. 2015). Since plant-associated fungi have a big impact on plant health and productivity, the knowledge of plant-fungal interactions has become an important topic for agriculture (Lo Presti et al. 2015).

Fungi that are associated with plants have very diverse lifestyles and different feeding strategies. Many interactions start with the fungus invading the plant, either by entering with hyphae through stomata or by directly penetrating plant cell walls. Direct penetrations are often enabled by a specialized organ called appressorium e.g. for *Ustilago maydis*, which is a biotrophic basidiomycete fungus (Giraldo & Valent 2013) or hyphopodium e.g. for arbuscular mycorrhizal (AM) fungi of the phylum Glomeromycota (Parniske 2008; Zeilinger et al. 2015), that apply high turgor pressure to break down plant cell walls. Some fungi also secret toxins and plant cell wall degrading enzymes (PCWDEs) to kill plant cells (Lo Presti et al. 2015). The plant can recognize fungal invasion with specialized pattern recognition receptors (PRRs) that are localized on the cell surface. These receptors bind conserved pathogen-, microbial- or damage-associated molecular patterns (PAMPs, MAMPs or DAMPs) like the fungal cell wall component chitin (PAMP) and can subsequently trigger an immune response that blocks the propagation of the fungal hyphae (Jones & Dangl 2006). However, some fungi can avoid recognition and activation of plant immune response by secreting effectors. Effectors can be small secreted proteins, secondary metabolites or small RNAs that modify host cell structure, metabolism and function in order to facilitate fungal colonization and reproduction (Bozkurt et al. 2012; Rafiqi et al. 2012; Weiberg et al. 2013; Keller et al. 2005). They suppress host immune response, manipulate host cell physiology and can also induce plant death (Lo Presti et al. 2015). However, specialized resistance proteins (R-proteins) inside plant cells can recognize fungal effectors and induce effector-triggered immunity (ETI). In this case, the effector is called avirulence (Avr) protein and its recognition leads to a strong hypersensitive response that can block further fungal growth (Jones & Dangl 2006).

Ecologically as well as agriculturally, one of the most important plant-fungus interactions are mycorrhizae, which are mutualistic symbioses between plant roots and fungi in the soil (Trappe et al. 2005; Zeilinger et al. 2015). Mycorrhizal fungi interact with the plants they are living in without harming them and both partners, fungi and host plants, benefit from each other. Mycorrhizal fungi improve the nutrient status and health of their host plants by influencing mineral nutrition, water absorption, growth and disease resistance, whereas in exchange the host plant provides carbohydrates and nitrogen for fungal growth and reproduction (Bonfante & Genre 2010). Due to this positive impact more than 90 % of all plant

species are associated with mycorrhizal fungi, which are a heterogeneous group of diverse fungal taxa (Bonfante & Genre 2010).

Endophytic fungi, e.g. members of the *Epichloë* genus, are symbionts and live associated with members of the *Poaceae* family. *Epichloë* species are wildly distributed mutualists and can protect their host against biotic and abiotic stresses (Schardl et al. 2013). In general, endophytes colonize living tissues of roots, stems or leaves for a long time without causing visible symptoms until senescence of the host plant (Zeilinger et al. 2015).

In contrast, plant-pathogenic fungi interact with their host plants by harming them to get the nutrients they need for growth and reproduction. A pathogen is generally defined as an organism that is able to cause a disease within a host. This lifestyle depends on different interaction strategies: biotrophic, hemibiotrophic and necrotrophic strategy (Lo Presti et al. 2015; Zeilinger et al. 2015). Necrotrophic pathogens kill host plant cells and feed on the dead host tissue. They penetrate and kill the host plant by secreting large amounts of plant cell wall-degrading enzymes (PCWDEs), reactive oxygen species (ROS) and/or toxic metabolites and proteins and colonize the dead cells (Lo Presti et al. 2015). Biotrophic pathogens depend on living host plant tissue as nutrient source and do not produce toxins but often secret effectors to suppress the host immune system. Biotrophic fungi can grow either intra- or intercellular in the host tissue (Zeilinger et al. 2015). Hemibiotrophic pathogens represent intermediates between the necrotrophic and biotrophic lifestyle, initially growing as biotrophs and later switching to a necrotrophic lifestyle (Lo Presti et al. 2015).

Considering this broad diversity of strategies to infect host-plants by pathogenic fungi, it is of common interest to learn more about the evolution of plant pathogens. How do pathogenic fungi evolve and what are the prerequisites to become a pathogen? Since many plants are always colonized by endophytic fungi, one possible hypothesis is that pathogens can evolve from endophytes. However, the reverse situation cannot be excluded since the earliest diverging branches of fungi already contains parasitic species (James et al. 2006). Additionally, a broader understanding of the ecology and evolution of pathogens is essential in order to characterize traits such as host sensing, exploration of host compounds, reproduction and interaction with other microbial species, but also for the improvement of strategies to control pathogens in agricultural ecosystems.

1.2 Three closely related *Zymoseptoria* species

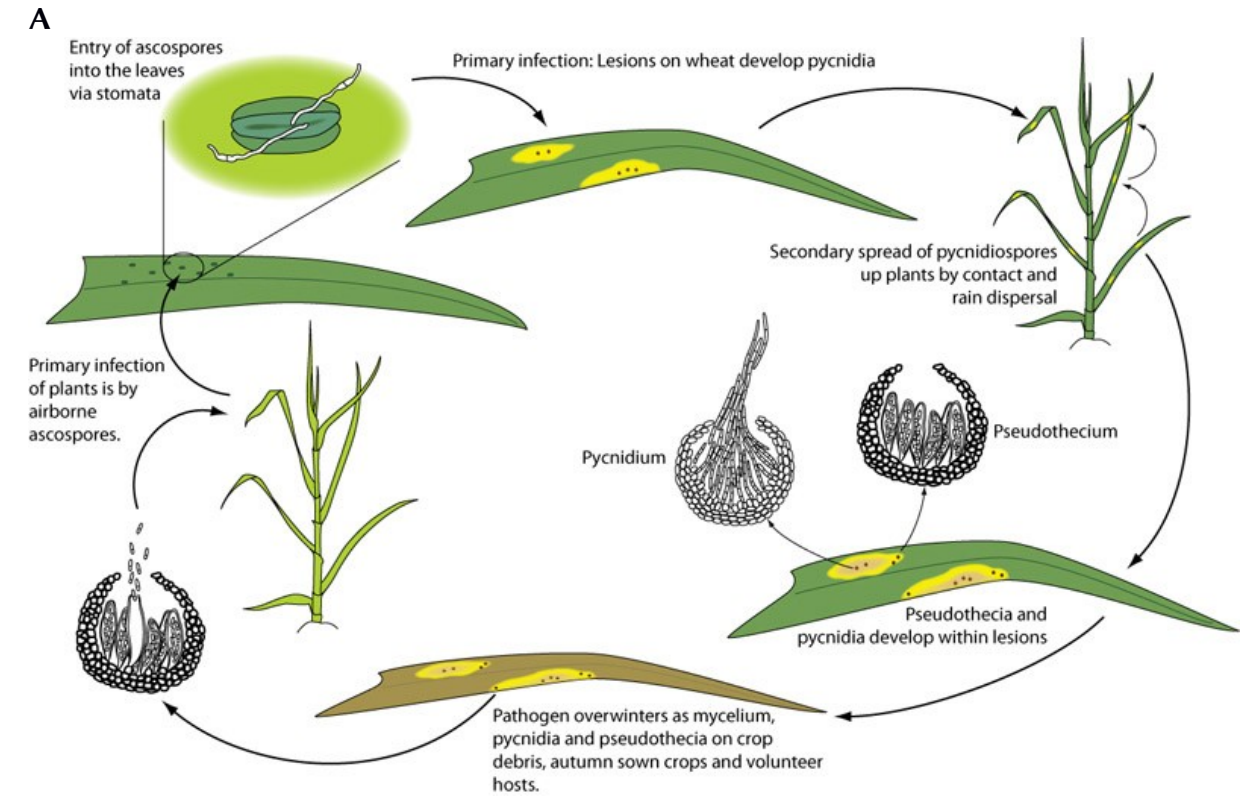
The most prominent member of the ascomycet genus *Zymoseptoria* is *Zymoseptoria tritici* (synonyms: *Mycosphaerella graminicola* and *Septoria tritici*). Through domestication of wheat in the Middle East, which started 11,000 years ago, this species emerged and today infects wheat all over the world (Stukenbrock et al. 2011).

Z. tritici is well known for causing the foliar disease Septoria tritici leaf blotch (STB) on wheat. This disease is characterized by necrotic lesions on leaves. The lesions contain black or brown fruiting structures in the substomatal cavities of the host, the asexual pycnidia, or underneath the host epidermis, the sexual pseudothecia (Ponomarenko et al. 2011). *Z. tritici* is a hemibiotrophic pathogen and its disease cycle starts with the attachment of air-borne ascospores (sexual) or splash-dispersed conidia (asexual) on the leaves (Figure 1). The spores germinate and develop infection hyphae that penetrate through stomata and gain entry into the sub-stomatal cavities. Once inside the plant the hyphae proliferate intercellulary in the mesophyll. After some proliferation the fungus changes from biotrophic to necrotrophic growth that leads to plant cells collapse, formation of necrotic lesions and eventually to the development of sexual and asexual fructifications. At the end of the disease cycle conidia (asexual fungal spores) and ascospores (sexual fungal spores) can be released and disseminated by rain and wind. They serve as inoculum to infect surrounding leafs and plants instantly and during subsequent growing seasons (Ponomarenko et al. 2011).

In Iran, closely related sister species of *Z. tritici* were found on uncultivated grasses (*Elymus repens, Dactylis glomerata* and *Lolium perenne)* (Stukenbrock et al. 2007; Stukenbrock et al. 2011). Two new *Zymoseptoria* species, *Z. pseudotritici* (formerly called *Mycosphaerella* S1) and *Z. ardabiliae* (formerly called *Mycosphaerella* S2), were introduced 2012 by Stukenbrock and colleagues and characterized as endemic for the Middle East (Stukenbrock et al. 2012).

Detached leaf infection assays on leaves of wheat, *Elymus repens*, *Dactylis glomerata*, *Lolium multiflorum* and *L. perenne* have shown that the three *Zymoseptoria* species can produce pycnida on the same hosts, but the degree of pycnidia coverage differed (Stukenbrock et al. 2011). Similarly, pycnidia for different isolates of *Z. pseudotritici* and Z. *ardabiliae* were formed on detached leaves of *D. glomerata* var. Loke and var. Belunga, *L. hybridum* var. Ibex, *L. perenne* var. Arvicola and *Arrhenaterum elatius* var. Arone at 27 days after inoculation. In contrast, no symptoms could be observed on individuals of these grasses in a simultaneously conducted plant experiment under greenhouse conditions (Haueisen et al., unpublished). Also, during infection experiments on living wheat leaves, *Z. ardabiliae* and Z. *pseudotritici* did not cause disease symptoms and microscopic analyses showed that infection hyphae are stopped after stomatal penetrations (Haueisen et al., unpublished). However, pycnidia of *Z. pseudotritici* and Z. *ardabiliae* isolates have been observed on leaves of compromised wheat plants, where intense light conditions induced early senescence and necrotic leaf tissues, which was also observed for mock treated wheat plants (Haueisen et al., unpublished).

Taken together, reproduction of *Z. pseudotritici* and Z. *ardabiliae* isolates by pycnidia formation was so far only observed on compromised or senescent grass leaves but actual infections and active induction of disease could not be shown experimentally yet. In contrast to the hemibiotrophic wheat pathogen *Z. tritici*, we still know very little about the lifestyles of *Z. pseudotritici* and Z. *ardabiliae* and how they colonize and reproduce inside grass leaves. Gaining insight in the lifestyles of *Z. pseudotritici* and Z. *ardabiliae* and how they interact with host plants will also improve our understanding of how *Z. tritici* evolved and adapted to be a pathogen of domesticated wheat.



B

Figure 1: **Septoria tritici blotch:**
A: Disease cycle of *Z. tritici* on wheat, from the entrance through the stomata, to the colonization of intercellular tissue area, to induction of cell death and formation of necrotic areas (STB) and to the creation of pycnidia in the substomatal cavity in necrotic areas and final release of spores to the surroundings (Ponomarenko et al. 2011); B: Leaf inoculated with *Z. tritici* showing the typical foliar disease STB. The black spots show the characteristic pycnidia.

1.3 Fluorescent strains to analyze fungal structures inside leaves by fluorescence microscopy

The use of fluorescing proteins as tools for fluorescence microscopy has become more and more important for researchers, since they allow the microscopic observation of individual cells or even whole organisms *in-vivo*. Fluorescent proteins have also proven to be very useful in fungi, as they allow to track the spread of fungi in natural systems and facilitate the analysis of fungal structures inside plant tissue to compare fungal behavior during the colonization of plants (Lorang et al. 2005). The most prominent fluorescent protein is the green-fluorescent protein (GFP) of the jellyfish *Aequorea victoria* which encoding region was the first to be cloned (Presher et al. 1992). The application of GFP as reporter or marker is very advantageous since the protein is extremely stable *in vivo* and requires only UV or blue light and oxygen to emit its green fluorescence (Lorang et al. 2005). The first filamentous fungus where the *gfp* gene was successfully integrated and expressed was *Ustilago maydis* (Spellig et al. 1996). Also, for the reference isolate of *Z. tritici* gfp-expressing mutants have been created and applied (Rohel et al. 2001), but cytosolically fluorescing strains of *Z. pseudotritici* and *Z. ardabiliae* are not available yet.

Creating *Z. pseudotritici* and *Z. ardabiliae* mutant strains showing green or red fluorescence in their cytoplasm will allow us to study and compare the different *Zymoseptoria* species during host interactions *in-vivo* and thereby facilitate the analysis of their lifestyles.

1.4 Objectives and Hypothesis

The aim of this project is to investigate the lifestyle of *Z. pseudotritici* and *Z. ardabiliae*. For this reason following questions are of our interest:

- Do *Z. pseudotritici* and *Z. ardabiliae* have the same lifestyle as their hemibiotrophic wheat infecting sister species *Z. tritici*?
- How do *Z. pseudotritici* and *Z. ardabiliae* actually colonize their wild grass hosts?

Based on the results of different detached leaf assays and the preliminary observations on senescent wheat leaves, we hypothesize:

- *Z. pseudotritici* and *Z. ardabiliae* colonize grass leaves in a saprotrophic or endophytic manner and do not possess the same pathogenic properties as *Z. tritici*.

To test our hypothesis, we will conduct plant infection experiments where we inoculate leaves of *Triticum aestivum* cultivar Obelisk and *Dactylis glomerata* var. Loke with isolates of the three *Zymoseptoria* species. For all plants, we inoculate the 2nd leaf but at different "ages" of the plant to test if natural senescence and associated necrosis of the leaves allows endophytic or saprotrophic growth and pycnidia formation of the different *Zymoseptoria* species. We will evaluate necrosis and pycnidia formation for each sub-experiment and combine this with microscopic analyses of inoculated leaves we collected during the time course of each sub-experiment.
Furthermore, we will create fluorescent strains for *Z. ardabiliae* and *Z. pseudotritici* to be able to observe with *in-vivo* microscopy plant colonization and the interaction between different *Zymoseptoria* species and isolates *in-planta*. To obtain these strains, we create specific plasmids carrying inserts with the genes encoding GFP (green fluorescence protein) or dsTomato (red fluorescence protein) and use them to transform the respective fungal isolates by *Agrobacterium tumefaciens*-mediated transformation.

2 Material and Methods

2.1 Material and ordering sources

2.1.1 Fungal and bacterial strains and plant species

The used fungal strains belong to the species *Zymoseptoria tritici* (Zt), *Z. ardabiliae* (Za) and *Z. pseudotritici* (Zp) and are shown in Table 1. For the plant experiment strains of all three species were used. While for the construction of plasmids with an encoding region for fluorescing proteins only genomic DNA (gDNA) of the strains Zp13 and Za17 was used.

The TOP10 strain (Invitrogen) of *E. coli,* which is a derivate of *E. coli K12,* was used for plasmid amplifications. The seeds that were used for the plant experiment are of *Triticum aestivum* (cultivar Obelisk) and of the non-domesticated grass *Dactylis glomerata* var. Loke.

2.1.2 Plasmids and oligonucleotides

All plasmids used and intended to be generated are summarized in Table 2 and shown in Figure 2.

All oligonucleotides that were used to create the plasmids are summarized in Table 3.

