PURE

Pesticide Use-and-risk Reduction in European farming systems with Integrated Pest Management

Grant agreement number: FP7-265865

Collaborative Project
SEVENTH FRAMEWORK PROGRAMME

D9.4
Endophyte strains and inoculation techniques

Due date of deliverable: M 36

Actual submission date: M 36

Start date of the project: March 1st, 2011 **Duration**: 48 months

Workpackage concerned: WP9

Concerned workpackage leader: Dr. Toby Bruce

Organisation name of lead contractor: INRA

Project co-funded by the European Commission within the Seventh Framework Programme (2007 - 2013)	
Dissemination Level	
PU Public	**PU**
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1) Summary

A large screening of wheat grain accessions, mainly from three sources: an Israel collection (ca. 100 accessions), a Dutch collection (ca. 100 accession) and a collection of ancient wheat species/cultivars from Denmark, was carried out to isolate and identify wheat endophytes. This screening resulted in the isolation of a large number of mainly *Fusarium (F. graminearum* and *F. avenaceum)* and *Alternaria* (*Lewia infectoria*), but also a small range of other, more rare, species, mainly from the Israel collection. A screening of wheat grain from agricultural fields, including fields from WP2, using next generation sequencing, resulted in the identification of a much larger diversity of fungal species (in total approximately 150 'species'). Statistical analysis of the next generation sequencing data showed that there was a remarkable co-existence pattern between *F. graminearum* and a range of other fungi, mainly *Alternaria, Epicoccum* and *Cladosporium* suggesting possible antagonistic effects. A closer examination of this interaction by double inoculating wheat grain showed that *F. graminearum* had a significant reducing effect on *L. infectoria.* We are currently investigating other combinations of endophytes/pathogens and depending on the outcome of this final screening we will use this information to study interactions between specific species combinations in field samples from WP2.

2) Introduction

To reduce amounts of pesticides used in controlling pathogens in wheat, endophytes may provide an alternative control strategy. There have already been some efforts to identify potential biological antagonists (Wagacha and Muthomi 2007). Similarly there has been some interest in using natural atoxigenic isolates of otherwise mycotoxin producing fungi to compete out their toxin producing counterparts. Atoxigenic *Aspergillus* strains have been successfully tested and proved to be highly effective in reducing the aflatoxin production in maize and cotton (Cotty 1994 and Dorner et al 1999) and strains are being field tested in Africa in maize (Bandyopadhyay et al 2005). Fungal endophytes are known from many hosts including cereals. The term 'endophyte' includes organisms that, during a period of their life, colonise the living internal tissues of their hosts without causing symptoms. Symbiotic endophytes vary with respect to their host specificity and mode of reproduction, some of

which are highly host specific and having seed transmission as the only mode of reproduction (Carroll 1988). Endophytes have been shown to protect their host against insects (Scott and Schardl, 1993), increase growth (Leuchtmann and Clay, 1988) and protect against various plant diseases (Clarke et al 2006). Fungal endophytes (*Epichloé*) are well studied in grasses (Schardl et al 2004) where they are used commercially for growth enhancement. However, the potential of endophytes in cereals is an under-explored area. In this task, we further explore the potential of using endophytes for control of wheat pathogens.

3) Objectives

Exploit fungal endophytes to improve disease management in European cereal-based systems

The objective of this task was to isolate and characterize fungal endophytes from organically grown wheat leaves and grain and to study the interactions between non-pathogenic endophytes and pathogens. This was done by:

- Screening a large number of wheat accessions for the presence of endophytes
- Studying interactions between a range of fungi in the field by using next generation sequencing
- Inoculating wheat grain with endophytes and testing for possible pathogen suppressing activity
- Testing selected endophyte/pathogen interaction in fields (from WP2)

4) Protocols

Samples of wheat grain

Samples of cereal grain were collected from a range of collections:

- Tel Aviv University, Israel: wild wheat grain samples from Israel, app. 100 accessions
- Wageningen University, Centrum vor Genetische Bronnen, The Netherlands: wild wheat grain samples worldwide, app. 100 accessions
- A range of grain samples were harvested from small plots at a Danish organic grower who has a number of ancient cultivars of wheat in his collection (app. 50 accessions).
- A collection of leaf samples from modern wheat cultivars grown in organic plots at Flakkebjerg, AU were included.

