Screening of microorganisms for *Venturia inaequalis* control by means of DGGE

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***Abstract - Venturia inaequalis* (Vi) causes apple scab. The control of the disease in organic apple production depends on the use of copper which will be restricted in future for environmental reasons. In the search for environmental friendly microbial biocontrol agents and stimulation of antagonistic populations *in situ* the molecular technique of DGGE fingerprinting is applied. Comparison of microbial fingerprints and pathogen development in leaf samples during winter and spring will be used to identify populations with antagonistic potential. Preliminary results identify possible antagonists. The identification based on the rRNA or ITS sequence retrieved from the DGGE fingerprints can help to focus on specific isolation or stimulation of a possible antagonist or a consortium of antagonists.[1]**

Introduction

Endophytic and epiphytic fungi and bacteria naturally occurring in and on apple leaves compete with Vi for nutrients and space as soon as the pathogen switches from its biotrophic stage to its necrotrophic stage. When colonising senescent leaves before mating, Vi may especially meet and interfere with endophytic populations of fungi and bacteria. This may result in reduced leaf colonisation and production of fruiting bodies for ascopore production by the pathogen.

Fingerprintings based on bacterial 16S-rRNA genes and ITS regions of fungal species have shown to be a powerful approach to analyse microbial communities. PCR-DGGE systems are applied to characterise the fungal and bacterial communities on and in apple leaves which are, to different extents, infested with Vi. Shifts in microbial populations will be addressed with disease management and other cultural practises. We will attempt to, correlatively, link the prevalence of the different microbials to Vi development and suppression. The most promising antagonistic populations will be singled out.

Materials and Methods

Various treatments were carried out in autumn in the organic orchard of PPO in Randwijk, The Netherlands. Treatments were replicated six times in different blocks. Each plot consisted of six trees. Leaves for determination of possible effects of treatments on micro-organisms and their possible interaction with Vi ascospore production were sampled as described below. Decomposition and ascospore formation was determined for all experimental plots.

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Sampling and DNA isolation

Samples of leaves (with and without sporulation of Vi), were obtained from the different treatments of the field experiment in 2003 and 2004. 1 ml of homogenated material was centrifuged and from 850 µl supernatant DNA was extracted using the Plant DNAeasy kit (Qiagen Benelux, Venlo, The Netherlands).

PCR amplification and analysis by DGGE

PCR of fungal ITS sequences was performed according to Anderson et al. (2003). PCR amplification of bacterial 16S rDNA genes was performed according to Postma et al. (2000). DGGE was performed with the phorU2 system (Ingeny, Leiden, The Netherlands) running 6% (wt/vol) polyacrylamide gels in 0.53 TAE containing a linear 35 to 65% denaturing gradient. The gels were electrophoresed for 16 h at 60°C and 100 V. Gels were stained for 30 min with SYBR Gold I nucleic acid gel stain (Molecular Probes Europe, Leiden, The Netherlands) and were photographed under UV light by a Docugel V system apparatus (Biozym, Landgraaf, The Netherlands).

Potential ascospore production on leaf residues

Leaf residues of 2 x 50 leaves per replicate fixed in a grid on the orchard floor during winter were collected at spring 2004 and 2005. A sub sample of maximum 7-17 g air-dried leaf residues of each sample were incubated in moist chambers for 21 days (20°C, 12 h light at 75 µE per day) to allow maturation (of a substantial fraction) of asci. The amount of ascospores potentially produced in differently treated leaf residues was determined by ascospore extraction in water by bubbling air (Heye and Andrews, 1995).

Analysis of DGGE fingerprints and statistics

Banding pattern analysis and comparison of gels is processed by GelcomparII software (version 1.61; Applied Maths, Woluwe, Belgium).

A unimodal distribution of species was used to explain the structure of species data (in this case, band positions and relative intensities) and environmental variables and treatments (CANOCO 4.5 software package, Biometris, Wageningen, The Netherlands). Blocks were implemented in the analysis and the experiment was treated as a split plot design because infected and symptomless leaf spots were sampled from treatments. Additional statistics were performed with Genstat software (8th Edition, Lawes Agricultural Trust, UK).

Results and Discussion

DGGE fingerprinting

In autumn 2003, leaves from nine treatments replicated in four blocks were sampled. A total of 72 DNA samples from Vi sporulating and symptomless spots were obtained and subjected to fungal and bacterial DGGE community analysis. Sporulating and symptomless leaf parts were taken in autumn 2004 from 15 treatments in six blocks resulting in 180 DNA isolations. A database was constructed containing bacterial and fungal community fingerprints combined with ascospore counts in spring, leaf degradation data and treatments.