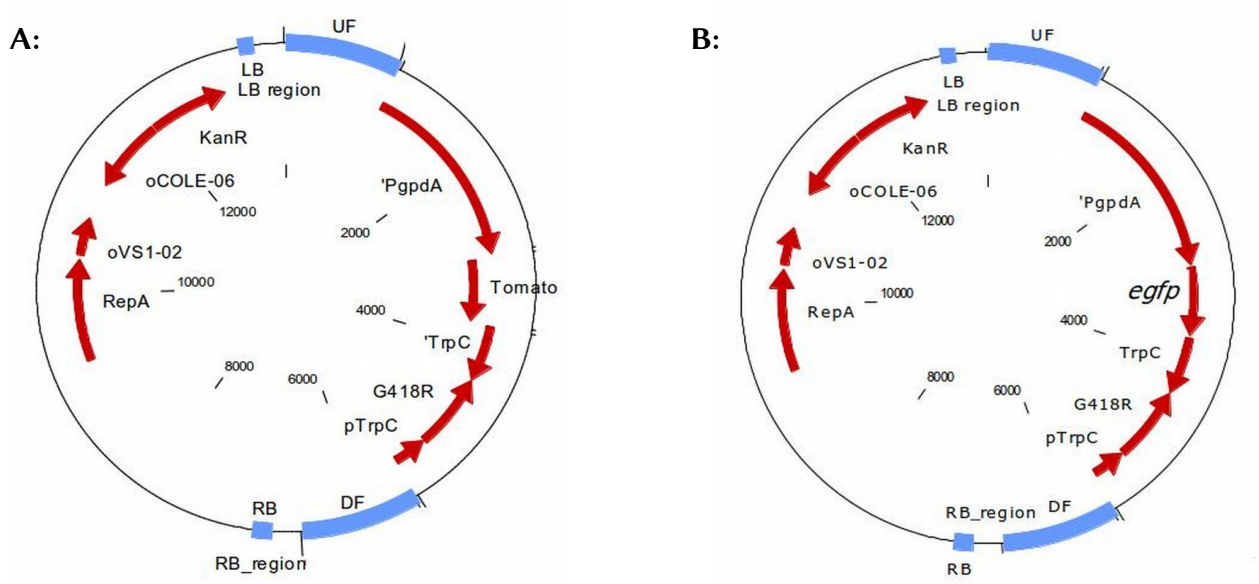


Figure 2: **Plasmids for integration of genes encoding GFP/dsTomato in *Zymoseptoria* spp. by *Agrobacterium tumefaciens*-mediated transformation:** A: plasmid map for pES94 and pES199 with backbone from pES61 with KanR: kanamycin resistance, UF and DF from a non-coding region in the genome of strain Za17, Insert with G418R: geneticin resistance encoding region, Tomato: red fluorescent protein encoding-region, PgpdA: promotor sequence of glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*, for the transcription of the gene encoding dsTomato, TrpC: terminator sequence for geneticin resistance gene of *A. nidulans*, pTrpC: promotor sequence for geneticin resistance gene of *A. nidulans;* B: plasmid map for pES93 and pES198 with backbone from pES61 with KanR: kanamycin resistance UF and DF from a non-coding region in the genome of strain Zp13, Insert with G418R: geneticin resistance encoding region, egfp: green fluorescent protein encoding region, PgpdA: promotor sequence for the transcription of the gene encoding GFP; TrpC: terminator sequence for geneticin resistance, pTrpC: promotor sequence for geneticin resistance gene.

2.1.3 Buffers and solutions

Standard buffers and solutions were prepared according to Ausubel et al. (1987) and Sambrock et al. (2001), and supplied by Difco (Augsburg), Ivitrogen (Karlsruhe), Merck (Darmstadt), Roche (Mannheim), Roth (Karlsruhe), and Sigma-Aldrich (Deisenhofer). Special buffers and solutions are mentioned in the according methods. If necessary all media, solutions and buffers were autoclaved for 5 minutes at 121 °C.

2.1.4 Enzymes and antibiotics

The restriction endonucleases were obtained from New England Biolabs GmbH (NEB, Frankfurt am Main). Standard PCR reactions were conducted using PHUSION-Polymerase of NEB. For fragment assembling high-fidelity DNA-Polymerase (NEB) was used.
Enzymatic digestions of DNA were conducted with the restriction enzymes NotI, EcoRV, NdeI, ApaI, ApaL, PvuI-HF, and XhoI according to manufactures' instructions.
Kanamycin was obtained from *Streptomyces* spp., supplied by Sigma-Aldrich.

2.1.5 Kits and other materials

To purify DNA from agarose gels the Wizard SV Gel and PCR Clean-Up System (Promega) was used. This kit was also used to purify the plasmids DNA after assembling. The GeneRuler 1kb DNA Ladder of New England Biolabs was used as a size marker on gels.
The Plasmid Midi Kit and the QIAprep Spin Miniprep Kit by QIAgen were used to extract plasmid-DNA from *E. coli.* All kits are listed in the appendix.

Table 1: *Zymoseptoria* spp. strains

Strain	Description	Mating type	Resistance	Provided by	Reference
Zt05	Wild type strain	mat 1-1	none	Karin Thygesen	Thygesen et al. 2009
Zt10	Wild type strain	mat 1-1	none	Bruce McDonald	-
Zp13	Wild type strain	mat 1-1	none	Bruce McDonald	Stukenbrock 2013
Zp72	Wild type strain	mat 1-1	none	Bruce McDonald	-
Za17	Wild type strain	mat 1-1	none	Bruce McDonald	Stukenbrock 2013)
Za48	Wild type strain	mat 1-1	none	Bruce McDonald	-

Table 2: Plasmids

Name	Application[1]	Resistance[2]	Reference
pES61	Backbone	Km	Petra Happel
pES91	cytoplas_GFP_GenR_mod_ZT	Km, G418R	Janine Haueisen
pPP56_pZero	source coding sequence dsTomato	--	Shaner et al. 2004
pES93	cytoplas_GFP_GenR_Zp13	Km, G418R	This study
pES94	cytoplas_GFP_GenR_Za17	Km, G418R	This study
pES198	cytoplas_Tomato_GenR_Zp13	Km, G418R	This study
pES199	cytoplas_Tomato_GenR_Za17	Km, G418R	This study

[2] Kanamycin (Km), Geneticin (G418R)

Table 3: Oligonucleotides

Name	Sequence 5' → 3'	Application[1,2]
oES2561	TACGAATTCTTAATTAAGATTCGGGCATCGGCAAGCAATC	Amplification of UF [R] region from gDNA of Zp13
oES2562	GTCGAAAGATCCCCGGGTACGCATGCTAAGGCGTGTTAAG	Amplification of UF [F] region from gDNA from Zp13
oES2563	CAGGTTGATGAGGGAAGAGGATTCCGGATCTCGTCGTATG	Amplification of DF [R] region from gDNA from Zp13
oES2564	TCGAGGGTACCGAGCTCGATATATCGCATACGCTGCTACC	Amplification of DF [F] region from gDNA from Zp13
oES2565	CCATCTTCAGTATATTCGGCGAGGCGGAGGCTTAATTAAC	Tomato [R] from pPP56_pZero
oES2566	GTTGACATGGGCCCCCACTTCTTACTTGTACAGCTCGTCC	Tomato [F] from pPP56_pZero
oES2567	TACGAATTCTTAATTAAGATCAGTGCAGTGGACGGAAACG	Amplification of UF [R] region from gDNA from Za17
oES2568	GTCGAAAGATCCCCGGGTACGGCCATCCATGCATCATCTC	Amplification of UF [F] region from gDNA from Za17
oES2569	CAGGTTGATGAGGGAAGAGGGATGCATGGATGGCCCTGTC	Amplification of DF [R] region from gDNA from Za17
oES2570	TCGAGGGTACCGAGCTCGATGCGTATCCCGGTTCCATGTC	Amplification of DF [F] region from gDNA from Za17
oES2575	CTTAACACGCCTTAGCATGCGTACCCGGGGATCTTTCGAC	PgpdA [R] from pES91
oES2576	GAGATGATGCATGGATGGCCGTACCCGGGGATCTTTCGAC	PgpdA-egfp-TrtpC-GenR-PtrpC [R] from pES91
oES2577	CATACGACGAGATCCGGAATCCTCTTCCCTCATCAACCTG	TrtpC-GenR-PtrpC [F] from pES91
oES2578	GACAGGGCCATCCATGCATCCCTCTTCCCTCATCAACCTG	PgpdA-egfp-TrtpC-GenR-PtrpC [F] from pES91
oES2579	GTTAATTAAGCCTCCGCCTCGCCGAATATACTGAAGATGG	PgpdA [F] fro pES91
oES2580	GGACGAGCTGTACAAGTAAGAAGTGGGGGCCCATGTCAAC	TrtpC-GenR-PtrpC [R] from pES91

[1]Oligonukleotides hybridize with the sense-strand [R] or with the complementary strand [F] of the corresponding gene.

[2] UF = 5' flanking region of target locus; DF = 3' flanking region of target locus

2.2 Plant infection assays

This protocol is a modified version of Poppe et al. (2015).

Seeds of bread wheat cultivar Obelisk and *Dactylis glomerata* var. Loke were placed into plastic boxes on wet filter paper and incubated in a phytochamber (20 °C; 16 h light period; lighting: 240 µmol $m^{-2}s^{-1}$; 90 % humidity) for pre-germination. Subsequently the seedlings of both plant species were planted respectively four and nine days after pre-germination start. All pots were placed in a phytochamber and grown for one, three or five weeks respectively (Figure 3). *Z. tritici* strains Zt05 and Zt10, *Z. pseudotritici* strains Zp13 and Zp72, and *Z. ardabiliae* strains Za17 and Za48 were first cultivated on fresh YMS medium, which is composed of 4g yeast extract, 4g malt extract, 4g sucrose and 16g agarose dissolved in 1000ml de-ionized water, and incubated at 18 °C for five days. Afterwards, colonies were picked, inoculated in 20 ml liquid YMS and incubated at 18 °C and 200 rpm for three days. From this pre-culture 5 ml were transferred to 100 ml of fresh YMS and incubated for two more days (18 °C, 200 rpm). These main cultures were used for preparation of the fungal inoculum. Spore concentrations were determined with a "Neubauer improved" cell counting chamber and the final concentration of fungal inoculum was adjusted to 1 x 10^8 cells/ml. Fungal cells were harvested by centrifugation (10 min at 4000 rpm) and resuspended in tap water containing 0.001 % Tween 20 (Roth, Karlsruhe, Germany). Plant infections were performed at three subsequent time points, respectively one, three and five weeks after planting (Figure 3, red arrows). The second leaf of each plant was inoculated by brushing the spore suspension on a marked area. After waiting a few minutes for the solution to dry, inoculated plants were placed into autoclaving bags and were incubated in the phytochamber for 48 hours at 100 % humidity and subsequently incubated at 20 °C with a 16 hours light period at 90 % humidity for the remaining days of the experiment (see Figure 3).

To collect data about the temporal disease development, treated leaves were screened manually for necrosis and pycnidia appearance every day starting at 7 days post inoculation (dpi).

Additionally, every seven days one leaf per treatment was collected and stored in 70 % ethanol (Figure 3, purple arrows). These leaves were then used for microscopy, to analyze the distribution of fungal hyphae in and on the leaves. Staining and microscopy will be explained further below.

At the end of each experiment (Figure 3, blue arrow) all treated leaves were cut, stuck on white paper, dried at 4 °C and scanned with HP Photosmart C4580 to obtain high quality images. The degree of necrosis and pycnidia on the leaves was evaluated manually by using a binocular-camera device (Leica S8AP0, Leica DFC480). To quantify necrosis and pycnidia coverage, a scoring scheme of six categories (0, 1-20, 21-40, 41-60, 61-80, 81-100) representing the percentage of leaf area covered with necrosis or pycnidia was used. Additionally, with the image processing software ImageJ a batch processing macro (Macro Settings_V15_20) was run on the images of the scanned leaves, with which automatically pycnidia number and necrosis coverage for each leaf was measured. For all experiments leaf inoculations and evaluations were done double-blind and the order of plants belonging to different treatment groups was randomized on the trays.

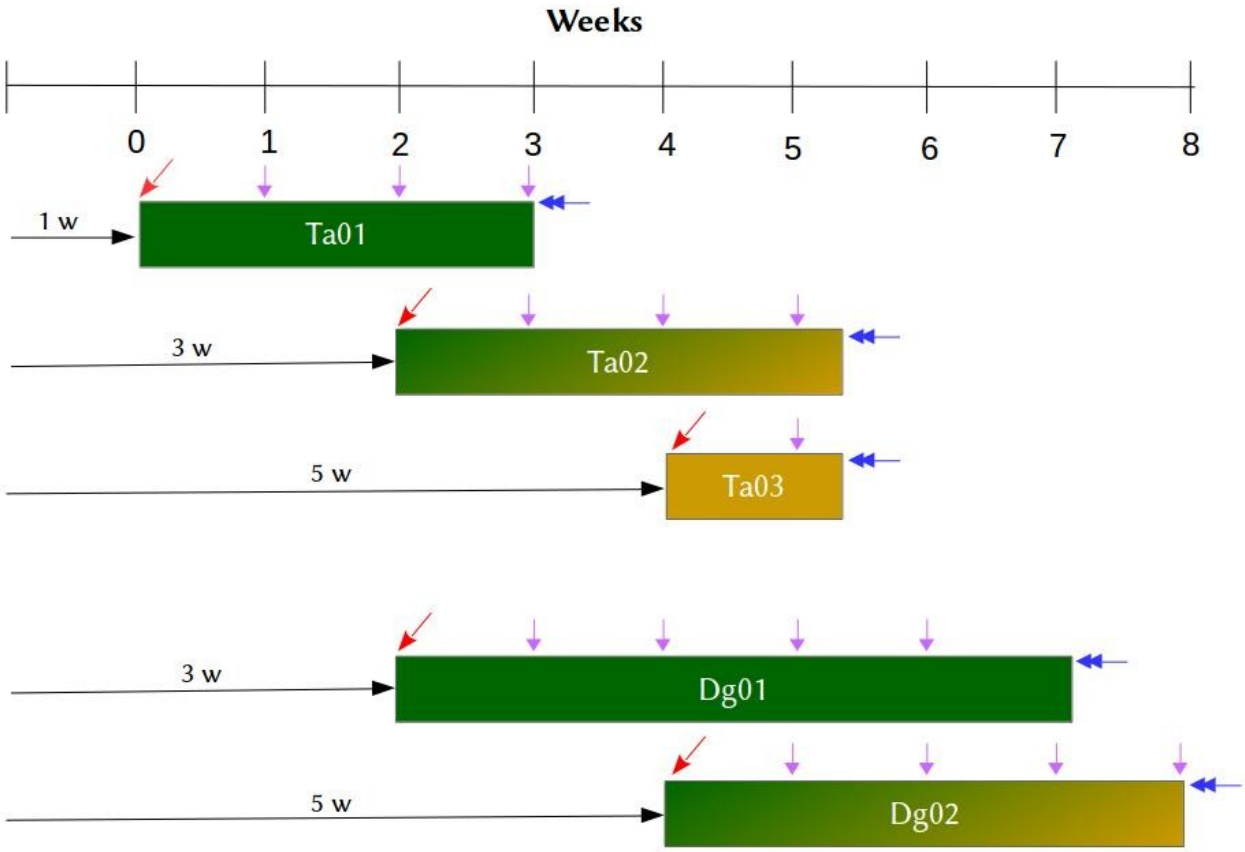


Figure 3: **Structure of plant infection experiment:** The figure shows an eight weeks period in which the five experiments conducted starting at different points of time and the condition of the plant leaves from inoculation with the 7 treatments to the end of each experiment; w: week; Ta01,Ta02,Ta03: *Triticum aestivum* plants observation period; Dg01, Dg02: *Dactylis glomerata* plants observation period; red arrow: inoculation time with treatment #1-6 and mock; purple arrow: cut of one leaf per treatment for microscopy; blue arrow: end and evaluation of experiment. Colors indicating condition of inoculated leaves: green: living tissue; green-brown: senescent tissue; brown/yellow: dead tissue.

2.2.1 Staining and confocal laser scanning microscopy

Prior to experimental procedure some leaves from all experiments were determined randomly for microscopic analysis. After collection of these harvested leaves they were destained for some days in 2 ml Eppendorf tubes in 100 % ethanol. Afterwards, they were incubated in 10 % KOH at 85 ℃ for 2:30 min and then washed 3 times with 1x PBS (pH 7.4). The staining solution was then added to the samples and vacuum infiltrated at 600 mbar inside a desiccator attached to a vacuum pump. Afterwards, the staining solution was collected for re-use and the samples were destained in 1x PBS and stored in the dark at 4 ℃. The staining solution was prepared using 20 µl/ml Propidium iodide, 10 µl/ml wheat germ agglutinin-fluorescein isothiocyanat (WGA-FITC), and 0.02 % Tween in 1x PBS (pH 7.4).

Microscopy was conducted using a Zeiss LSM880 confocal laser scanning microscope. The filter wavelengths used for the detection were 490-579 nm (for WGA-FITC) and 579-696 nm (for Propidium iodide). The fluorophores were excited by an argon and a diode-pumped solid-state (DPSS) laser.