In a survey we have analysed a high number of samples (~500) (grain, leaves) from conventionally grown wheat, including samples from WP2. These samples have been characterized by next generation sequencing.

Sample characterization

Cultivation:

Samples were surface sterilized to ensure only endophytes were isolated, except samples from conventionally grown fields that were used for survey/interaction studies. Several methods for surface sterilisation were tested, including washing in sodium hypochlorite followed by EtOH treatment.

Grains were incubated in petri dishes with suitable media (Potato Dextrose Agar) for several weeks and fungal colonies were transferred to new media to obtain pure colonies. Colonies were then sorted based on morphology. Representatives from each group were sequenced using their ITS DNA region for identification in the NCBI GenBank.

Next generation sequencing (NGS):

Grain and leaf samples from both conventionally and organically grown wheat were analysed by NGS. Initially, DNA was extracted: wheat grain samples (100 g) were homogenized in a blender and approximately 5 g were further ground in liquid N_2, and then a subsample of this was used for DNA extraction. In short, 100 mg ground material was mixed with water and CTAB buffer before incubation. Thereafter, proteinase K solution was added before an additional incubation. Samples were centrifuged and the supernatant was transferred to a tube with chloroform and centrifuged. The upper phase was transferred to a new tube and precipitated. The pellet was dissolved in 1.2M NaCl, and chloroform was added before centrifugation. The upper phase was precipitated with isopropanol and centrifuged. The pellet was washed in ethanol, air dried and resuspended in TE buffer. The DNA samples were further purified using a DNeasy kit (QIAGEN) according to the manufacturer's instructions except that the lysis and QIAshredder steps were omitted.
To generate ITS1 amplicons for next generation sequencing ITS1-F and ITS2 were used as primers. The two primers were tag encoded using the forward primer 5'-CGTATCGCCTCCCTCGCGCCATCAG-MID-ITS1F-3' and the reverse primer 5'-CTATGCGCCTTGCCAGCCCGCTCAG-ITS2-3'. The pooled amplicons were electrophoresed and a visible smear of PCR products at approximately 280-360 bp was cut from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN). The sample pools were sequenced by Eurofins MWG on a GS Junior 454 Sequencer. Sequence quality filtering, clustering and BLAST searches were performed at the University of Oslo (http://www.bioportal.uio.no) using the CLOTU application. This package returns a matrix containing information on the sequence abundance in each operational taxonomic unit (OTU) in each sample. All sequences from the 90 tagged samples were analysed together to identify OTUs. Sequences, in which primers and tag could not be identified and sequences which were shorter than 150 bp., were discarded. Remaining sequences were clustered using BLASTclust in the CLOTU package at 99 % similarity and 90 % coverage. As a further quality measure, singletons (OTUs containing only one sequence in the global dataset (90 samples)) were discarded as these often are a result of low quality reads. To identify OTUs, BLAST searches were performed in GenBank using a set of randomly selected sequences from each OTU.

Interaction studies
Due to a high abundance/incidence of *Fusarium* ssp and *Lewia infectoria* in our samples and due to the possible interaction between *F. graminearum* and *L. infectoria* (as seen in the NGS studies), it was decided to initially focus on this possible interaction in subsequent studies. After initial *in vitro* experiments on artificial medium, a seed inoculation method was developed that could be used on sterilized/non-sterilized seeds: a suspension of *F. graminearum* spores is added in one end of the seed and a suspension of spores of *L. infectoria* is added in the other end. The seeds are then incubated at moist conditions and individual seeds are taken out at different time points. The biomass of each species is measured using real time PCR using an *Alternaria* assay and a *F. graminearum* assay (Nicolaisen et al 2009).