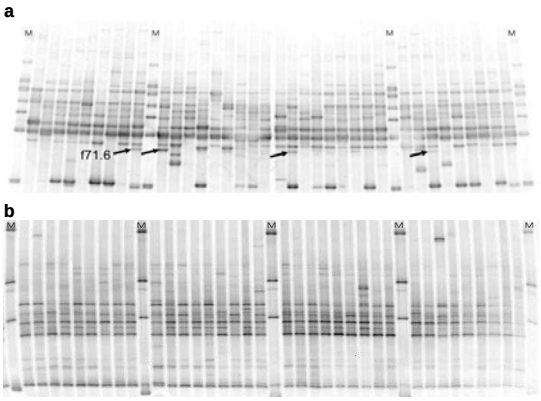


Figure 1. Example of fungal community ITS (a) and 16S-rRNA bacterial (b) DGGE fingerprints of 36 samples from 2003 (M marker lanes for correction and comparison purposes): image analysis of band positions and band intensities indicates composition and abundance of the fungal community members. Fungal ITS sequence f71.6 is indicated by arrows in figure a.

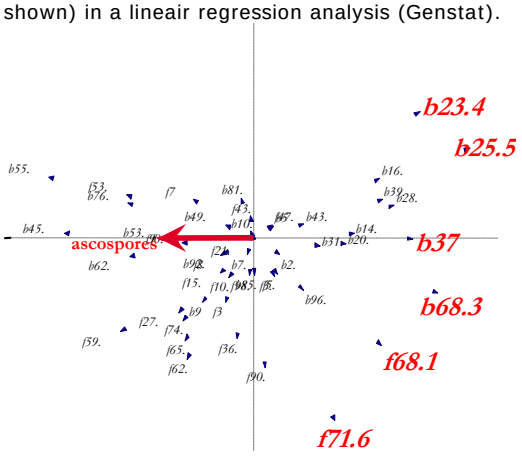
Statistical analysis

Treatments could not be correlated with reduced numbers of ascospores. From the sampling of 2003 none of the treatments showed a relation with the species composition. Data from 2004 indicates that the control treatments and 1% aminosol treatments in October show coherent microbial communities $p<0.05$ (Table 1). Other treatments did not reveal a consistent composition of the microbial community ($p>0.05$) The 28 October treatment with 1% Aminosol is correlated to a fungal species band f56.1 (Graph not shown). Other band positions, bacterial and fungal, are not singled out to team up with treatments.

Table 1. Treatments and their p-values correlation with the microbial community fingerprints derived from canonical correspondence analysis (CANOCO)

Treatments 2004	Date	p value
Control 1		0.01
Control 2		0.012
Urea 5%	19 Oct	0.424
Urea 5%	28 Oct	0.966
$Ca(OH)_2$ 5%	28 Oct	0.838
Beet pulp 1%	19 Oct	0.932
Beet pulp 1%	28 Oct	0.724
Beet pulp 5%	28 Oct	0.108
Aminosol 1%	30 Sep	0.484
Aminosol 1%	19 Oct	0.002
Aminosol 1%	28 Oct	0.002
Aminosol 1% + Resistim	19 Oct	0.61
Aminosol 1% + Resistim	28 Oct	0.478
Aminosol-PS 1%	19 Oct	0.876
Aminosol-PS 1%	28 Oct	0.574

Fingerprint data of 2003 and 2004 were combined to analyze the relation between amounts of ascospores and individual species ($p=0.002$). Two fungal bands at positions 68.1 and 71.6 and 4 bacterial bands at positions 23.4, 25.5, 28.4, 37 in the normalised fingerprints are negatively correlated to ascospore amounts (Graph 1). The negative correlation of band f71.6 with ascospore amounts was confirmed to be the strongest by a forward selection procedure (not shown) in a lineair regression analysis (Genstat).



Graph 1. CANOCO analysis of fungal and bacterial band positions and intensities from autumn leaf samples in relation to numbers of ascospores in spring. Each number represents a band position. Prefix b indicates a band from bacterial fingerprints, prefix f indicates an ITS band from fungal community fingerprints. Potential antagonistic band positions are enlarged.

Conclusions

The correlation of the treatment of 1% aminosol at October 28 and fungal species band f56.1 indicates that this treatment can stimulate a specific fungal population. Furthermore there is a possible relation of a fungal ITS sequence f71.6 with reduced numbers of ascospores. Future sequencing of bands from fingerprints will give a general identification of the species which possibly counteract with the development of Vi in apple leafs.

Results on manipulation of quantity and quality of endophytic and epiphytic populations by cultural practises and specific organic treatments on ascospore production can be used to develop management systems enhancing these functional microbial groups. Isolates of endophytic fungi and bacteria can be identified and potentially applied as antagonists to leaves.

Acknowledgement

This work is funded by the European Commission (Project No 501452; REPCO) and the Dutch Ministry of Agriculture, Nature and Food quality.

References

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