2.3 Creation of fluorescent fungal strains

2.3.1 Preparation of genomic DNA

Genomic DNA of strains Zp13 and Za17 was required to amplify the DNA sequences flanking the genomic region – upstream (UF) and downstream flank (DF) - that serves as target for the integration of the fluorescent protein and antibiotic resistance encoding genes. For this reason, *Z. pseudotritici* and *Z. ardabiliae* cultures were inoculated in liquid YMS medium and grown at 18 ℃ and 200 rpm. Fungal cells of 30 ml YMS culture were harvested by centrifugation (13,000 rpm for 10 time). Afterwards, the fungal cell tissue was destroyed and genomic DNA was isolated. To destroy the tissue a spoon of glass beads, 500 µl TE-Phenol/Chloroform and 500 µl *Zymoseptoria* lysis buffer, which is composed of 10 ml Triton X100, 10 ml 10% SDS, 2 ml 5M NaCl, 1 ml 1M TrisHCl and 2 ml 0.5M EDTA filled up to 100 ml with water, were added and the solutions were incubated by shaking for 30 minutes using a Vibrax-VXR shaker. After phase separation (14.800 rpm, 15 min) ca 400 µl of the upper phase was transferred to 1 ml 100 % ethanol and DNA was precipitated at -20 ℃ for 20 min. The solution was subsequently centrifuged (14.800 rpm, 5 min) and the supernatant was discarded. The DNA pellet was dried and dissolved in 50 µl of TE-RNase A and incubated shaking at 50 ℃ for 10 minutes.
DNA concentration and quality was estimated by Nanodrop and agarose gel electrophoresis.

2.3.2 Polymerase chain reaction

For the amplification of specific DNA fragments polymerase chain reaction (PCR) was used. PCR consists of: Initial denaturation, followed by 35 cycles of denaturation – annealing – elongation and a final elongation. The Phusion polymerase (NEB), which possesses proof reading and has a reduced mutation rate, was used to amplify the different fragments for plasmid construction. The standard protocol used for one reaction of a total volume of 50 µl looks as follows:

1.0µl	template DNA	(gDNA Za17/Zp13, pDNA pES91, pDNA pPP56_pZero, pDNA pES61)
10.0µl	5xHF-buffer	
1.5µl	DMSO	
0.5µl	Fwd. primer	(Table 3)
0.5µl	Rev. primer	(Table 3)
0.5µl	dNTPs	
0.5µl	Phusion polymerase	
35.5µl	H_2O	

PCR reactions were conducted using S1000^{TM} Thermocycler of Bio-Rad or T-Personal Thermocycler of Biometra with the following program:

hotstart 98 °C

step 1	98 °C	-	3 min	
step 2	98 °C	-	8 s	
step 3	65 °C	-	20 s	35 cycles
step 4	72 °C	-	30 s per 1 kb	
step 5	72 °C	-	8min	
step 6	15 °C	-	∞	

2.3.3 Gel electrophoresis and purification of PCR products

To separate DNA specifically according to its size gel electrophoresis was used. For the generation of 0.8 % agarose gels the respective amount of agarose was solved by boiling in 1x TAE. After cooling the medium, Midori Green, which intercalates in the DNA and emits low-energy photons upon excitation with UV light, was added. The finished agarose solution was then poured into a gel tray and was let solidify at room temperature. After solidification the tray was transferred into a gel chamber and covered with 1x TAE. DNA was than loaded into the gel chamber at the cathode side and exposed to an electric field of 100-130V for 45-60 minutes. Under these conditions the DNA migrates according to its size in the direction of the anode. By using GelDoc™ XR+ (Bio-Rad) the binding of Midory Green to DNA was visualized with UV-light.

In order to purify the fragments of interest, PCR products were run on gels and fragments of interest were cut out and cleaned up with the Wizard SV Gel and PCR Clean-Up Kit (Promega) (Protocol A in the Appendix). After purification the concentration of PCR products was estimated with Nanodrop and on another agarose gel.

2.3.4 Gibson Assembly

To create the four different plasmids (Table 2) with an encoding region for GFP or dsTomato the different DNA fragments obtained by PCR were assembled by Gibson Assemby. This method is used to assemble DNA fragments under isothermal conditions (50 ℃) by using the enzymatic activity of a 5'-exonuclease which generates long overhangs, a Phusion DNA-polymerase which fills the gaps of the annealed single strand regions, and a DNA-ligase which removes any nick formed in the DNA. At first the amount of fragments and vector needed for the plasmid creations had to be calculated using the Gibson-Script. For this calculation fragments concentration and length (bp) is needed. The length was measured by the program Clone Manager before while the concentration has to be measured with Nanodrop. To reach a total amount of 20 µl for each reaction the different amount of each fragment and vector were added to 15 µl of Gibson Master Mix which is composed of the three enzymes needed, an isothermal-reaction mix and water. These mixtures were incubated for 60 minutes at 50 ℃ in a PCR-cycler (T-Professional Thermocycler; Biometra) and the obtained plasmids were subsequently used for the transformation of *E. coli*.

2.3.5 Transformation of *E. coli* and purification of plasmid DNA

For the amplification of plasmids, transformation of chemically competent *E. coli* TOP10 cells was conducted. *E. coli* cells were thawed on ice, mixed with the respective plasmid DNA and incubated for 30 minutes on ice. Subsequently *E.coli* cells were treated with a heat shock (45 sec at 42 ℃) that allows the DNA to enter the cells. Thereafter 5 volumes dYT medium, which is composed of 1.6 g tryptone, 1 g yeast extract and 0.5 g NaCl solved in 100 ml de-ionised water, was added. For regeneration, cells were incubated on Thermomixer for 45 minutes at 37 ℃ with gentle shaking. Transformed cells were plated on dYT-Kanamycin selection medium and incubated at 37 ℃ overnight. Only *E. coli* cells carrying a plasmid with an encoding-reagion for kanamycin resistance will survive on these plates (Protocol B in the Appendix).

For the purification of plasmid DNA different methods were applied.

The QIAGEN Plasmid Midi Kit was used to purify the plasmid pES61 and pES91. Prior to purification, one colony of each *E.coli* strain incorporating one of the two plasmids was picked and grown in liquid dYT medium at 200 rpm and 37 ℃ overnight. The following day the purification was conducted as described in the protocol (Protocol D in the Appendix).

Boiling Prep is another method adopted to purify plasmid-DNA, with which DNA is not cleaned as good as with the aforementioned Kit, but good enough to test if the right plasmids were build. This was conducted following the protocol of Petra Happel (Protocol C in the Appendix).

To test if the right plasmid-DNA was obtained, digestion with different enzymes was conducted and tested on gels by gelelectrophoresis.

The last Kit for DNA-purification QIAprep Spin Miniprep Kit (QIAGEN) was used to purify the plasmid pES94 (Protocol E in the Appendix). Again colonies were picked and inoculated in 10 ml dYT+Kanamycin before purification and incubated shaking at 37 ℃ overnight.

To test if the right plasmid-DNA was obtained, restriction digestion with different enzymes was conducted and tested on gels by gelelectrophoresis.

3 Results

In this bachelor thesis the lifestyle of the pathogenic fungus *Z. tritici* was compared with the lifestyle of its wild grass associated sister species *Z. pseudotritici* and *Z. ardabiliaae.* Therefore, a comparative plant inoculation experiment was conducted and evaluated with different methods.

3.1 Temporal disease development

The leaves of the two plant species *T. aestivum* and *D. glomerata* inoculated with the six *Zymoseptoria* strains and the mock control were screened for first appearance of necrosis and pycnidia formation every day to analyze the temporal disease development. As shown in Figure 3, the plants were inoculated at three subsequent points of time (Ta01; Ta02/Dg01; Ta03/Dg02). Thus, it was possible to observe how the strains of the three species behave on leaves at different developmental stages (living tissue, senescent tissue, dead tissue).

In the first experiment with *T. aestivum* (Ta01, Figure 3) it was observed that on plants inoculated with *Z. tritici* strains and Zp13 necrosis formed first. On plants inoculated with Za48 or the mock control, necrosis appeared only at the end of the experiment (Figure 4). A Wilcoxon rank sum test was conducted to compare the temporal appearance of necrosis on fungi treated leaves with the necrosis appearance on mock-treated leaves. As a result, a significant difference was estimated, except for thous leaves inoculated with Za48. Furthermore, in the course of the daily screening, pycnidia formation was only observed on leaves treated with *Z. tritici* strains starting 13 days after inoculation.

In the second experiment with *T. aestivum* (Ta02, Figure3), in which most of the leaves were in a senescent stage, no pycnidia formation was observed and the onset of necrosis formation was early after the inoculation and similar for most treatments (Figure 5). Only for thous leaves inoculated with spores of Zt05 and Za48 appearance of necrosis was significantly earlier than for mock treated leaves (Wilcoxon rank sum test; Zt05 p=0.017; Za48 p=0.0005).

As for the third experiment with *T. aestvum* (Ta03, Figure 3), all leaves were completely necrotic already by the time of inoculation with the different treatments. By daily manual inspection no pycnidia formation was observed (Figure 6). This experiment had to be stoped 10 days after the inoculation because of a contamination inside our phytochamber. We observed symptoms of a *Blumeria graminis* f.sp. tritici infection on some of the wheat leaves.

With the wild grass *D. glomerata* only two experiments were conducted (Dg01 and Dg02; Figure3) since this plant species showed slower growth than wheat.

In the first experiment (Dg01, Figure 3) necrosis development was very variable. Only on leaves inoculated with Za17 the necrosis development was significant (Wilcoxon test: p=0.0087), as the necrosis development started much sooner than the one of the other treatments. On leaves of *D. glomerata* formation of pycnidia was only observed for Za48 (Figure 7). As for the second experiment (Dg02, Figure 3), necrosis development was observed between 20 and 25 dpi, except for leaves inoculated with spores of

Za17 where appearance of necrosis was observed soon after inoculation with the fungal spores. However, we found no significant difference between the appearance of necrosis on leaves inoculated with spores of *Zymoseptoria* spp. and mock-treated leaves. We did not observe the formation of pycnidia for any treatment on *D. glomerate* leaves during the daily manual screening (Figure 8).