5) Results

Screening wheat accessions for the presence of endophytes
A high number of endophytes were isolated on different media (mainly PDA) from a range of wheat accessions from different collections that represent the northern European zone and the southern zone (Israel). After initial culturing, colonies were purified and grown as single colonies. Colonies were initially sorted based on morphological traits such as colour, spore

production and form, and growth form and vigour. After this initial sorting, representative colonies were identified by extracting their DNA and sequencing the ITS region. This showed that the majority of colonies belonged to different species from the genus *Fusarium* (*F. graminearum/ F. culmorum, F. avenaceum/ F. tricinctum*, and a few other *Fusarium* species) together with species from the genus *Alternaria* (mainly *Lewia infectoria* and *Alternaria alternata/A. tenuissima*). However, also rarer species were occasionally found in the material. Representative isolates from each of these species were stored for subsequent studies.

Studying interactions in the field by using next generation sequencing

To study fungal interactions in the wheat plant grown under field conditions, we used NGS to identify and quantify fungal species in wheat leaves (growth stage 73) and grain at harvest. This method can identify a large amount of fungal 'individuals' in one sample, in our setup where several samples were pooled, we were able to identify app. 2000 'individuals' from each wheat sample. Using this approach, a huge diversity of fungi were identified (approximately 150 fungal taxonomic units (approximately 'species')), and these fungal communities were highly responsive to agronomical management of crops such as fungicide applications, previous crop and tillage. Furthermore, a statistical analysis showed that *F. graminearum* ('1' in figure 1) had a distinct negative correlation with a range of other fungi, e.g. *Lewia infectoria, Cladosporium cladosporioides, C. herbarum, and Epicoccum nigrum* in figure 1., indicating a possible antagonism between *Fusarium* and these species, although this negative correlation could also be caused by contrasting preferences for environmental conditions.

Inoculating wheat grain with endophytes and testing for possible pathogen suppressing activity

Based on the classical isolations and the interaction studies using NGS we focused on the possible *Fusarium graminearum*/ *Lewia infectoria* interaction, as these species were found to be present in most accessions and based on NGS studies there was a significant negative correlation between these two species in wheat grain and also in wheat leaves. Furthermore, there are reports indicating that these two species interact (Andersen et al. 1996). An inoculation method on wheat grain was developed: a spore suspension from each of the two isolates was produced and this suspension was added in each end of the wheat grain. The inoculated grain was incubated in a moist petri dish and grains were taken at different time points (0, 3, 7, 10 and 14 days) and the biomass was determined for both species by real time PCR. This showed that while the biomass of *F. graminearum* was rapidly increasing, the biomass of *Lewia infectoria* remained almost constant during the time period, indicating that *F. graminearum* has an inhibitory/antagonistic effect on *L. infectoria* (Figure 2 and 3). However, as *F. graminearum* is considered a pathogen itself, this interaction is therefore unfortunately not considered a viable option for biocontrol. We are currently looking into other interactions such as the interaction between *F. graminearum* and *Epicoccum nigrum*, which has previously been suggested as a candidate for biocontrol of pathogenic fungi (Favaro et al. 2012) using a similar approach.

Testing selected endophyte/pathogen interaction in fields

Unfortunately, we did not yet identify one endophyte that can suppress *F. graminearum* or any other wheat pathogen although we still do have some candidates. We are currently looking into the *F. graminearum*/*Epicoccum nigrum* interaction by the inoculation assay described above. Depending on the outcome of these study, we will further look into the interaction in field samples from WP2 (AU Flakkebjerg) by determining the biomass of the pathogen and the endophyte.

6) Conclusion

Using two approaches, we isolated and characterized the fungal mycoflora found within wheat seeds and leaves. A classical cultivation based method resulted in isolation of mainly *Fusarium* and *Alternaria* species, but also a few rarer species, whereas a sequencing approach revealed much higher diversity (Figure 1). Both the results of the cultivation studies and statistical analysis of the sequence data pointed to an interaction between *F. graminearum* and *L. infectoria*. This interaction was thus chosen for closer examination. Simultaneous inoculation of sterilized wheat seeds showed that there was an antagonistic effect of *F. graminearum* on *L. infectoria*, thus proving the concept that suppression can occur. However, as *F. graminearum* is a pathogen itself, it cannot be used as a biocontrol agent. Therefore, we are currently looking at other pathogen/endophyte combinations based on the NGS results. Potentially interesting combinations will be further tested at field level using samples from WP2.

