

Interactions between yeasts, fungicides and apple fruit russetting

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Abstract

The effect of inoculations with yeasts occurring on apple surfaces and fungicide treatments on the russetting of Elstar apples was studied. Captan, dithianon and a water treatment were implemented to study the interaction between the fungicides, the inoculated yeast species and *Aureobasidium pullulans*, and the development of russet. All yeast inoculations aggravated russet, but *Rhodotorula glutinis*, *Sporidiobolus pararoseus* and *A. pullulans* did so to a greater extent than the other species. Both captan and dithianon significantly reduced russetting. Denaturing gradient gel electrophoresis analysis showed that inoculations with *R. glutinis* and *S. pararoseus* seemed to suppress other yeast species present on the apple surface.

Introduction

Russetting of 'Elstar', one of the most important apple cultivars in The Netherlands, results in substantial economic losses as the result of reduced fresh-market value (Gildemacher, 2000) and storability (Walter, 1967; Tromp *et al.*, 1976). Russetting is the formation of cork cells in the apple skin as a reaction to the death of epidermal and hypodermal tissue (Meador & Taylor, 1987). The characteristics of the cuticle and epidermis determine the susceptibility of a given apple variety to russetting (Eccher, 1978). The most vulnerable period for russet formation is between 1 and 4 weeks after the end of flowering (Creasy, 1980; Creasy & Swartz, 1981). The diminishing susceptibility after this period is probably related to the development of a thicker cuticle during the first eight weeks after full bloom (Miller, 1982). Weather conditions are an important factor in russet development (Walter, 1967; Creasy, 1980), making the occurrence of russet irregular over the seasons (Vogl *et al.*, 1985; Gildemacher, 2000). Nitrogen excess (Hatch, 1975) and boron deficiency (Smith *et al.*, 1987) as well as water stress (Catzeflis, 1979) can augment russet, whereas the use of gibberellins can diminish it (Taylor, 1978; Wertheim, 1982). Bremer & Büneman (1982) and Jones *et al.* (1994) have shown that various fungicides may reduce russet as well.

The inoculation of apples with the dimorphic ascomycetous fungus *Aureobasidium pullulans* and the basidiomycetous red yeast *Rhodotorula glutinis* was found to increase the

formation of russetting on apples (Matteson Heidenreich *et al.*, 1997) and on pears (Spotts & Cervantes, 2002). Goffinet *et al.* (2002) demonstrated that *A. pullulans* is able to use cutin from the apple skin as a sole carbon source. It is possible that as a result of degradation of the apple cuticle the apple skin epidermis is exposed, which triggers the formation of periderm (cork) tissue, which shows in the form of russetting.

The first research objective was to re-identify a selection of yeast isolates obtained from apple fruit surfaces (Gildemacher *et al.*, 2004), by sequence analysis of the D1/D2 domain of the 26S rRNA gene and the internal transcribed spacer (ITS) 1 and 2 regions of the rRNA gene. The second objective was to investigate whether yeast species that were found to occur commonly on russeted Elstar apples (Gildemacher *et al.*, 2004) play a role in russet formation. The third objective was to study the interaction between scab fungicides, yeast community structure and russet formation. Denaturing gradient gel electrophoresis (DGGE) was used to study the effect of the inoculations and fungicide treatments on the apple fruit fungal communities.

Materials and methods

Inoculations and russet assessment

Apple trees of cultivar Elstar at Randwijk experimental station (The Netherlands) were used as experimental trees.

The trees, with an average height of 2.5 m on M 9 rootstock, were planted in 1996 in a 1.25 × 3.00 m configuration with Delcorf and Everest as pollinators within the row. The experiment was conducted in 2000, and the experimental design was a randomized complete split-plot design with ten replicates. The experimental field comprised five double rows of Elstar, alternating with double rows of Jonagold. On every single Elstar row one replicate was laid out. The main plots consisted of four experimental trees, flanked by two buffer trees on either side. The main factor was fungicide treatment with three variates, namely captan (Captosan 80 WG, 1.2 kg active ingredient ha⁻¹), dithianon (Delan flowable 75%, 0.375 kg active ingredient ha⁻¹), and a water-sprayed control. Within every main plot, five marked flower/fruit clusters on each of three experimental trees were periodically sprayed per inoculation treatment. Part of the yeast isolates, identified previously using morphological and physiological characteristics only (Gildemacher *et al.*, 2004), were re-identified in this study using sequencing of the D1/D2 domains of the 26S rRNA gene and the ITS 1+2 regions of the rDNA following standard approaches (see e.g. Gupta *et al.*, 2004) to ensure correct identification. Yeast isolates were selected on the basis of their dominant presence on apple surfaces and their ability to produce large amounts of extracellular proteases, lipases (including cutinases) and pectinases (Gildemacher *et al.*, 2004). *Aureobasidium pullulans* was included because other studies had found that it induced russet (Matteson Heidenreich *et al.*, 1997; Goffinet *et al.*, 2002; Spotts & Cervantes, 2002). This resulted in the following inoculation treatments: (1) *A. pullulans* (CBS 102662), (2) *Metschnikowia pulcherrima* (CBS 8783), (3) *Cryptococcus victoriae* (CBS 8784, CBS 8785), (4) *Sporidobolus pararoseus* (CBS 8792), (5) *Rhodotorula glutinis* (CBS 8790), (6) a mix of these, excluding *A. pullulans*, (7) buffer solution only, and (8) a non-treated control. Marked buds, flowers and fruitlets were sprayed with hand-pumped plant sprayers of 500 mL until fully wet while holding a plastic tray underneath to protect underlying clusters. Inoculations were executed in 2000 on April 10 (mouse-ear stage), April 20, May 1, 12 and 23 and June 6 (fruit set), and were timed between the fungicide sprayings (Fig. 1).