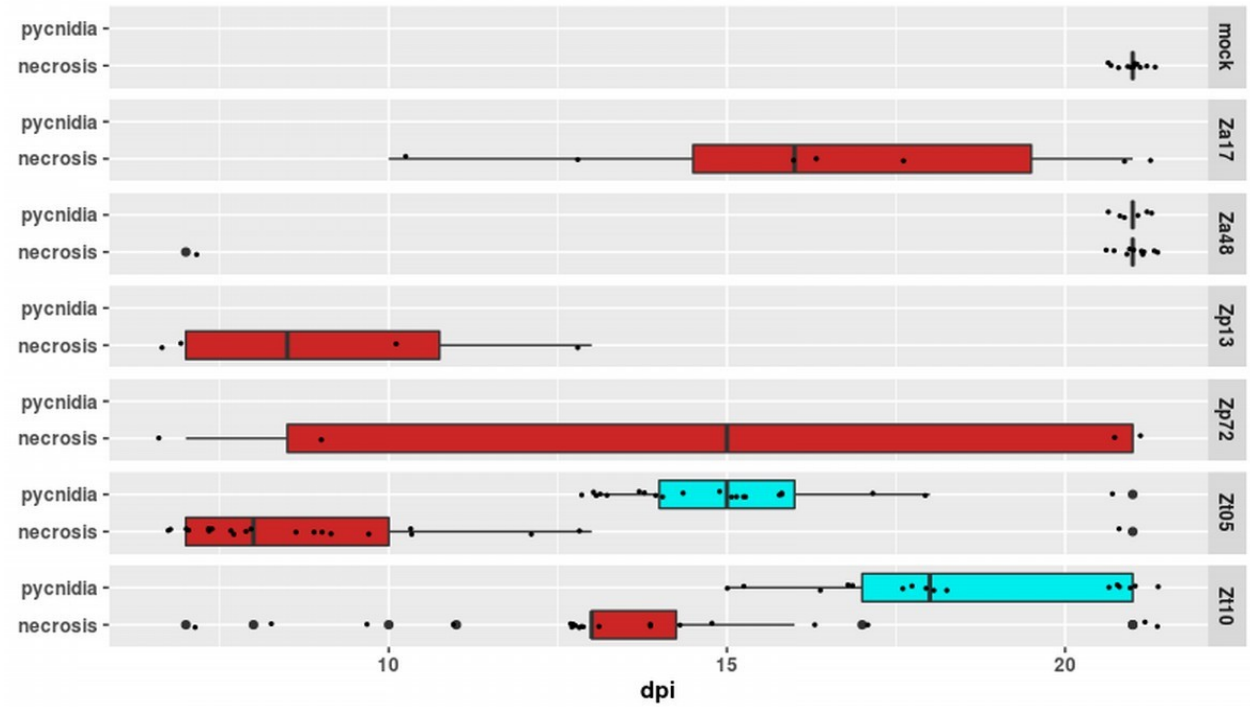


Figure 4: **Temporal disease development on wheat; Ta01**

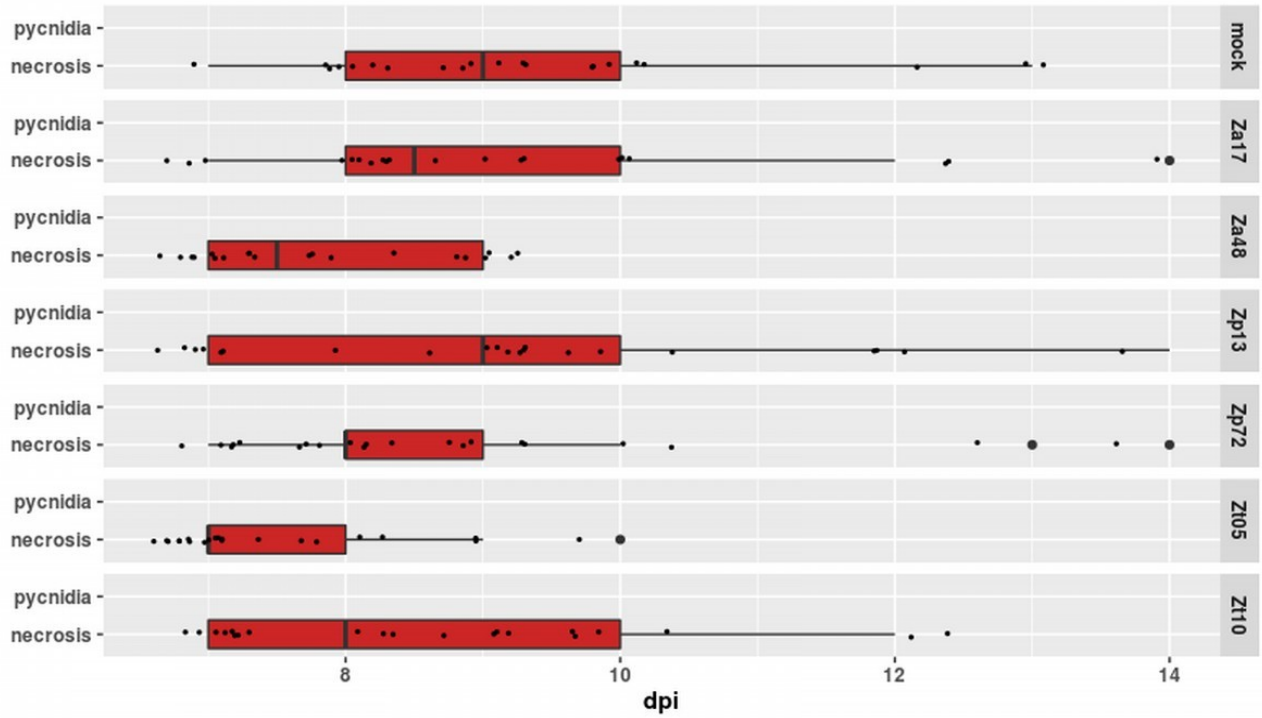


Figure 5: **Temporal disease development on wheat, Ta02**

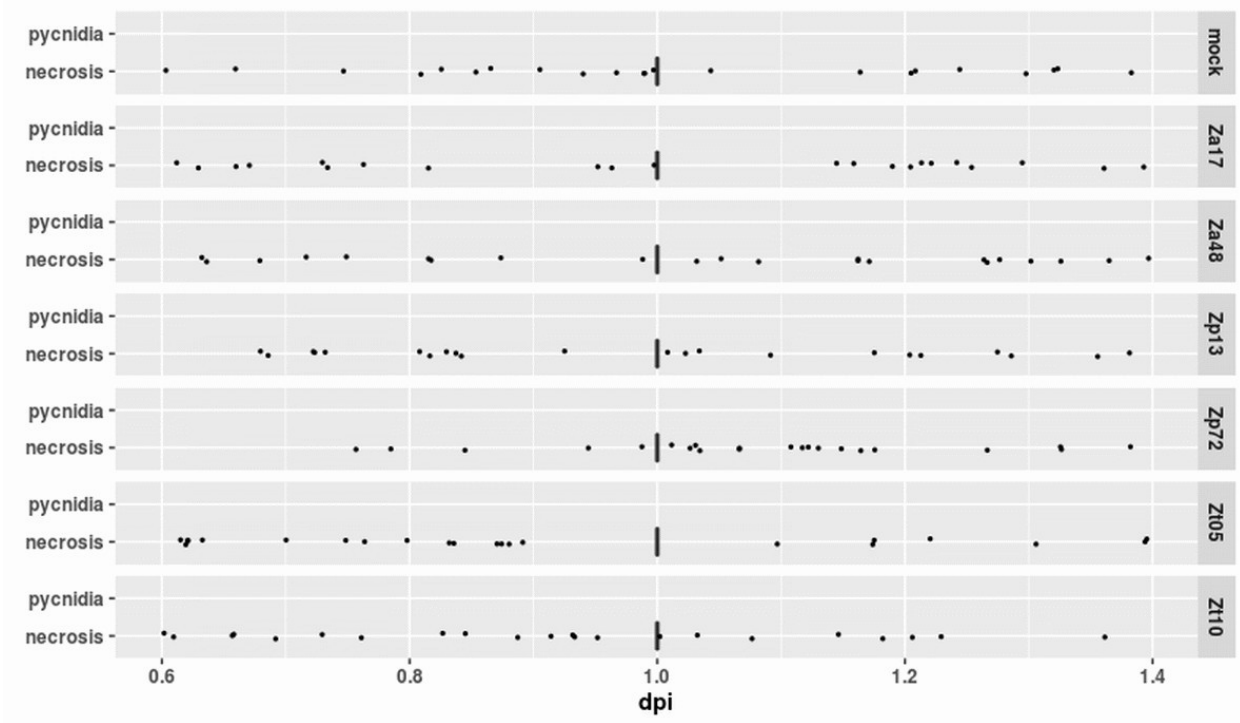


Figure 6: **Temporal disease development on wheat; Ta03**

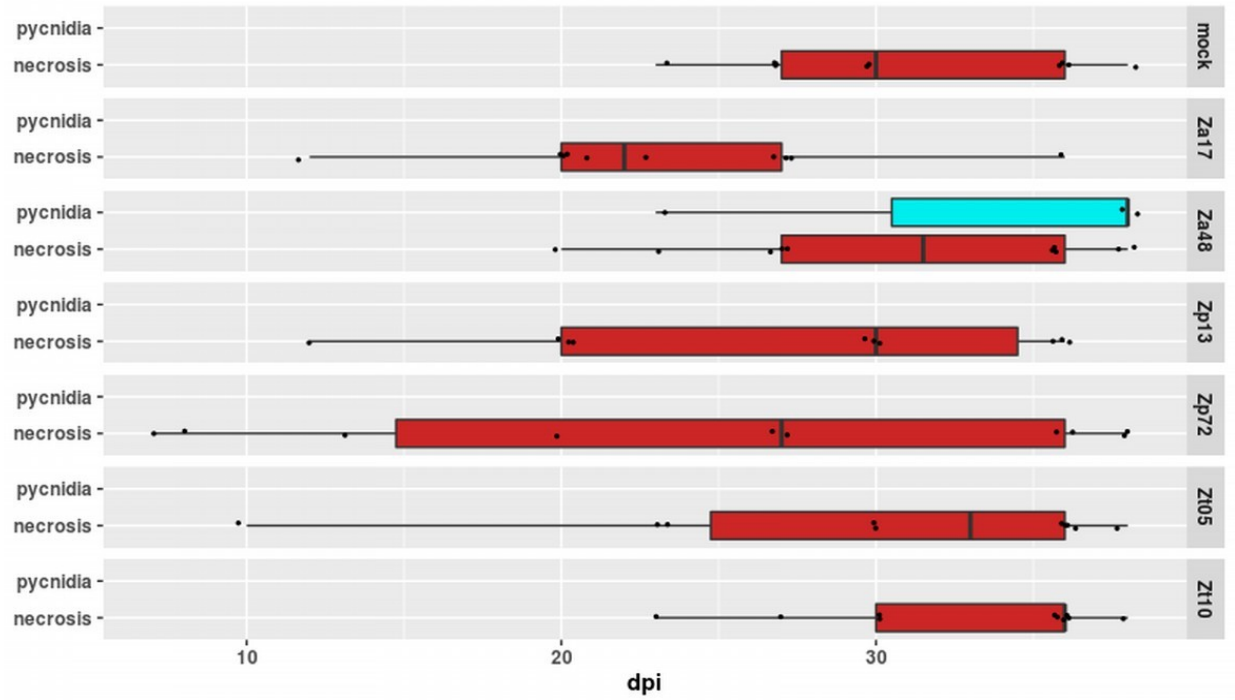


Figure 7: **Temporal disease development on *D.glomerata,* Dg01**

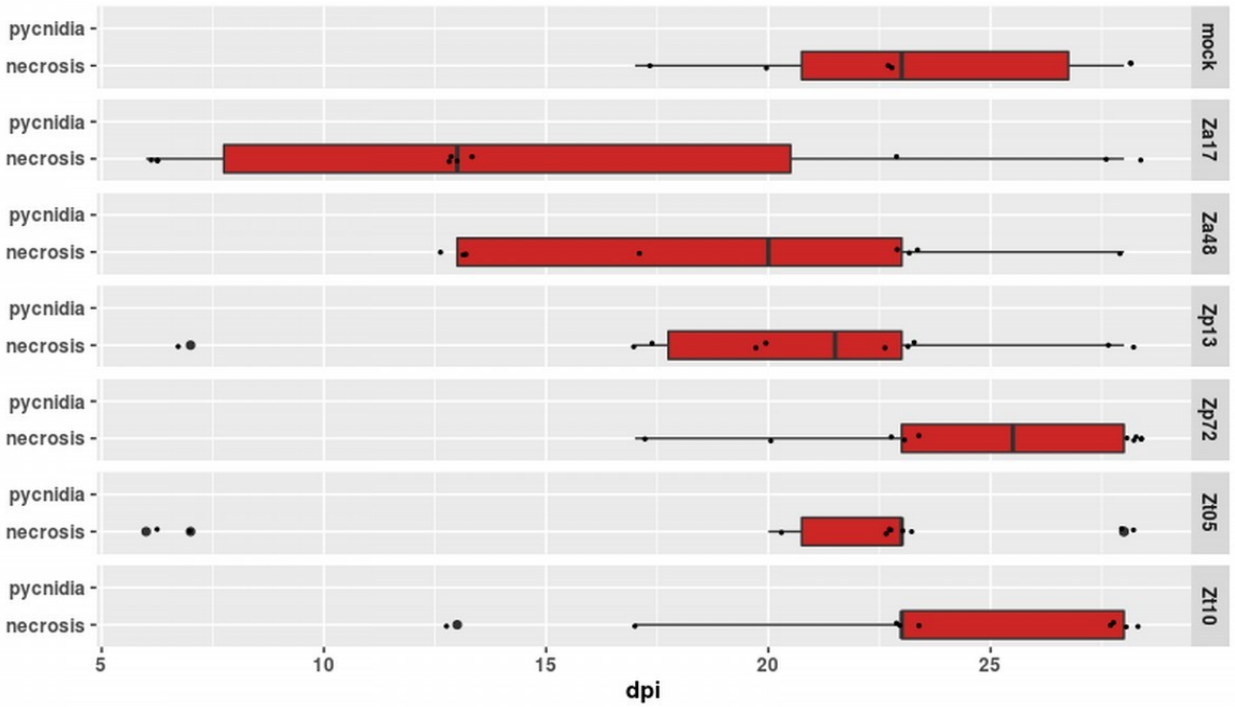


Figure 8: **Temporal disease development on *D. glomerata,* Dg02**

3.2 Virulence phenotypes of *Zymoseptoria* ssp. strains on leaves of wheat and *D. glomerata*

3.2.1 Results of manual visual evaluation of inoculated leaves

At the end of each plant experiment, all inoculated leaves were collected and virulence phenotypes were evaluated manually with the help of a binocular. The inoculated regions of the leaves were thereby screened for coverage with necrosis and pycnidia. A scoring scheme as described in the method chapter was applied.

In Ta01 finished at 21 dpi, all leaves (n=21 per treatment) inoculated with spores of Zt05 were fully necrotic while Zt10 inoculated leaves varied in necrosis levels and were covered with individual necrotic lesions (Figure 11, A). Plants inoculated with Za48 also showed a high necrotic level; half of the leaves were completely necrotic. These results overlap with pycnidia coverage, since on leaves inoculated with these three strains pycnidia formation was observed. All leaves inoculated with Zt05 were covered by pycnidia and six of them showed a high level of pycnidia coverage (40 % - 100 %), while for leaves inoculated with Zt10 16 were covered by pycnidia but only two of them showed a high coverage (40 % - 60 %) (Figure 9, A and B). As for leaves inoculated with Za48, six of them were covered with pycnidia (Figure 9, C). The other strains and the mock control showed less necrosis and no pycnidia coverage (Figure 11, A).

In Ta02 finished at 24 dpi, leaves of all treatment groups (n=21 per treatment) were fully necrotic and no pycnidia were found (Figure 11, B).

For Ta03 that ended at 10 dpi, all leaves (n=23 per treatment) were fully necrotic and coverage with pycnidia was observed for leaves inoculated with Zt05, Zt10, Za17 and Za48 (Figure 9, D-F). Half of the leaves inoculated with Za48 and Zt05 showed pycnidia formation, but only one (or two respectively) samples had a pycnidia coverage that scored 40 % - 60 % (Figure 11, C).

For Dg01 that ended at 38 dpi, we found that almost all inoculated leaves (n=10 per treatment) showed high levels of necrosis and we observed pycnidia on three leaves inoculated with Za48 and one leaf inoculated with Zp72 (Figure 10; Figure 11, D).

Similarly, in Dg02 finished at 28 dpi, almost all leaves (n=10 per treatment) were fully necrotic but no pycnidia were observed (Figure 11, E).

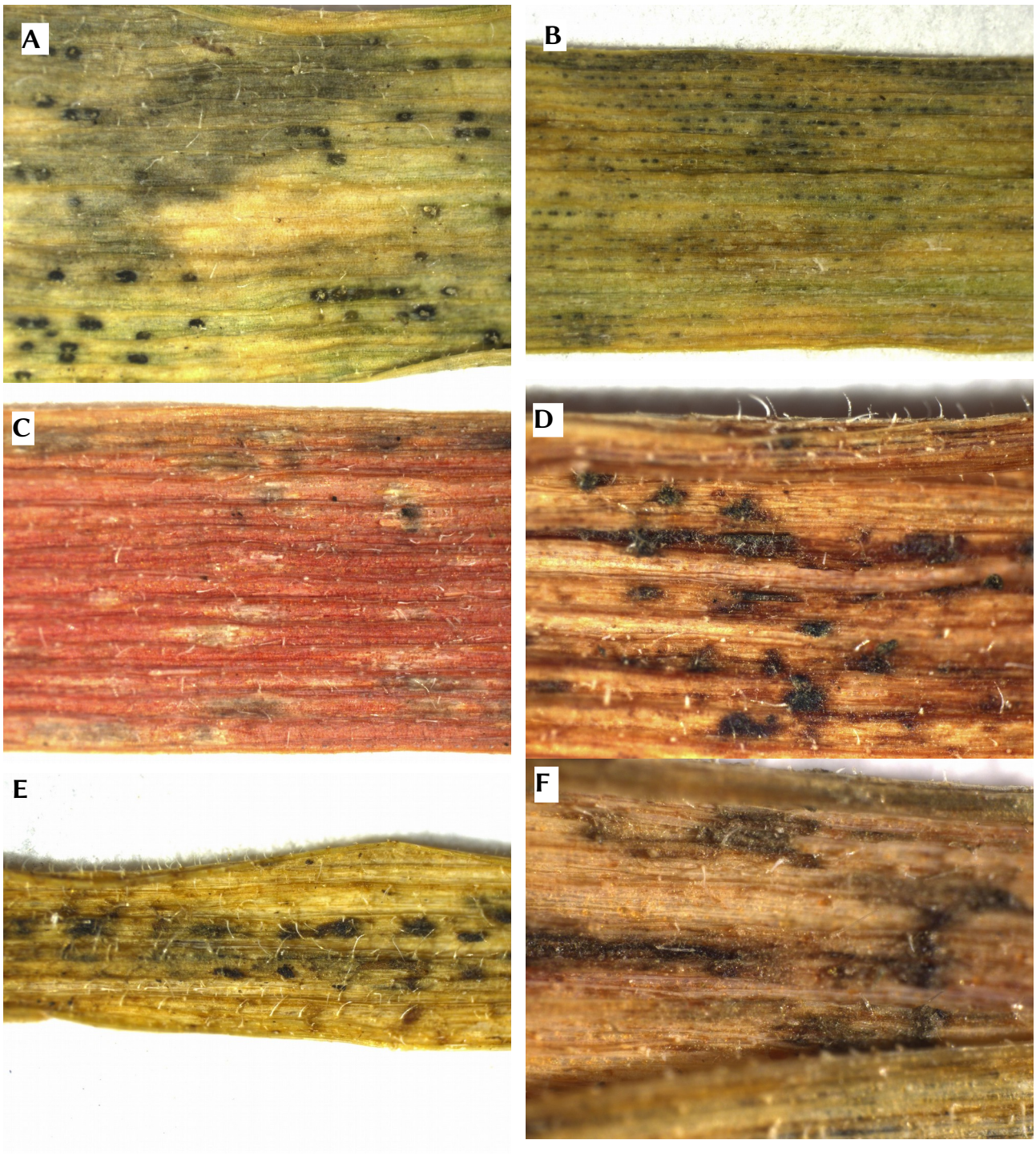


Figure 9 : **Images of wheat leaves treated with different *Zymoseptoria* spp. strains:**
A and B: Typical pycnidia formation on leaves inoculated with *Z. tritici* strains in Ta01 experiment;
C: Structures found on leaves inoculated with Za48 in the Ta01 experiment that we counted as pycnidia;
D, E and F: Structures that were counted as pycnidia on leaves inoculated with Za48 (D), Zt05 (E) and Zt10 (F) by manual visual evaluation in Ta03 experiment.

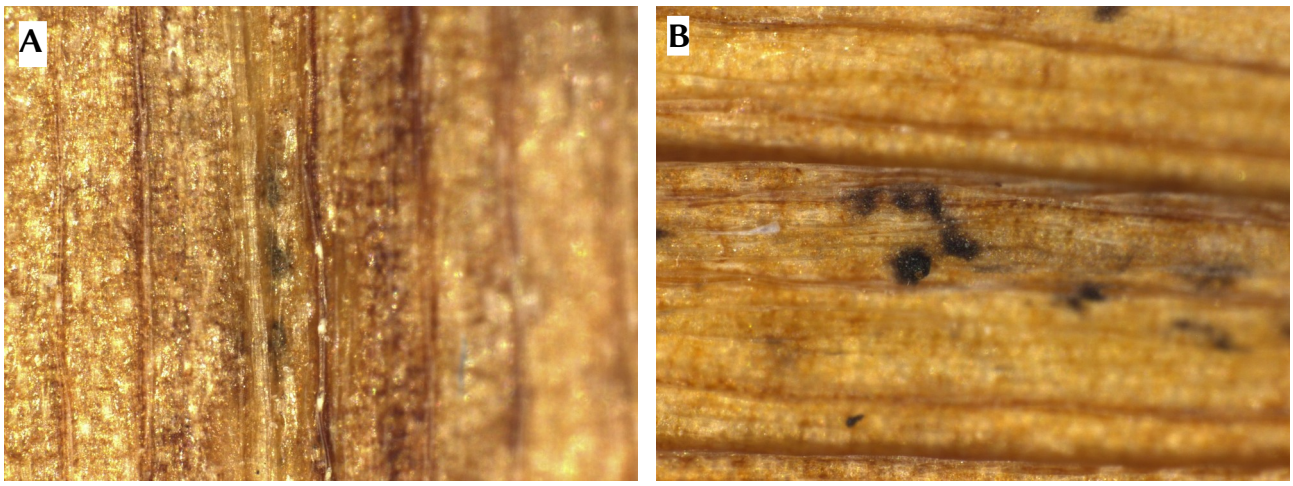
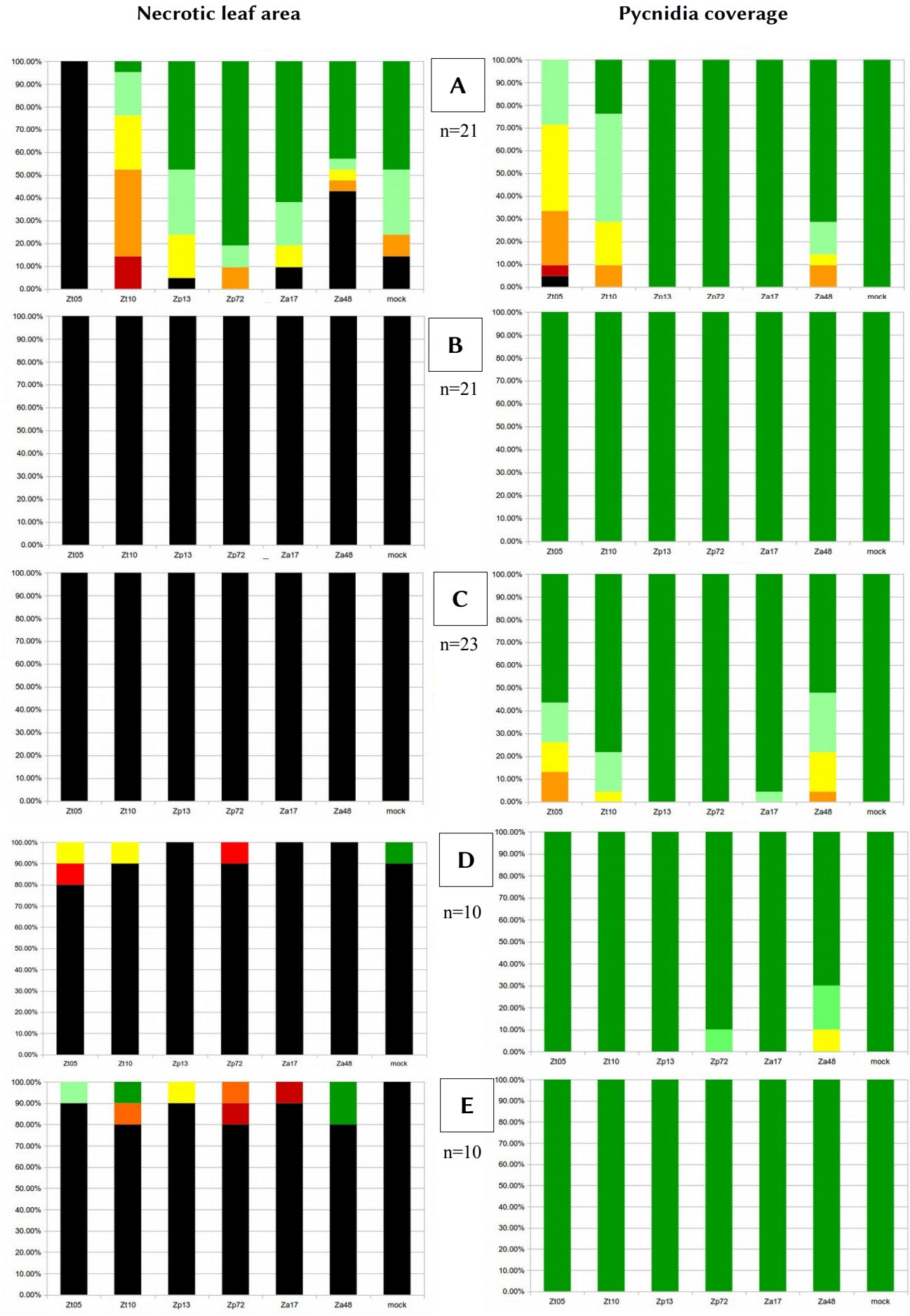


