Characterization of the barley (*Hordeum vulgare*) phyllosphere

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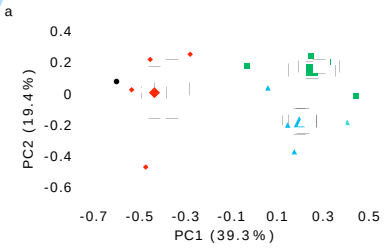
Microbial interactions in the phyllosphere are poorly characterized but could provide a valuable approach to directly or indirectly control diseases or improve tolerance‡. The aim of this work is to develop and use molecular techniques to characterise the dynamics of microbial populations on barley leaves. We focus pathogen, *Pectobacterium atrosepticum* (Pba) the causal agent of potato blackleg disease, on the barley on the effects of barley leaf-associated microbes and a particular non-host foliar disease *Rhynchosporium secalis* (Rs)◇.

Material and methods

- Barley was grown under field conditions with no treatment applied. Leaves were sampled at growth stage 30 and stored at -20°C.
- The composition of bacterial communities were determined using Terminal-Restriction Fragment Length Polymorphism (T-RFLP) based on 16S ribosomal DNA and analysed using Principal Component Analysis (PCA).
- Culturable microbes were isolated on nutrient agar and CzV8CM supplemented with streptomycin and incubated at 18°C.
- *in planta* effect of leaf-isolated micro-organisms was studied by pre-treating barley with microbes two days before Rs infection.
- Pba was detected using species specific primers▫ and its effects on Rs were assessed *in vitro* and under field conditions, by growing barley on previously Pba-infected or non-infected fields.

Establishing microbial isolation technique

- Sonication as a non-invasive method for extracting microbes for characterisation

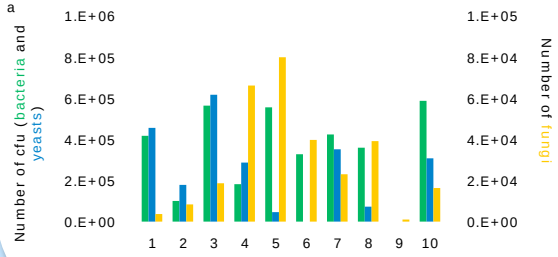


b

	S	H	1-D	E	Hv
◆	17.5[a]	2.168[a]	0.846[a]	0.766[a]	3/4
■	18.4[a]	2.238[a]	0.888[b]	0.770[a]	2/4
▲	24.4[b]	2.729[b]	0.939[c]	0.855[b]	4/4

Figure1: a) PCA of bacterial epiphytical T-RFLP profiles isolated using three methods: dDIP† (◆), sonication (■) and washing (▲) with negative control (●) tested at two DNA concentrations: pure and 10-fold dilution (plain and empty respectively). b) Richness (S), diversity (H, 1-D), evenness (E) and barley DNA presence (Hv) of T-RFLP samples (ANOVA, F<0.001%).

- Surfactants selectively improve removal of microbes from leaf surface



b

	Treatments
1	- Sterile Distilled Water
2	- PBS (washing)
3	- PBS + 0.001% Tween
4	- PBS + 0.1% Tween
5	- PBS + 0.1% SDS
6	- PBS + 0.1% Triton
7	- PBS + EDTA
8	- PBS + 0.0025% hypochloride
9	- PBS + 0.25% hypochloride
10	- PBS (sonication)

Figure2: a) Number of culturable microbes (bacteria, yeasts and filamentous fungi) isolated from leaf surface using 10 methods. b) List of methods tested for epiphytical microbial isolation from the leaf surface.

The barley leaf surface ecology

- The leaf surface varies in its chemistry and structure

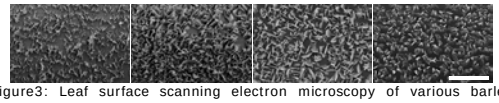


Figure3: Leaf surface scanning electron microscopy of various barley cultivars (from left to right): Optic, Cellar, Bowman and Bowman 2015 wax mutant (scale: 5nm).