7) References

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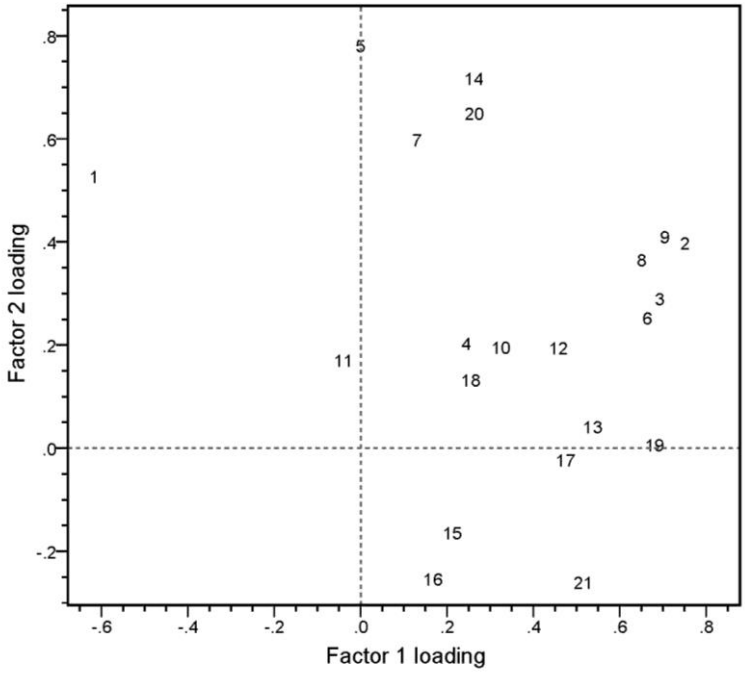
8) Annex I



OTU	BLAST Id
1	*F. graminearum* group (Fgr) (pathogen)
2	*Lewia infectoria*
3	*Cladosporium herbarum*
4	*Didymella exitialis*
5	*Fusarium avenaceum* (Fav) (pathogen)
6	*Epicoccum nigrum*
7	*Microdochium nivale (*pathogen)
8	*Alternaria alternata*
9	*Cladosporium cladosporioides*
10	*Pyrenophora tritici-repentis* (pathogen)
11	*Fusarium poae*group (Fpo) (pathogen)
12	*Mycosphaerella graminicola*(pathogen)
13	*Botrytis cineria*
14	*Phaeosphaeria nodorum* (pathogen)
15	*Cryptococcus victoriae*
16	*Sporobolomyces sp*
17	*Stemphyllium*
18	*Cryptococcus tephrensis*
19	*Alternaria sp.*
20	*Phaeosphaeria avenaria f.sp. triticae* (pathogen)
21	*Dioszegia hungarica*

Figure1. A PCA plot of the 21 most abundant sequence types of the wheat grain fungal flora in approximately 100 grain samples showing co-occurrence patters among different fungi. Numbers in the figure correspond to numbers in the table.