Figure 10: **Images of *D. glomerata* leaves treated with different *Zymoseptoria* spp. strains:**
A: Pycnidia found on Dg01 leaf inoculated with Zp72; B: Pycnidia found on Dg01 leaf inoculated with Za48.

Figure 11: **Virulence phenotype:** evaluation by eye at the end of each experiment.
left: necrotic leaf area; right: pycnidia coverage; A: Ta01; B: Ta02; C: Ta03; D: Dg01; E: Dg02; Colors: green = 0 %, turquoise = 1 % - 20 %, yellow = 21 % - 40 %, orange = 41 % - 60 %, red = 61 % - 80 %, black = 81 % - 100 %; these scores describe the necrosis or pycnidia coverage level of each leaf.


Necrotic leaf area
Pycnidia coverage
A
n=21
B
n=21
C
n=23
D
n=10
E
n=10
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Zt05
Zt10
Zp13
Zp72
Za17
Za48
mock

3.2.2 Results of automated image analysis

To further support the results of the manual visual evaluation, the leaves were scanned. From the images obtained necrosis percentage per leaf and pycnidia number per cm² was measured automatically with a batch processing macro utilizing the image processing software ImageJ.

A selection of overlay leaf images used for this analysis, in which the identified necrotic areas and pycnidia are marked, can be found in the appendix (Figure 16).

The results are as follows:

For all experiments the necrosis coverage percentage on all treated leaves was estimated between 90 % and 100 %, which is shown by the boxplots collected in the appendix (Boxplots 1 in the Appendix).

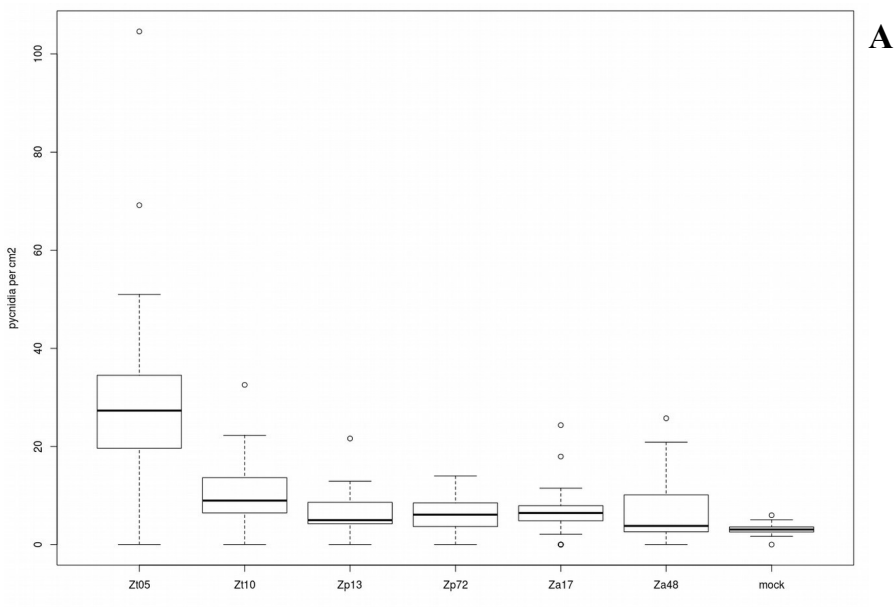
The pycnidia number per cm² calculated on the leaves inoculated with the different treatments in the five experiments corresponds to the results obtained with the manual visual evaluation.

In Ta01 a high number of pycnidia was identified on leaves infected with Zt05 (on average: 32 pycnidias per cm²). On leaves treated with Zt10 pycnidia were also found, but on average only 11 per cm². As for the other treatments, the pycnidia number measured was vary little and near the one of the mock control (Figure 12, A).

In Ta02 the number of pycnidia found on leaves treated with Zt05 was less then the one in Ta01, on average 21 pycnidia per cm² were counted. The pycnidia density on leaves treated with Zt10 and the mock control is comparable to the results of the first experiment. On leaves treated with the other *Zymoseptoria* strains the pycnidia density measured was higher then in Ta01, especially for leaves inoculated with Za48 spores; on average 15 pycnidia per cm² (Figure 12, B).

In Ta03 the number of pycnidia measured on the leaves treated inoculated with Za48 reached an average number of 62 pycnidia per cm². Moreover, the pycnidia number measured on leaves inoculated with Zt05 was greater then in Ta02 (30 pycnidia per cm² on average), and leaves inoculated with Zt10 showed more pycnidia in comparison to both the other experiments. For the other treatments no change was shown (Figure 12, C).

As for the results obtained for *D. glomerat* the automated image analysis showed nearly no difference between the two experiments. In both experiments the average number of pycnidia on leaves treated with Za48 was 5 pycnidias per cm², but some leaves had also over 15 pycnidia per cm². On leaves inoculated with the other treatments the number of pycnidia was measured approximately at 2 pycnidia per cm². In this experiment the software also identified a small number of pycnidia on the mock-treated leaves (Figure 13).



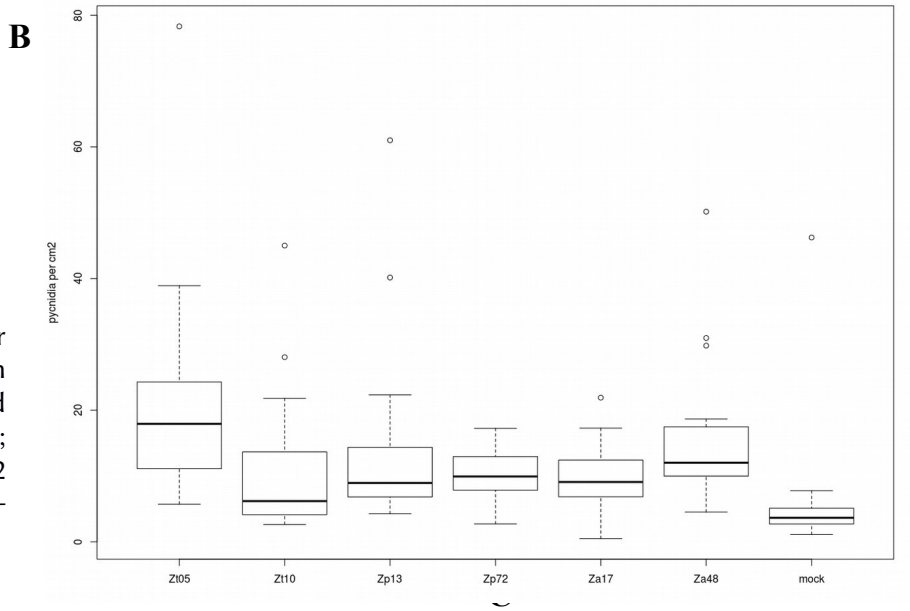
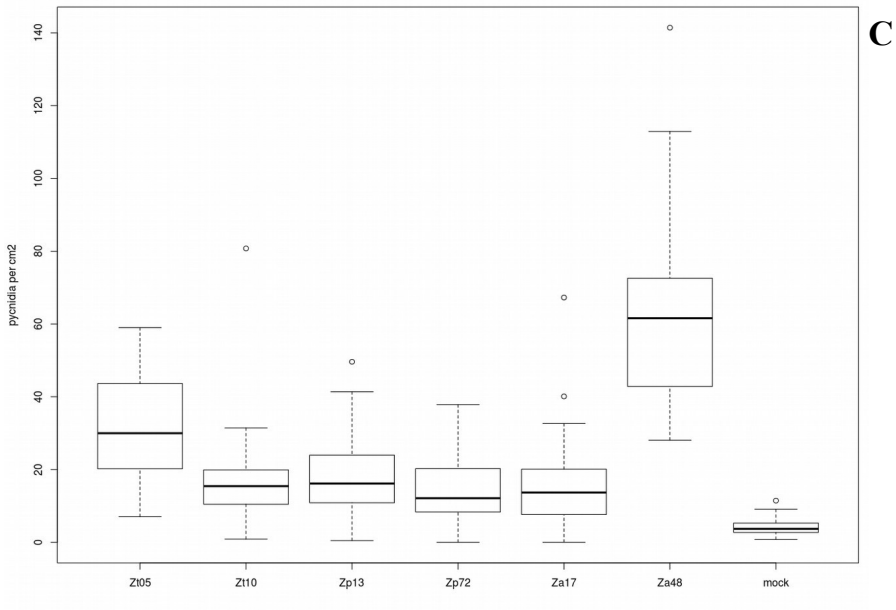


Figure 12 :
Automated image analysis: Boxplots showing the number of pycnidia per cm² found on the wheat leaves inoculated with the different treatments; A: Ta01 experiment; B: Ta02 experiment; C: Ta03 experiment.



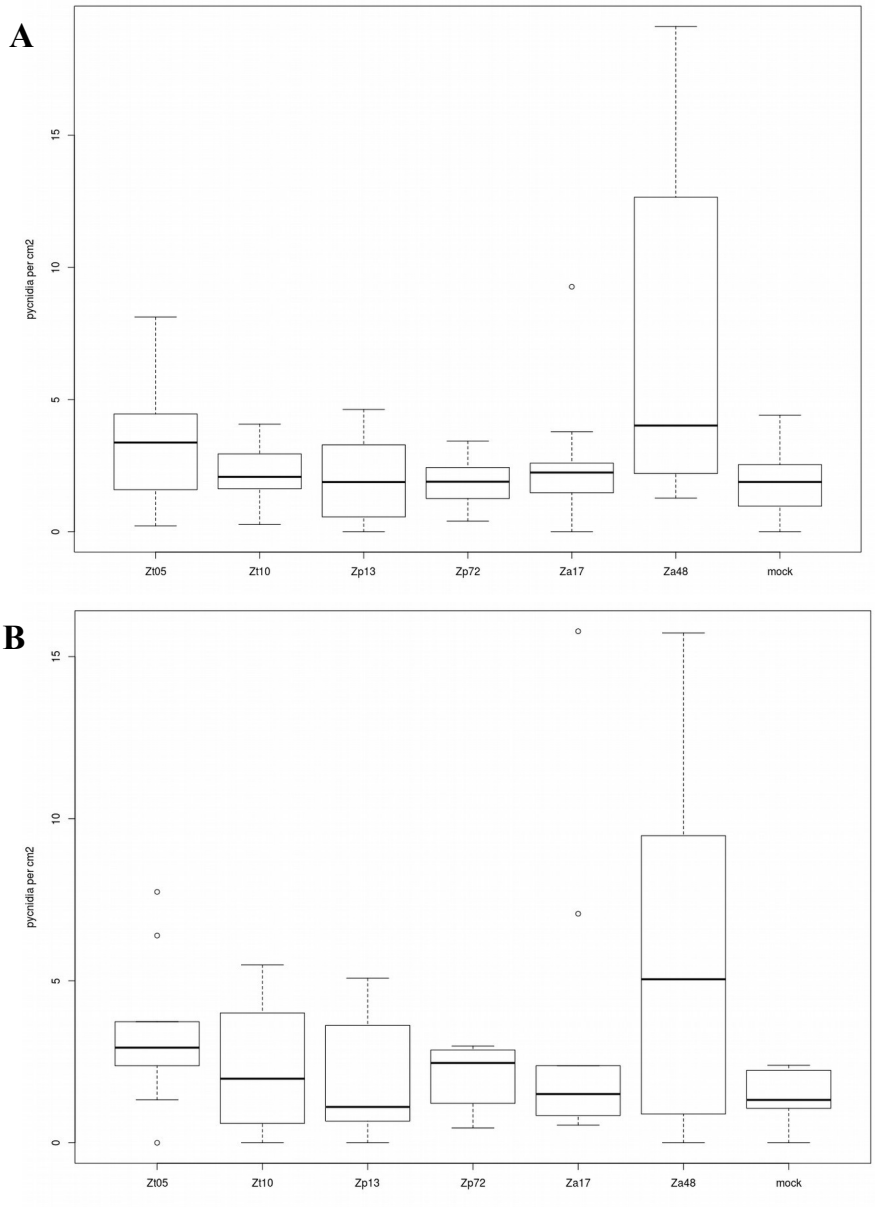


Figure 13: **Automated image analysis:** Boxplots showing the number of pycnidia per cm² found on *D. glomerata* leaves inoculated with the different treatments; A: Dg01 experiment; B: Dg02 experiment.

3.3 Microscopic analysis of *Zymoseptoria* spp. inoculated wheat leaves

During the three wheat inoculation experiments (Ta01, Ta02, Ta03) one leaf per time point and treatment was collected every 7 days. The material was fixed, de-stained and afterwards fungal hyphae and plant cells were stained with WGA-FITC / Propidium iodide staining solution. A selection of the fluorescent stained samples was subjected to confocal laser scanning microscopy where plant - fungus interactions, in particular the extent of colonization and reproduction of *Zymoseptoria spp.* inside wheat tissue, were analyzed.

For leaves collected during Ta01 we observed frequent stomatal penetrations by *Z. tritici* hyphae that emerged from spores on the leaf surface (Zt05 at 7 dpi). Infection hyphae entered through stomata and colonized the wheat mesophyll tissue intercellularly (Figure 14, E). At 14 dpi plant cells in some leaf areas were already collapsed and shrunken. In addition both initial and fully developed pycnidia of strain Zt05 were present in substomatal cavities. At 21 dpi, all host cells in the analyzed leaf areas were found to be dead and the necrotic mesophyll tissue was completely occupied by fungal mycelium and numerous maturated pycnidia that released pycnidiospores (Zt05) (Figure 14, F).

In contrast, in leaves collected during Ta02 and Ta03 growth and reproduction of *Z. tritici* (Zt05 and Zt10) inside wheat mesophyll was found to be reduced. In the 7 dpi sample of Ta02 leaf cells were intact and we observed fungal hyphae entering the plant mesophyll. With regard to the later samples, 14 and 21 dpi of Ta02 and 7 dpi of Ta03, we mainly saw strong proliferation of *Z. tritici* mycelium on the leaf surface and rarely colonization inside the tissue that was completely necrotic in the three samples. Pycnidia were not found in samples of Ta02 and Ta03 for *Z. tritici* strains that were analyzed by confocal microscopy.