For the preparation of the yeast inocula, yeast isolates were grown on four to six Petri dishes containing YPGA (4% D-glucose, 1% yeast extract, 0.5% Bacto peptone and 2% agar) at 20 °C for 7–10 days. Yeast biomass was scraped cautiously from the medium and transferred to 50 mL of

buffer solution (0.5 M KH₂PO₄, 0.5 M K₂HPO₄, pH 6.5). The suspension was diluted to 1 L, and the number of yeast cells counted with a Bürker haemocytometer and adjusted to approximately 2 × 10⁹ cells mL⁻¹.

The indicated fungicides were used in the experimental plots during the entire season. Trees were sprayed until runoff, at a rate of 1000 litres per hectare – approximately 0.4 L per tree. Spraying started on 29 March 2000 and was executed roughly every ten days, depending on Welte scab warning system (Batzer *et al.*, 2000) data, until the end of the scab hazard period on 27 July. This resulted in 13 sprays (Fig. 1).

On 30 and 31 August, fruits were ripe, and all fruits from the marked clusters were harvested and kept at 3 °C until classification on russet on 6 and 7 September. All individual fruits harvested were scored for russet separately for the stalk and calyx half of the apple. Five russet grades were distinguished, and weighing factors were attached to every russet grade. A russet index was calculated as the average russet score per fruit, similar to the method used by Wertheim (1982) and also previously by Gildemacher *et al.* (2004). The russet grades with corresponding weighing factors in brackets were: smooth (1), slight russet (3), moderate russet (5), strong russet (7), and very strong russet (9). The russet index expresses the severity of russet in a quantity of apples both in terms of the fraction of fruits affected and as the density of the cork formation on individual fruits in one single figure, which can then be analysed statistically.

Russet index data were analysed by two-way analysis of variance, followed by pairwise comparison of differences using *t*-tests to identify significant differences between treatments.

DGGE

Four-week-old apples that had not been inoculated with yeasts nor with the dimorphic ascomycete *A. pullulans* were sampled and analysed by DGGE as described below. From individual apples, smooth and somewhat rough parts of the skin were analysed separately. Six weeks after the end of flowering, a fruit sample was taken that was inoculated with the yeasts. One fruitlet was picked randomly from each of the marked clusters and combined, resulting in 54 samples (3 fungicide treatments × 9 inoculations × 2 repetitions). The samples were kept in plastic bags at –18 °C until

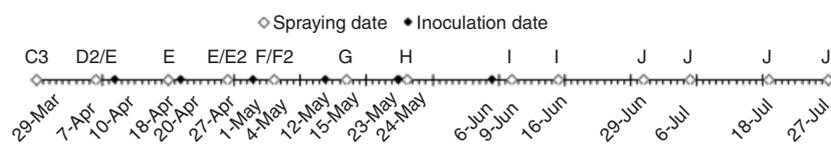


Fig. 1. Dates of scab-control fungicide application and inoculation of apple flower/fruit clusters with yeast suspensions, with the corresponding phenological stages of the apple trees indicated by letters according to EPPO (1989).

transport to the laboratory for DGGE analysis. Russeted and smooth pieces of skin were taken from all fruits in a combined sample, resulting in a total of 108 samples for DGGE analysis.

With a razor blade, clean skin pieces of *c.* 3 × 3 mm were prepared. Lyophilization of the apple skin was carried out inside Petri dishes under vacuum conditions (5–10 µm Hg), at –50 °C, for 18 h, using a Lyph-Lock 1L System (Labconco, Beun & de Ronde, Abcoude, The Netherlands). The apple skin (500 µL) was lyophilised a second time in sterile 1.5-mL micro-centrifuge tubes. Two hundred µL of sterile sand was added to the tubes to pulverize the apple skin. DNA extraction was carried out with CTAB buffer according to O'Donnell *et al.* (1997).