- Bacterial communities changes with location and host cultivar

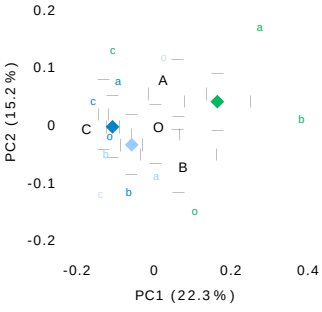


Figure4: PCA of bacterial epiphytical T-RFLP profiles isolated from four spring-type barley varieties (O: optic, A: optic glossy mutant 48-82, B: optic glossy mutant 37-54, C: cellar) at GS30 grown on two locations (1: ◆ and ◆, 2: ◆) and sown at two dates (winter: ◆ and ◆, spring: ◆). Error bars represent standard error. Each cultivar data point represents the average of three biological replicates, error bars are not represented.

- *Rhynchosporium secalis* detection

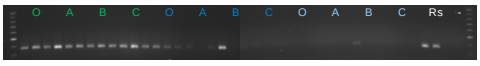


Figure5: PCR detection of Rs in field samples.

Biological control agents against *Rhynchosporium secalis* (Rs)

- Leaf isolated microbes mostly increase Rhynchosporium symptoms

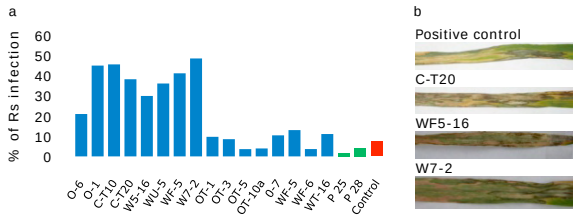


Figure 6: a) Percentage mean infection level of *Rhynchosporium secalis* on both leaves and main stem after pre-treatment with selected yeast (blue) or bacteria (green). Error bars represent standard deviations. b) Macroscopic observations of Rhynchosporium symptoms on pre-treated leaves with yeasts.

- Pba presence correlates with lower Rs infection in field trials.

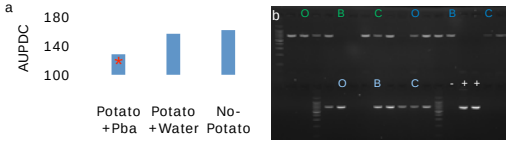


Figure 7: a) Disease assessment of Rs on susceptible barley grown under various field conditions (ANOVA, P<0.01%)°. b) species specific PCR detection of Pba from fig4 samples.

- Pba inhibits Rs spore germination and hyphal growth by direct contact potentially involving the Type 6 Secretion System, toxins and quorum sensing.

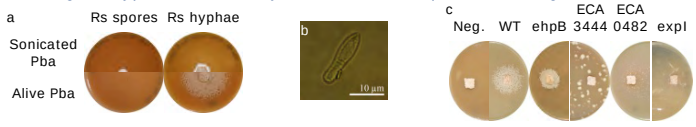


Figure 8: a) Pba effect bioassay on Rs spores and hyphae (6 and 18dpi resp.) Pba was mixed with cooled agar at 10^5 cells/mL. b) Observation of Pba fixed on Rs spores under light microscope () c) Pba effect bioassay on Rs hyphae using various Pba pathogenicity mutants: ephB (phenazine mutant), ECA3444 (Type 6 Secretion System mutant), ECA0482 (potential toxin mutant), expI (quorum sensing mutant).

Conclusions

- Sonication is a reliable method to study the barley phyllosphere at a molecular level.
- The composition of microbial communities on the barley phylloplane can be affected by location, cultivar and physicochemical properties.
- Various microbes live on the phylloplane and can assist or compete with Rs pathogenicity.
- Pba is present on leaves of most barley cultivars from the field and has the ability to control Rs.

Future work

- Further identify microbes (bacteria, yeasts and fungi) present on the leaf surface with molecular tools and characterize the chemistry of leaf waxes and leachate.
- Identify effects of treatments (fungicide, elicitor, combination) on microbial dynamics in the field.
- Intensively characterise the Pba-Rs interaction using confocal microscopy and micro-array.

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References

‡ Newton *et al.* (2010) *Ann Appl Biol*, *in press*.
◇ Newton *et al.* (2004) *New Phytol*, 163: 133-138.
▫ De Boer and Wart (1995) Phytopathology, 85: 854-858.
† Suda *et al.* (2008) *Microbes Environ*, 23: 248-252.