However, we also identified fungal hyphae colonizing both the in- and outside of these necrotic leaves and the fruiting structures that are morphologically clearly distinct from *Zymoseptoria* species. Similarly, these structures were also found on wheat leaves collected during Ta02 and Ta03 at 14 and 21 dpi and 7 dpi respectively, which were all inoculated with spores of *Z. pseudotritici* and *Z. ardabiliae.*

For wheat leaves inoculated with *Z. pseudotritici* strains Zp13 and Zp72 and with *Z. ardabiliae* strain Za48 we observed spore germination and development of infection hyphae on the leaf surface (Figure 14, A/C). We saw frequent stomatal penetrations in leaf samples collected at 7 to 21 dpi during Ta01 and at 7 dpi during Ta02. Hyphae were entering the leaves through stomata but no colonization further than the mesophyll cells surrounding the substomatal cavities was found (Figure 14, B/D). Leaves that were harvested later during Ta02 and Ta03 and that had become necrotic, growth of *Zymoseptoria* spp. hyphae inside mesophyll tissue was also not observed. In the leaf samples that we analyzed by confocal microscopy, we did neather find pycnidia nor formation of pycnidia precursors for *Z. pseudotritici* and *Z. ardabiliae* strains.

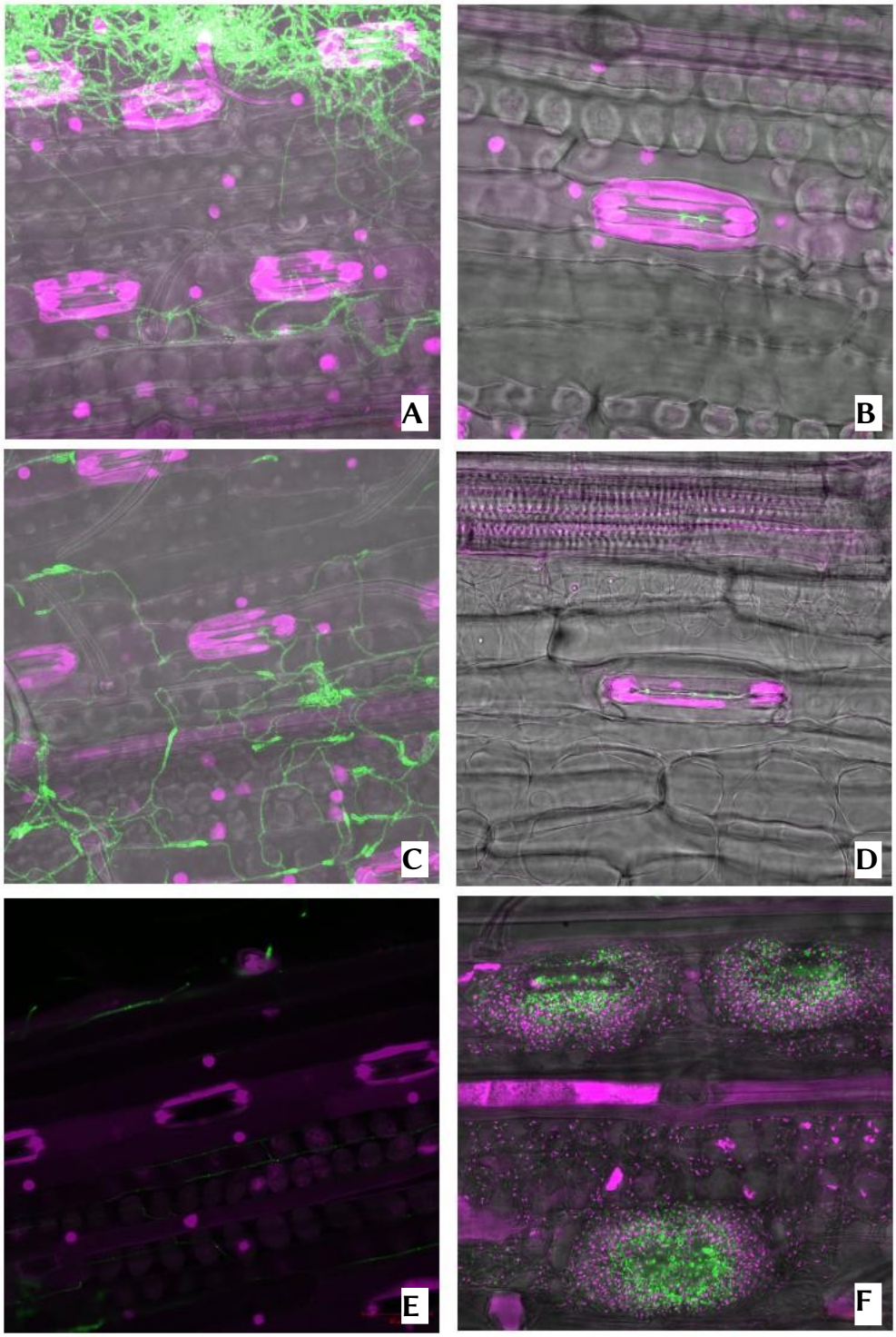


Figure 14: **Confocal laser scanning microscopy images of *T. aestivum* leaves inoculated with *Zymoseptoria* spp. spores.** Images show individual focal planes that have been acquired as part of larger z-stacks or maximum intensity projections. Green color: emission of WGA-FITC representing fungal structures, magenta color: emission of Propidium iodide representing nucleic acid and cell walls, grey color: transmitted image. A: proliferation of Za48 hyphae on leaf surface in Ta01 at 7dpi and first attempt to penetrate through stomata; B: penetration attempt of Za48 through stomata at 14 dpi in Ta01; C: proliferation of Zp13 hyphae on leaf surface in Ta01 at 7dpi and first attempt to penetrate through stomata; D: penetration attempt of Za48 through stomata at 21 dpi in Ta01; E: Zt05 biotrophic colonization of leaf in Ta01 at 7 dpi, and extracellular hyphae; F: Zt05 pycnidia formation in leaf substomatal cavities in Ta01 at 21 dpi.

3.4 Fluorescent *Zymoseptoria* spp. strains

In order to express cytosolically fluorescent proteins as markers in cells of *Zymoseptoria* spp. strains, binary plasmids have to be generated that contain the 5' and 3' flanking regions of the target genomic locus, the gene for the respective fluorescent protein, a selection marker gene and all the required elements for amplification in *E. coli* and *Agrobacterium tumefaciens*. Eventually, transformation of single cells of *Zymoseptoria* spp. is mediated by *A. tumefaciens* and elements of the plasmid insert are integrated by homologous recombination at the target genomic locus in *Zymoseptoria* spp. We planned four plasmids for transformation of *Z. pseudotritici* strain Zp13 and *Z. ardabiliae* strain Za17. The plasmids consist of a backbone (pES61) with an encoding region for kanamycin resistance and an insert with an encoding region for a fluorescent protein and one for the geneticin resistance. The kanamycin resistance gene is used as a selection-marker for the two bacterial species the plasmid are transformed in, while the geneticin resistance gene is used as a selection-marker for transformation in the fungal strains when the plasmids are correctly sequenced. The plasmids pES93 and pES94 have an encoding region for the green fluorescent protein, while the plasmids pES198 and pES199 have an encoding region for the red fluorescent protein dsTomato. The UF and DF also differ in the four plasmids: pES93 and pES198 have flanking regions obtained from gDNA of Zp13, while pES94 and pES199 have flanking regions obtained from gDNA of Za17. These flanking regions were obtained out of non-coding regions between a gene and a transposable element of both gDNA. All required DNA fragments for the construction of the different plasmid inserts were successfully amplified by PCR, which was tested with gelelectrophoresis that showed the right bands length of the different plasmid fragments. Nevertheless, assembly of insert fragments and backbones by Gibson Assembly did not result in the correct constructs. We were not able to confirm correct plasmids by analysing restriction digestion patterns. For plasmid pES94, intended for transformation of Za17, we nevertheless found one promising candidate construct (pES94#26) (Figure 15, A), but fragment patterns of restriction digestions with NdeI/ApaI, ApaL, PvuI-HF, Xho1 showed slightly incorrect fragment sizes (Figure 15 B). However, the limited time of this bachelor thesis did not allow final verification of pES94 candidate and generation of putative candidates for pES93, pES198 and pES199.

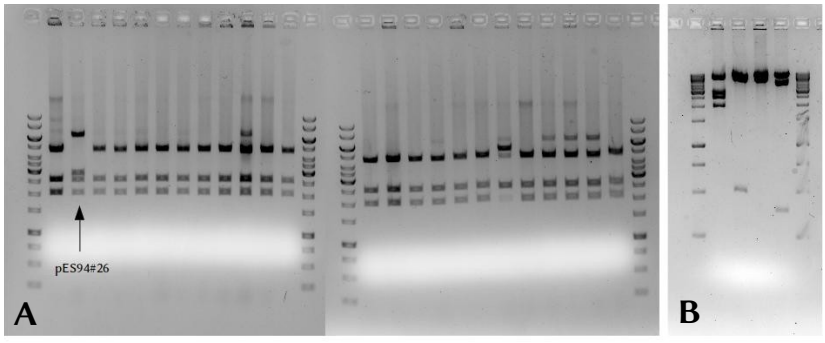


Figure 15: **Gelelectrophoresis digestion pictures.** A: Digestion with NdeI and ApaI of assembled plasmid pES94, colonies #25 to #48. The arrow shows the only plasmid that seemed to be right, since it had four fragments of the length of: 6100bp, 2800bp, 2400bp and 1900bp as expected. → pES94#26; B: Test digestion, to control if the right plasmid formed. The enzyme used are: 1. NdeI/ApaI, 2. ApaL, 3. PvuI-HF, 4. Xho1.

4 Discussion

Fungi can interact in many different ways with plant species and the interaction of one fungal species can also differ between individual isolates and different host genotypes (Eyal et al. 1985). This interaction is influenced by the physiological condition of the host as well e.g. by its nutritional status (Kracher et al. 2016).

The aim of this thesis was to investigate the lifestyles of *Zymoseptoria pseudotritici* and *Z. ardabiliae,* two species that are closely related to the hemibiotrophic wheat pathogen *Z. tritici*. We compared the behavior of the three different *Zymoseptoria* species during their interaction with wheat and *Dactylis glomerata* leaves at different host developmental stages to assess whether the wild-grass associated *Z. pseudotritici* and *Z. ardabiliae* have similar lifestyles as their pathogenic sister species *Z. tritici* and to also investigate how they colonize and reproduce inside their non-domesticated grass hosts.

In order to infect and reproduce in a host plant, a pathogen has to overcome several plant immunity layers (Jones & Dangl 2006). When trying to enter the plant tissue, via stomata or with the help of an appressorium, the plant receptors recognize pathogen-associated molecular patterns (PAMPs) such as chitin and induce an immunity response. This response can be avoided by the fungus by secreting effectors that are small molecules interfering with the perception of PAMPs by the plant receptors (Lo Presti et al. 2015). The plant itself can produce resistance proteins that recognize pathogen effectors or monitor its effector-targeted proteins and stop the fungal invasion in this way (Jones & Dangl 2006). Successful infections are possible only if the fungus has the ability to overcome the host plant immunity system and is adapted to grow and reproduce inside the host tissue (Haueisen & Stukenbrock 2016).

The pathogen *Z. tritici* establishes a symptom-less colonization on living wheat tissue by masking its pathogen-associated molecular pattern (PAMP) chitin with a LysM-domain that suppresses PAMP recognition by the plant receptors (Marshall et al. 2011). After this initial biotrophic phase the fungus switches its lifestyle to a necrotrophic growth whereby it kills the host cells and gains the nutrition needed (Horbach et al. 2011; Rudd et al. 2015). In this necrotrophic stage, that starts around 11 days after inoculation, *Z. tritici* forms pycnidia in the substomatal cavities (Ponomarenko, Goodwin, and Kema 2011).

In our experiments we observed successful infection of *Z. trtici* during Ta01 experiment, but reduced symptom formation during Ta02 and Ta03 and no symptoms were observed on *Dactylis glomerata* leaves.

These results indicate that for full virulence on wheat, *Z. tritici* requires a phase of biotrophic colonization during which leaves are healthy and not yet compromised or senescent. It also shows that *Z. tritici* is not adapted to *D. glomerata* and hence its specialization to wheat can be confirmed.

The two sister species of this wheat pathogen, *Z. pseudotritici* and *Z. ardabiliae,* are endemic species in Iran and were only isolated from non-cultivated grasses. However, previous analyses conducted with the Detached Leaf Assay (DLA) showed that they were able to produce pycnidia on different plant leaves, among others on wheat leaves (Stukenbrock et al. 2011, Haueisen and Eschenbrenner (unpublished)).

With this type of assay, it was difficult to distinguish the fungal or plant inducted necrosis from natural senescence. Due to this it remained an open question whether fungal growth and pycnidia formation were only possible on already compromised or necrotic leaves, or if *Z. pseudotritici* and *Z. ardabiliae* require a biotrophic colonization phase prior to reproduction in necrotic tissue.
With this question in mind, we tested their behavior on living plants with leaves at different developmental stages.
Our results showed no infection of wheat leaves by *Z. pseudotritici* and *Z. ardabiliae* strains in Ta01. For this reason we conclude that *Z. pseudotritici* and *Z. ardabiliae* do not need a biotrophic phase to colonize wheat. On senescent or already dead leaves in Ta02 and Ta03 some pycnidia-like structures were observed by visual evaluation and with the automated image analysis assay, but by examining some *Z. pseudotritici* and *Z. ardabiliae* inoculated leaves with Confocal laser scanning Microscopy (CLSM) no fungal structures were found inside the leaves. Perhaps more time would have been needed to allow complete maturation of this pycnidia-like structures.
Because of the contamination with *Blumeria graminis* f.sp. *tritici* we were not able to finish analyzing the development of these structures with the leaves still attached to the wheat plants. However, we do not expect infections with *B. graminis* on the inoculated leaves in Ta03. *B. graminis* is an obligath biotrophic fungal pathogen that requires living host cells for successful infections (Horbach et al. 2011). Wheat leaves that were inoculated for Ta03 were already necrotic at the time of inoculation and did not show any symptoms of *B. graminis* infections prior to the necrosis.
In the Dg01 experiment a few pycnidia were observed by visual evaluation and with the automated image analysis, but no CLSM was conducted. This is why we can not be sure, if the structures we observed were actually pycnidia and if so whether they were pycnidia of the two *Zymoseptoria* spp. strains or perhaps of contaminating saprotrophic fungi. This might be a possibility since necrotic leaves represent a food source for many saprotrophic species and our experiments were not conducted in a sterile environment. Interactions such as the competition between contaminating species and *Zymoseptoria* spp. cannot be excluded. These saprotrophic fungi might as well have inhibitory effects on *Zymoseptoria* species.

This has been the first project in which leaves of different aging stages were inoculated, in order to test if natural senescence and associated necrosis of the leaves allow endophytic or saprotrophic growth and pycnidia formation of the different *Zymoseptoria* species.
Nevertheless, even with this experimental setup it was difficult to differentiate between fungal induced necrosis that accompanies the switch from biotrophic to necrotrophic growth (Ponomarenko et al. 2011), natural senescence of leaves and leaf necrosis as a result of plant hypersensitive response (Jones & Dangl 2006).
In the Ta01 experiment leaves inoculated with all treatments showed necrosis. There was a significant difference between the onset of necrosis on leaves inoculated with the mock control and the one on leaves inoculated with the fungal strains. In turn there was no significant difference between the onset of necrosis on leaves inoculated with different fungal strains. For this reason we could not determine the

cause of the necrosis on fungi inoculated leaves. We could only hypothesize that the necrosis observed on wheat leaves inoculated with *Z. tritici* was caused by the fungus, since pycnidia formed shortly after the onset of necrosis, while on leaves inoculated with *Z. pseudotritici* and *Z. ardabiliae* strains the necrosis was induced by the plant. This second hypothesis originated due to the presence of yellow areas on the leaves in the regions where the fungi presumably tried to enter the plant. These symptoms are typically shown when the plant immune system resists fungal colonization by recognizing their PAMPs and inducing a hypersensitive response involving the release of reactive oxygen species (ROS), which stops the proliferation of fungal hyphae in the plant (Zeilinger et al. 2015). This is still difficult to confirm, since the 2nd wheat leaf normally starts to age quite soon and plant-induced necrosis can be confused with natural senescence. A possible improvement of this plant experiment could be to inoculate younger leaves of one plant besides the 2nd leaf. This would allow the comparison of the necrosis development and therefore a better analysis of the necrosis type. The ROS production, which changes in association with cell death and defense response of the plant, could also be measured by staining leaves with nitroblue tetrazolium (NBT) or diaminobenzidine (DAB) (Adam et al. 1989; Thordal-Christensen et al. 1997). This method was previously used on leaves inoculated with *Z. tritici* which showed high levels of H_2O_2 production during the onset of visible disease (Shetty et al. 2007).

In the experiments conducted on *D. glomerata,* we wanted to observe the same aging development of the leaves as in the wheat experiments. Since *D. glomerata* plants showed a slower growth, the natural senescent stage of the leaves was achieved later. Only on a few necrotic leaves some fungal pycnidia were observed. A possible improvement of this plant experiments could be to prolong the experimental period on *D. glomerata,* giving the fungi more time to establish an interaction with this plant species.