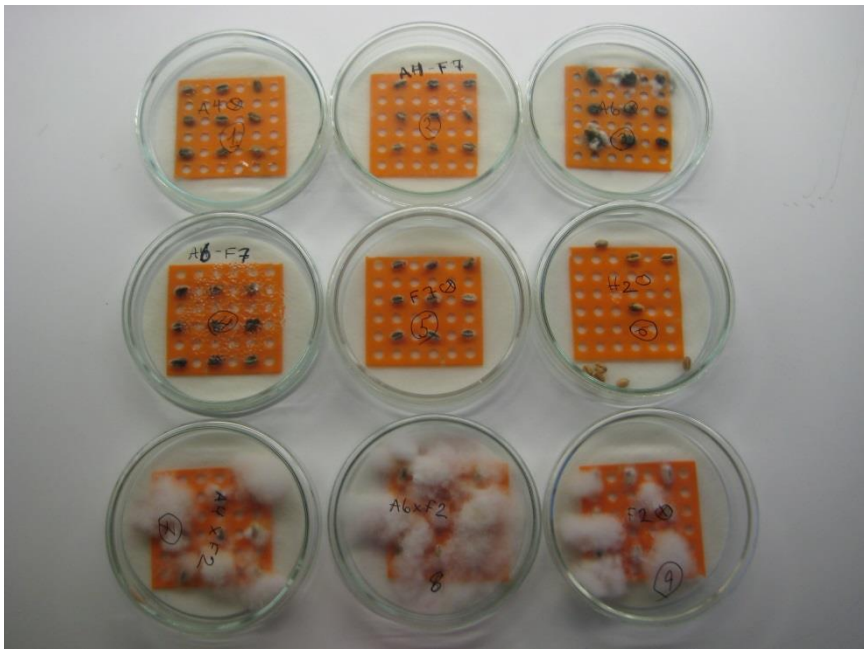


Figure 2. A setup for testing fungal interactions on wheat grain showing different combinations of *F. graminearum* isolates and *L. infectoria* isolates. This picture is from day 10. The lower row shows inoculation with a non-sporulating, but vigorously growing *Fusarium* isolate.

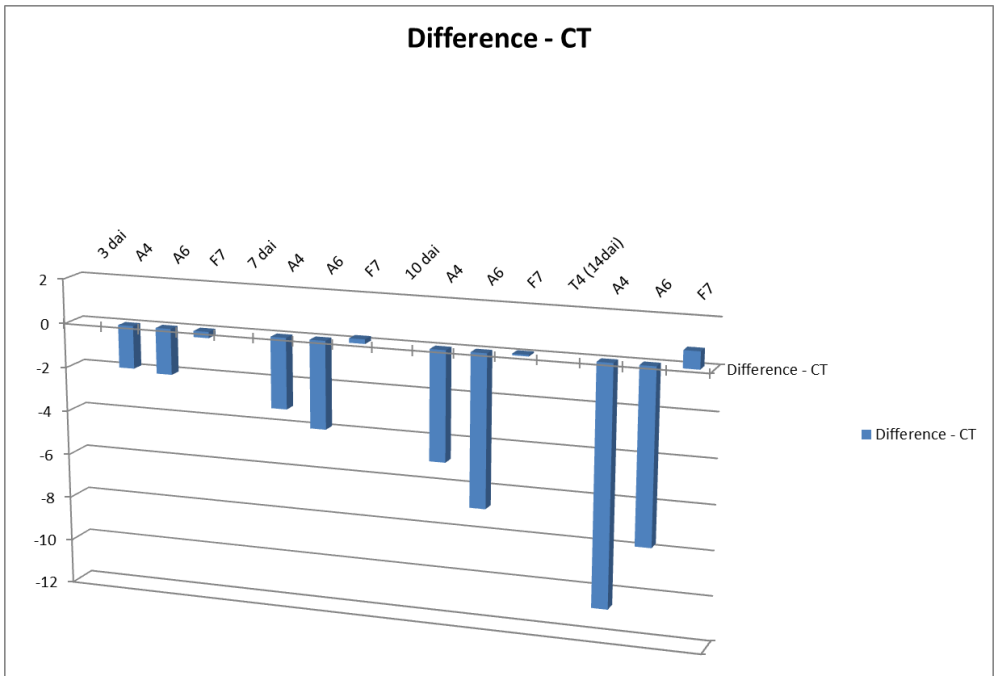


Figure 3. Graph showing the Ct difference between *F. graminearum* and *L. infectoria* biomass grown in dual culture over time (14 days). Ct values are corrected for fungal growth in pure culture to normalize for growth vigor of each isolate. The graph shows that *F. graminearum* has a much higher corrected growth rate than *L. infectoria* in dual culture.