There are further methods that could be applied to optimize these experiments.

Besides prolongating the duration of the plant experiments to evaluate whether real pycnidia formed on the leaves treated with *Z. pseudotritici* and *Z. ardabiliae* or not, the Pycnidia-Induction method could be applied. Due to the high humidity inside the Petri disks, in which the leaves are positioned, the pycnidia maturation and the induction of the release of pycnidiospores can be accelerated (Poppe et al. 2015). Furthermore, CLSM could be used to analyze more samples to obtain meaningful results. It should be used especially on *D. glomerata* leaves inoculated with the wild-grass associated fungi since no CLSM analysis on them was conducted yet. This would allow a better evaluation of the pycnidia structures observed so far. Nevertheless, with this method it is difficult to distinguish hyphae of *Zymoseptoria* spp. from contaminating fungal hyphae, since WGA-FITC staining is not specific to *Zymoseptoria* but stains all chitin-containing structures. For even better comparative microscopy, strains expressing specific fluorescent markers such as GFP or dsTomato are required.

The high sensitivity of the automated image analysis assay misled us concerning the pycnidia recognition, as impurities on the leaf surface were counted as pycnidia including the leaves inoculated with the mock control. Additionally, the software only recognizes pycnidia based on gray values and shape whereby too big, too small or not yet dark colored pycnidia were not detected. The use of CLSM allows a better

analysis of the morphology of fungal structures inside the leaf tissue.

To additionally optimize these experiments, a higher sample size is needed, especially one of *D. glomerata* for which we only had 10 replicates per treatment. Furthermore, experiments should be repeated at least three times. Moreover, different plant cultivars should be used, since as previously demonstrated for *Z. tritici* strains on wheat, the genotype-genotype interaction of each strain can be different with each cultivar (Eyal et al. 1985). *Z. pseudotritici* and *Z. ardabiliae* were only isolated in Iran (Stukenbrock et al. 2012), thus for the analysis of the interaction between these fungal strains and *D. glomerata* some cultivars from Iran could be used.

4.1 Fluorescent *Zymoseptoria* spp strain creation

In this thesis none of the four planned plasmids could be generated, whereby *A. tumefaciens*-mediated transformation of *Zymoseptoria* spp. strains was not yet possible.

During the process of plasmid generation several problems occurred. Although we amplified the correct fragments by PCR, we might have mixed up fragments of similar size during the purification on agarose-gels. Thereby Gibson-Assembly could not have worked because of incompatible fragment overhangs. Furthermore, Gibson-Assembly reaction requires accurate concentration ratios between fragments while small mistakes, e.g. during pipetting or during Nanodrop measurements, can lead to inefficient reactions.

The 5' and 3' flanking regions (UF and DF) that are part of the plasmid inserts consist of repetitive DNA from the target loci in the fungal genomes. Due to the similarity of their sequences it is possible that predominately not their 3' overhangs anneal but rather other regions are detected, because of exonuclease activity. This erroneous hybridization of UF- and DF- DNA fragments can prevent the correct annealing of all fragments which is why the correct plasmid cannot be assembled.

The creation of these plasmids and the proximate *A. tumefaciens*-mediated transformation of *Z. pseudotritici* and *Z. ardabiliae* is still essential for the understanding of these species' interaction with their host plants or other fungi.

For further experiments the creation of these plasmids might be achieved by conducting an overlap PCR with the fragments for the plasmid insert regions, whereby a long DNA fragment (the complete insert) is created out of the single insert fragments. In this way using Gibson Assembly only two fragments have to be assembled: backbone and insert. This would lower the error rate in the fragments assembling.

Another method could be to plan and create new plasmids where UF and DF do not consist of repetitive DNA which might further prevent wrong assembling.

4.2 Conclusion

Our initial hypothesis that *Z. pseudotritici* and *Z. ardabiliae* colonize grass leaves in a saprotrophic or endophytic manner and do not possess the same pathogenic properties as *Z. tritici* could only partially be confirmed.

We can confirm that *Z. pseudotritici* and *Z. ardabiliae* do not possess pathogenic properties on *D. glomerata,* and compared to *Z. tritici* do not possess pathogenic properties on wheat.

Since no pycnidia were found on senescent or dead wheat leaves, but only some similar structures by visual evaluation, we cannot determine by now if these two fungal species have a saprotrophic or endophytic colonization manner on wheat. Further investigation is therefore required.

The observation of some pycnidia on necrotic *D. glomerata* leaves inoculated with *Z. pseudotritici* and *Z. ardabiliae* could lead to the hypothesis that they have a saprotrophic lifestyle. However, more analysis is needed, since confocal microscopy was not yet conducted on *D. glomerata* and the pycnidia observed might be caused by contaminating saprotrophic fungi. Thus, on *D. glomerata* the lifestyles cannot be determined yet. The different necrosis types could also not be differentiated yet.

By creating fluorescent strains of these two fungal species, the demonstrated experimental approach in this thesis can be improved and the interaction between these two fungi and their host plants might be better understood.

In conclusion, this thesis was the first one describing a new experimental approach, in which also senescent and dead leaves are treated with the fungal strains. Better results could be achieved by further adapting the methods we used, as suggested above.

Zusammenfassung

Pilze leben in kontinuierlicher Wechselwirkung mit Pflanzen und anderen Organismen. Diese Interaktion kann eine positive (mutualistische) bis negative (parasitische/pathogene) Wirkung in dem Partner (Wirt) hervorrufen.

Der hemibiotrophe Pilz *Zymoseptoria tritici* ist ein Beispiel für einen pathogenen Organismus, der auf Weizenblätter spezialisiert ist. Es kolonisiert erst harmlos seinen Wirt (biotrophe Phase) und dann, nach einer bestimmten Zeit, tötet es die Blattzellen der Pflanze, um an die intrazellulären Nährstoffen zu gelangen (nekrotrophe Phase). Dabei entstehen nekrotische Wunden in den Blättern, in denen sich viele kleine schwarze Punkte bilden, die die Fruchtkörper des Pilzes (Pyknidien) in dem substomatären Hohlraum darstellen.

Diese Wundenbildung ist als Septoria Tritici Blotch (STB) Krankheit bekannt und wegen dem weltweit verbreiteten domestizierten Weizen in jedem Land zu finden.

Zwei mit dieser Art nah verwandte Arten, *Z. pseudotritici* und *Z. ardabiliae,* sind hingegen endemisch für das Land Iran und wurden bis her nur aus Wildgräsern isoliert.

In dieser Bachelorarbeit wurde die noch unbekannte Lebensweise dieser zwei Arten mit der des Weizenpathogen verglichen mit der Hypothese, dass diese keine Pathogene sind, sondern endophytisch oder saprotroph leben.

Dafür wurden drei Experimente mit Weizen und zwei Experimente mit dem Wildgras *Dactylis glomerata* durchgeführt, bei denen jeweils das zweite Blatt der Pflanzen mit einer Sporen-suspension inokuliert wurde. Diese Pflanzenexperimente wurden zeitlich versetzt durchgeführt, um die Interaktion der Pilze mit Blättern in unterschiedlicher Lebensstadien zu untersuchen.

Parallel dazu wurde versucht im Labor Plasmide herzustellen, die ein Gen für ein fluoreszierendes Protein (GFP oder dsTomato) tragen. Durch die Transformation des Plasmids durch *Agrobacterium tumefaciens* in zwei Stämme der Wildgras-assoziierten Pilzarten könnte man fluoreszierende Stämme herstellen, die dazu beitragen würden das Verständnis über die Interaktion zwischen den Pilzen untereinander und zwischen den Pilzen und den Wirtpflanzen zu erweitern.

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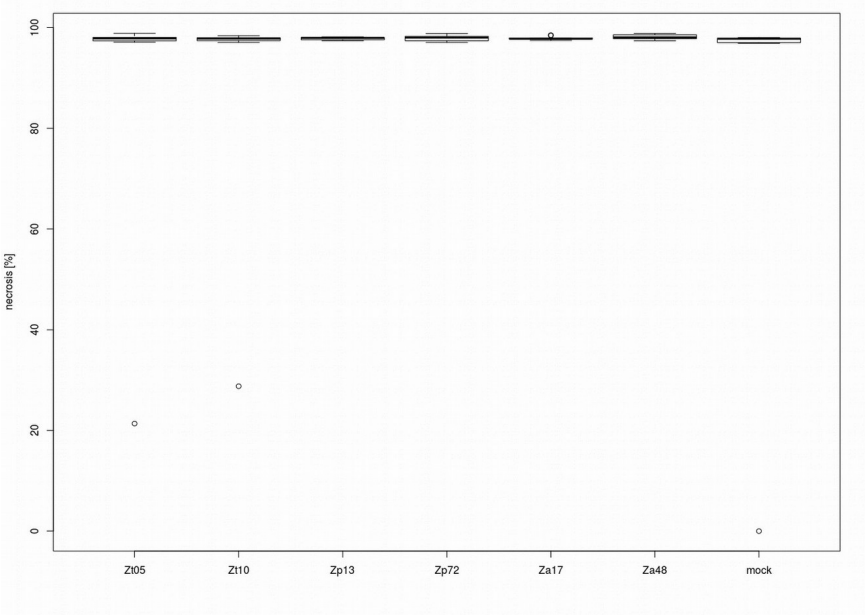
Lastly I thank my friends and my parents for their emotional support and motivation and the sometimes necessary distraction. A special thank you to Lukas Werner, who accompanied me for the entire journey and to Sophia Storm, who helped me out in the last intense weeks.

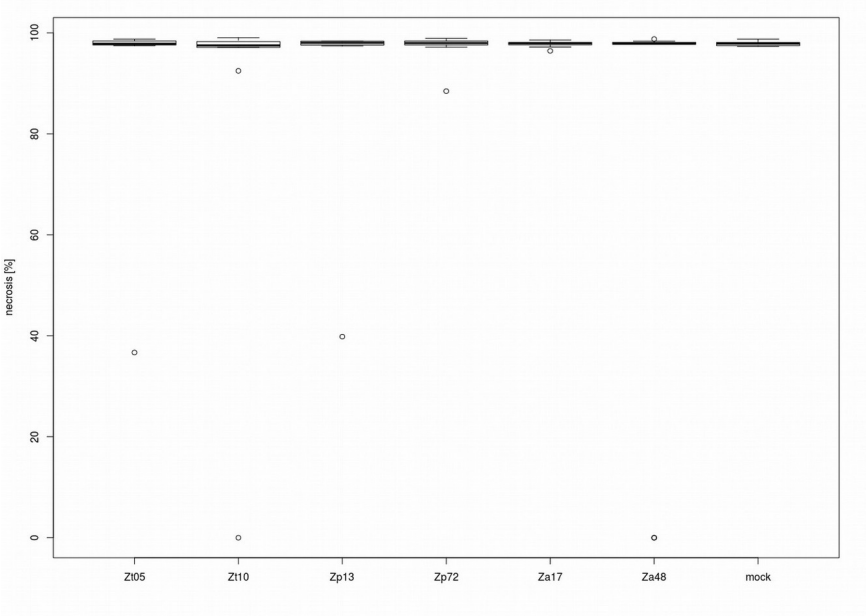
Appendix

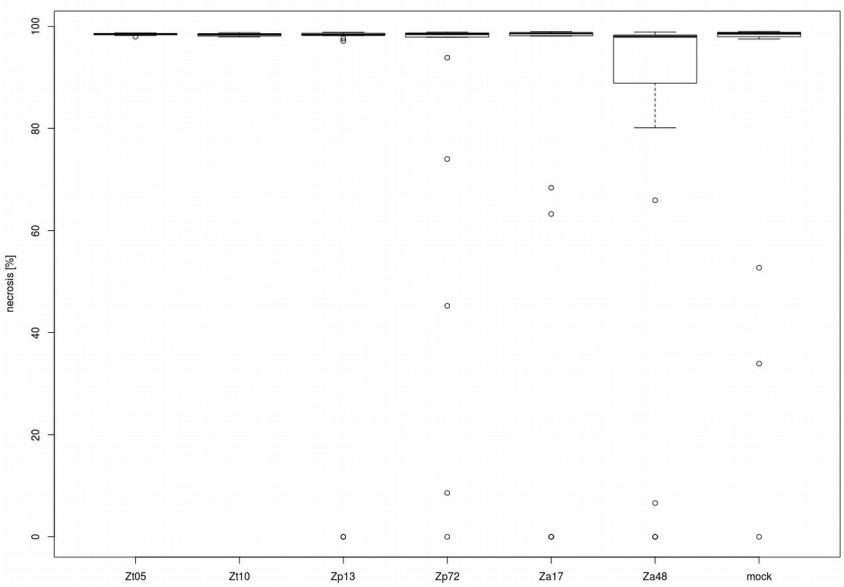
Boxplots **1 Evaluation of necrosis levels with the image analysis assay**

Dg01



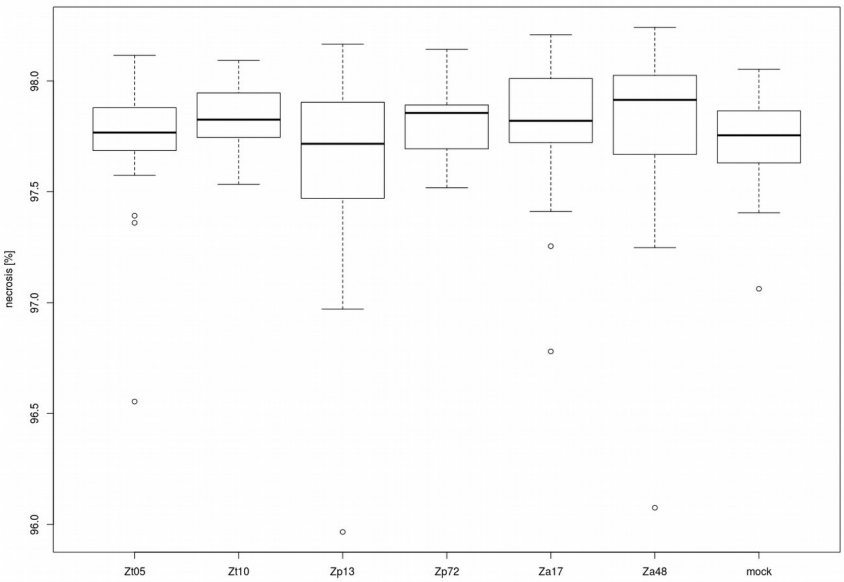
Dg02



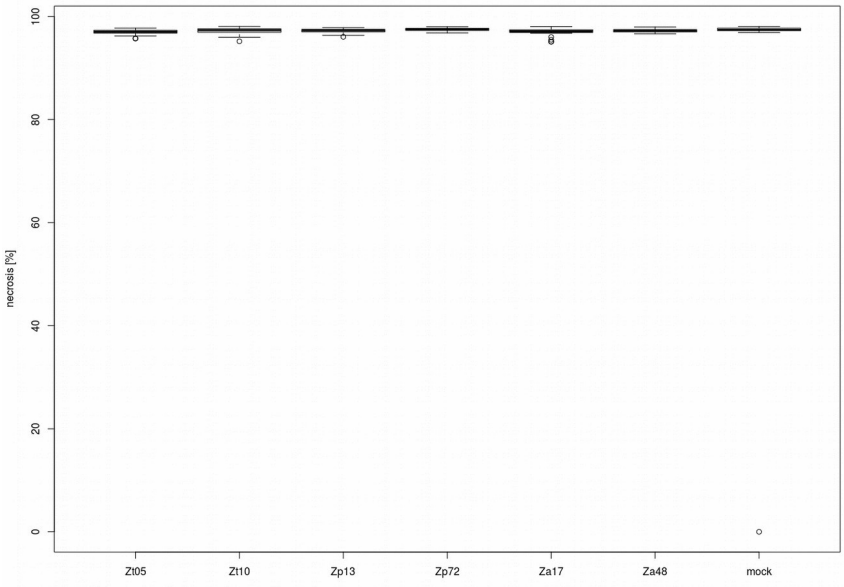


Ta01

Ta02



Ta03



Protocol **A**

Wizard® SV Gel and PCR Clean-Up System

INSTRUCTIONS FOR USE OF PRODUCTS A9260, A9281, A9282, AND A9285.

Quick PROTOCOL

DNA Purification by Centrifugation

Gel Slice and PCR Product Preparation

A. Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10µl Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C until gel slice is completely dissolved.

B. Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA

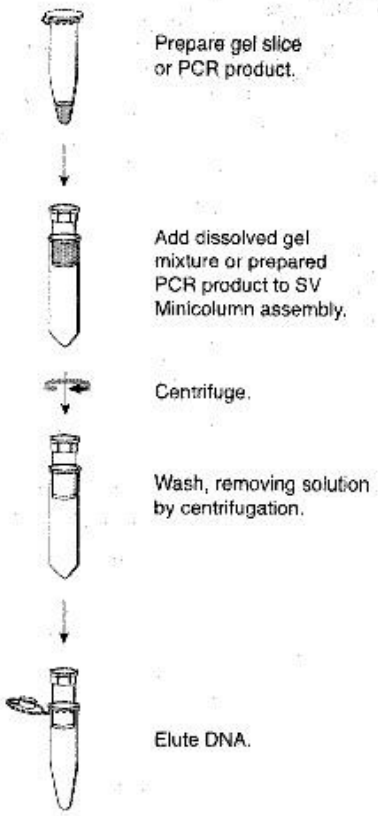
1. Insert SV Minicolumn into Collection Tube.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Washing

4. Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × *g* for 5 minutes.
6. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × *g* for 1 minute.
9. Discard Minicolumn and store DNA at 4°C or –20°C.



3760MA07_2A

Additional protocol information is available in Technical Bulletin #TB308, available online at: www.promega.com

ORDERING/TECHNICAL INFORMATION:
www.promega.com • Phone 608-274-4330 or 800-356-9526 • Fax 608-277-2601

Printed in USA. Revised 11/09
Part #9FB072

Protocol **B**

Transformation of E. coli

by Petra Happel modified by AN

E. coli

- Thaw chemically competent E. coli on ice for 10 minutes
- Pre cool Eppis for reactions on ice
- Mix carefully for each transformation reaction:

	Retrafo:	**Topo-Cloning:**	**Ligation:**
DNA amount	1µl (0,5µg)	3µl	10-20µl
E.coli	15µl	50µl	50µl

- Incubate **on ice for 30 minutes** (or 5 minutes for retrafo)
- **45 seconds, 42°C** heat shock
- **2 minutes on ice**
- + **5 Volumes dYT** medium
- **30 minutes at 37°C** with gentle shaking (this step can be left out for retrafos on ampicillin)
  Keep in mind that for some antibiotics the E. coli cells need longer regeneration time.
- **Plate** on dYT-Amp plates (or appropriate antibiotic if needed)
  (For Topo Cloning distribute 60µl X-Gal (2%) on the plate and let solvents vaporize)
- Incubate at **37°C over night**

Protocol **C**

Created: 28.03.13 by Petra Happel

Boiling Prep (Plasmid-DNA-Preparation)

- **Inoculate a colony** in test tubes in **2ml dYT + antibiotic**, 37°C, over night
- Thaw Lysozyme, heat thermomixer to 95°C
- Transfer culture to **2ml Eppis**, centrifuge for **2 min. at 13,000rpm**
- Discard supernatant
- Add **200µl STET buffer**
- Add **20 µl Lysozyme**
- **Vortex** until pellet is dissolved
- **60 sec, 95°C**, Thermomixer
- Centrifuge **13,000rpm, 10min**
- **Remove pellet** with a toothpick
- Add **20µl MiniIII** (=3M NaOAc)
- Add **500µl 2-Propanol** and mix thoroughly by inverting the tube
- Centrifuge **13,000rpm, 10min**
- Discard supernatant
- Wash pellet: add **500µl 70% Ethanol**, let stand a few minutes and discard Ethanol
- Centrifuge for a few seconds to collect residual Ethanol at the bottom of the Eppi, discard Ethanol
- Add **100µl TE-RNase A**
- Incubate at Thermomixer 37°C, 15min

Protocol: Plasmid or Cosmid DNA Purification Using QIAGEN Plasmid Midi and Maxi Kits

This protocol is designed for preparation of up to 100 µg of high- or low-copy plasmid or cosmid DNA using the QIAGEN Plasmid Midi Kit, or up to 500 µg using the QIAGEN Plasmid Maxi Kit. For additional protocols, such as for purification of very low-copy plasmids or cosmids of less than 10 copies per cell, see page 29 or visit www.qiagen.com/goto/plasmidinfo .

Low-copy plasmids that have been amplified in the presence of chloramphenicol should be treated as high-copy plasmids when choosing the appropriate culture volume.

Maximum Recommended Culture Volumes*

	QIAGEN-tip 100	QIAGEN-tip 500
High-copy plasmids	25 ml	100 ml
Low-copy plasmids	100 ml	500 ml

* For the QIAGEN-tip 100, the expected yields are 75–100 µg for high-copy plasmids and 20–100 µg for low-copy plasmids. For the QIAGEN-tip 500, the expected yields are 300–500 µg for high-copy plasmids and 100–500 µg for low-copy plasmids.

Important points before starting

- New users are advised to familiarize themselves with the detailed protocol provided in this handbook. In addition, extensive background information is provided on our plasmid resource page www.qiagen.com/goto/plasmidinfo .
- If working with low-copy vectors, it may be beneficial to increase the lysis buffer volumes in order to increase the efficiency of alkaline lysis, and thereby the DNA yield. In case additional Buffers P1, P2, and P3 are needed, their compositions are provided in Appendix B: Composition of Buffers, on page 44. Alternatively, the buffers may be purchased separately (see page 49).
- Optional: Remove samples at the steps indicated with the symbol ☞ in order to monitor the procedure on an analytical gel (see page 41).
- Blue (marked with a ▲) denotes values for QIAGEN-tip 100 using the QIAGEN Plasmid Midi Kit; red (marked with a ●) denotes values for QIAGEN-tip 500 using the QIAGEN Plasmid Maxi Kit.

Things to do before starting

- Add the provided RNase A solution to Buffer P1 before use. Use one vial of RNase A (centrifuge briefly before use) per bottle of Buffer P1, to give a final concentration of 100 µg/ml.
- Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.
- Pre-chill Buffer P3 at 4°C.
- Optional: Add the provided LyseBlue reagent to Buffer P1 and mix before use. Use one vial LyseBlue (centrifuge briefly before use) per bottle of Buffer P1 to achieve a 1:1000 dilution. LyseBlue provides visual identification of optimum buffer mixing thereby preventing the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. For more details see "Using LyseBlue reagent" on page 14.

Procedure

1. **Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approx. 8 h at 37°C with vigorous shaking (approx. 300 rpm).**

   Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. **Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids, inoculate ▲ 25 ml or ● 100 ml medium with ▲ 25–50 µl or ● 100–200 µl of starter culture. For low-copy plasmids, inoculate ▲ 100 ml or ● 500 ml medium with ▲ 100–200 µl or ● 250–500 µl of starter culture. Grow at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm).**

   Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately 3–4 x 10^9 cells per milliliter, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium.

3. **Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 min at 4°C.**

   ⊗ If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. **Resuspend the bacterial pellet in ▲ 4 ml or ● 10 ml Buffer P1.**

   For efficient lysis it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer P1.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle before use to ensure LyseBlue particles are completely resuspended. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. **Add ▲ 4 ml or ● 10 ml Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4–6 times, and incubate at room temperature (15–25°C) for 5 min.**

   Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO_2 in the air.

   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

6. **Add ▲ 4 ml or ● 10 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for ▲ 15 min or ● 20 min.**

   Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and KDS. The lysate should be mixed thoroughly to ensure even potassium dodecyl sulfate precipitation. If the mixture still appears viscous, more mixing is required to completely neutralize the solution.

   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

7. **Centrifuge at ≥20,000 x *g* for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly.**

   Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed in non-glass tubes (e.g., polypropylene). After centrifugation the supernatant should be clear.

   Note: Instead of centrifugation steps 7 and 8, the lysate can be efficiently cleared by filtration using a QIAfilter Kits or Cartridges (see www.qiagen.com/products/plasmid/LargeScaleKits).

8. **Centrifuge the supernatant again at ≥20,000 x *g* for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly.**

   This second centrifugation step should be carried out to avoid applying suspended or particulate material to the QIAGEN-tip. Suspended material (causing the sample to appear turbid) can clog the QIAGEN-tip and reduce or eliminate gravity flow.

   ☞ Remove a ▲ 240 µl or ● 120 µl sample from the cleared lysate supernatant and save for an analytical gel (sample 1) in order to determine whether growth and lysis conditions were optimal.

9. **Equilibrate a ▲ QIAGEN-tip 100 or ● QIAGEN-tip 500 by applying ▲ 4 ml or ● 10 ml Buffer QBT, and allow the column to empty by gravity flow.**

   Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

10. **Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow.**

    The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be centrifuged again or filtered before loading to prevent clogging of the QIAGEN-tip.

    ☞ Remove a ▲ 240 µl or ● 120 µl sample from the flow-through and save for an analytical gel (sample 2) in order to determine the efficiency of DNA binding to the QIAGEN Resin.

11. **Wash the QIAGEN-tip with ▲ 2 x 10 ml or ● 2 x 30 ml Buffer QC.**

    Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove all contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.

    ☞ Remove a ▲ 400 µl or ● 240 µl sample from the combined wash fractions and save for an analytical gel (sample 3).

12. **Elute DNA with ▲ 5 ml or ● 15 ml Buffer QF.**

    Collect the eluate in a 15 ml or 50 ml tube (not supplied). Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps.

    Note: For constructs larger than 45–50 kb, prewarming the elution buffer to 65°C may help to increase yield.

    ☞ Remove a ▲ 100 µl or ● 60 µl sample of the eluate and save for an analytical gel (sample 4).

    ⊗ If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. **Precipitate DNA by adding ▲ 3.5 ml or ● 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant.**

    All solutions should be at room temperature in order to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

14. **Wash DNA pellet with ▲ 2 ml or ● 5 ml of room-temperature 70% ethanol, and centrifuge at ≥15,000 x *g* for 10 min. Carefully decant the supernatant without disturbing the pellet.**

    Alternatively, disposable conical-bottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

15. **Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris-Cl, pH 8.5).**

    Redissolve the DNA pellet by rinsing the walls to recover all the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry at 260 nm and quantitative analysis on an agarose gel. For reliable spectrophotometric DNA quantification, A_{260} readings should lie between 0.1 and 1.0.

Agarose gel analysis

We recommend removing and saving aliquots during the purification procedure (samples 1–4). If the plasmid DNA is of low yield or quality, the samples can be analyzed by agarose gel electrophoresis to determine at what stage of the purification procedure the problem occurred (see page 41).

Protocol: Plasmid DNA Purification using the QIAprep Spin Miniprep Kit and a Microcentrifuge

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of *E. coli* in LB medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on page 34.

Please read "Important Notes" on pages 12–18 before starting.

Note: All protocol steps should be carried out at room temperature (15–25°C).

Procedure

1. **Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.**

   Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. **Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.**

   Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3. **Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.**

   To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥ 5 ml) may require inverting up to 10 times. The solution should become cloudy.

   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

4. **Centrifuge for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge.**

   A compact white pellet will form.

5. **Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.**
6. **Centrifuge for 30–60 s. Discard the flow-through.**
7. **Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.**

   This step is necessary to remove trace nuclease activity when using *endA*+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5®α do not require this additional wash step.

8. **Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.**
9. **Discard the flow-through, and centrifuge at full speed for an additional 1 min to remove residual wash buffer.**

   Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10. **Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.**

Protocol: Plasmid DNA Purification using the QIAprep Spin Miniprep Kit and 5 ml Collection Tubes

The QIAprep Spin Miniprep procedure can be performed using 5 ml centrifuge tubes (e.g., Greiner, cat. no. 115101 or 115261) as collection tubes to decrease handling. The standard protocol on pages 19–20 should be followed with the following modifications:

Step 4: Place a QIAprep spin column in a 5 ml centrifuge tube instead of a 2 ml collection tube.

Step 6: Centrifuge at 3000 x *g* for 1 min using a suitable rotor (e.g., Beckman® GS-6KR centrifuge at ~4000 rpm). (The flow-through does not need to be discarded.)

Steps 7 and 8: For washing steps, centrifugation should be performed at 3000 x *g* for 1 min. (The flow-through does not need to be discarded.)

Step 9: Transfer the QIAprep spin column to a microcentrifuge tube. Centrifuge at maximum speed for 1 min. Continue with step 10 of the protocol.

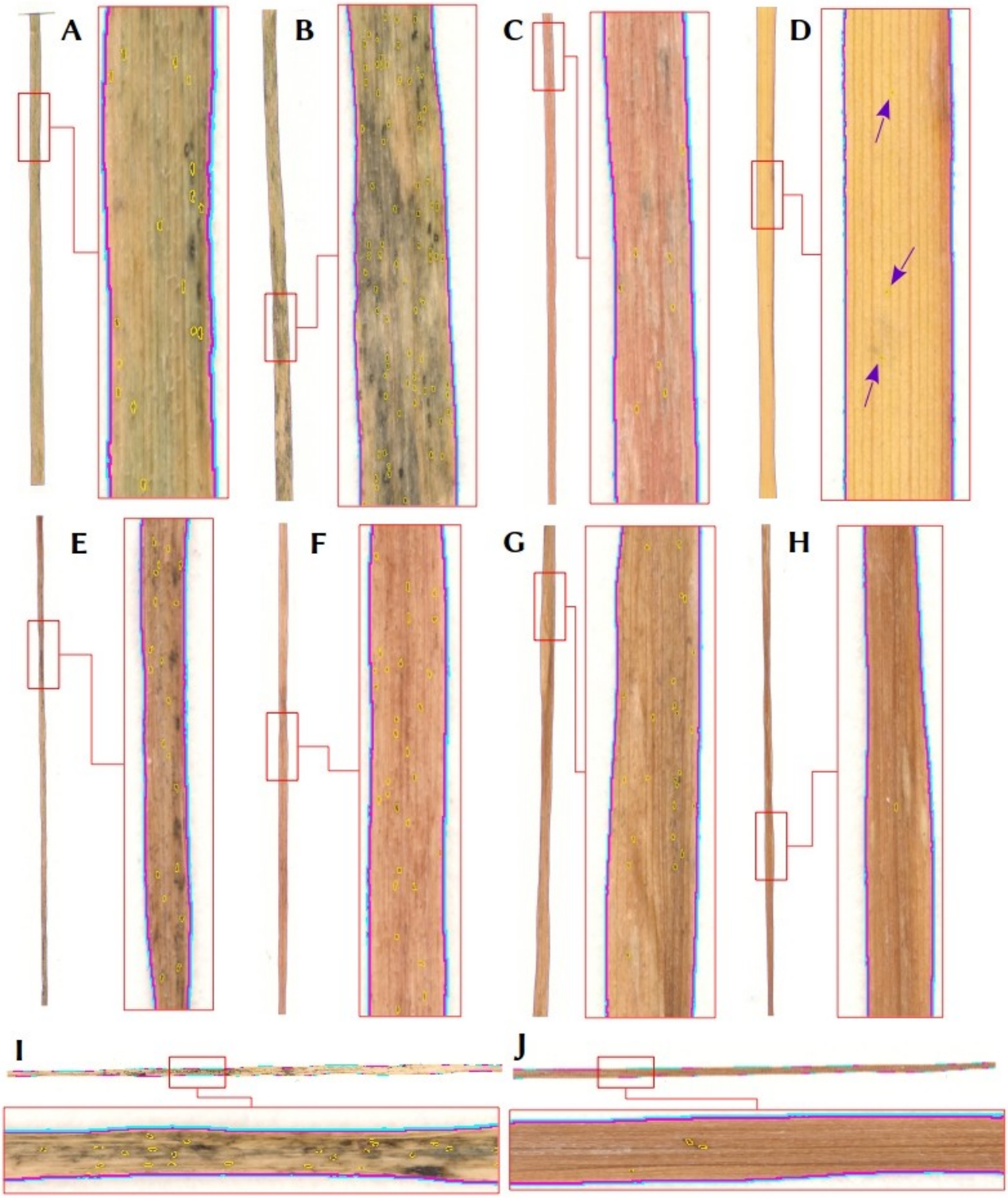


Figure **16**: **Selection of overlay leaves images obtained by automated image analysis:** A: Zt05 inoculated wheat leaf in Ta01, no recognition of big pycnidia by the software; B: Zt05 inoculated wheat leaf in Ta01, high pycnidia coverage; C: Za48 inoculated wheat leaf in Ta01, wrong pycnidia recognition; D: mock inoculated wheat leaf in Ta01, arrows show were pycnidia were found by the software were no pycndia were observed by visual evaluation; E: Za48 inoculated wheat leaf in Ta03, pycnidia-like structures were not marked but wrong marked area; F: Zp13 inoculated wheat leaf in Ta03, wrong pycnidia recognition; G: Za48 inoculated *D. glomerata* leaf in Dg01, wrong pycnidia recognition; H: Zp13 inoculated *D. glomerata* leaf in Dg01, nearly no pycnidia were mesured; I: Za05 inoculated wheat leaf in Ta03, many pycnidia measured where no pycnidia were; J: Zp72 inoculated *D. glomerata* leaf in Dg02, nearly no pycnidia were mesured.

Declaration

I herewith declare, that I have written this thesis independently and by myself. I used no other sources, than those listed. I have indicated where I used quotations. I assure that this thesis has not been submitted for examination elsewhere.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Die eingereichte schriftliche Fassung der Arbeit entspricht der auf dem elektronischen Speichermedium.

Weiterhin versichere ich, dass diese Arbeit noch nicht als Abschlussarbeit an anderer Stelle vorgelegen hat.

Ort, Datum

Unterschrift