In two PCR steps, the 18S rRNA gene fragment suitable for DGGE analysis was amplified (Kowalchuk, 1998). In the first PCR reaction, the fungus-specific NS1/NS8 (NS1 = 5'-GTAGTCATATGCTTGTCTC-3'; NS8 = 5'-TCCGCAGGTTACCTACGGA-3') primer pair produced a DNA fragment of *c.* 1.7 kb. Twenty-five µL of PCR product was obtained using the following PCR program (Thermolyne Amplitron II, Barnstead/Thermolyne Corporation, Beun & de Ronde, Abcoude, The Netherlands): pre-dwell at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 30 s; annealing at 50 °C for 45 s; extension at 72 °C for 75 s; and a post-dwell at 72 °C for 5 min. In the subsequent nested PCR the NS1GC/NS2+10 primer pair (NS1GC = 5'-CGCCCGCCGCGCGCGGGCGGGCGGGCGGGGCACGGGCCAGTAGTCATATGCTTGTCTC-3'; NS2+10 = 5'-GAA TTACCGCGGCTGCTGGC-3') was used, producing a DNA fragment of approximately 500 bp (Kowalchuk, 1998). Fifty microlitre of PCR product was obtained by running the following PCR program: a pre-dwell at 94 °C for 2 min; 25 cycles of denaturation at 94 °C for 30 s; annealing at 62 °C for 45 s; extension at 72 °C for 70 s; and a post-dwell at 72 °C for 5 min.

DGGE (Dcode Universal Mutation Detection System, BIORAD, Veenendaal, The Netherlands) was carried out using a 6% polyacrylamide gel and a urea and formamide denaturation gradient ranging between 25% and 40%. Perpendicular gradient gels were run to determine the exact denaturing concentration of the DNA from the fungi used for the inoculation of apples (results not shown). Fifty microlitre of the nested PCR product was pre-run at a constant voltage of 10 V for 20 min and run at a constant voltage of 150 V for 4 h.

Results and discussion

Molecular identification of yeast isolates

In a previous study (Gildemacher *et al.*, 2004), yeast isolates were identified phenotypically, using traditional morpholo-

gical and physiological methods. Recently, it has become clear that this may result in erroneous identifications, and hence a selection of these yeast isolates was re-identified using sequence analysis of the D1/D2 domain and the ITS 1+2 regions of the rRNA gene – a process that yielded interesting results. The *Metschnikowia pulcherrima* isolates (CBS 8783, CBS 10125 = isolate 1A8, CBS 10127 = isolate R1A5, and isolate 3B5) showed 98–99% BLAST similarity with sequences of both DNA fragments from that species, except isolate CBS 10127, which showed a 97% BLAST similarity for the ITS regions with *M. fructicola*. For the purpose of this work, all these isolates are considered to belong to the *M. pulcherrima* species complex and hence are listed under this name. One isolate (CBS 10130 = 2F2) showed a 99–100% BLAST match of both DNA sequences with those of *Candida sake*. Among the red yeasts some isolates turned out to belong to *Rhodospiridium babjevae* (CBS 10136 = 2B4, CBS 10126 = G2D3, CBS 10138 = 1A9, and isolate 1A7; with 99–100% BLAST matches for both DNA fragments). Single isolates were re-identified as *Erythrobasidium hasegawianum* (CBS 10139 = 1A5; 99% BLAST match for both D1/D2 and ITS), *Rhodotorula (R.) glutinis* [CBS 8790; 100% BLAST match for both D1/D2 and ITS, and correctly identified previously (Gildemacher *et al.*, 2004)], *R. aff. pinicola* (CBS10126 = G2D3; 100% BLAST match with D1/D2, but showing a 98% BLAST match with the ITS sequence from *R. laryngis*), *R. fragariae* (CBS 10128 = 3J8; 100% BLAST match for both D1/D2 and ITS). The ballistoconidium-forming red yeasts belonged to *Sporidiobolus pararoseus* (CBS 8792 and isolate 1B1; 98–100% BLAST matches for both D1/D2 and ITS). This latter species had been identified as *Sporobolomyces roseus* in the previous study (Gildemacher *et al.*, 2004), and this species ranked second in the list of ITS matches. Isolates identified previously as *Cryptococcus (C.) laurentii* (Gildemacher *et al.*, 2004), turned out to belong to the recently described species *C. victoriae* (CBS 8784, CBS 8785, CBS 10131 = 1D3, CBS 10132 = R3I-S2, CBS 10133 = 1C8, CBS 10134 = 1F4, CBS 10135 = G1B1; with 98–100% BLAST matches for both sequences). Isolates identified by physiological means as *C. albidus* turned out to represent an undescribed anamorphic stage of a *Tremella* species (CBS 10129 = G1B6 = DQ242634; CBS 10259 = 2C1 = DQ242630; CBS 10260 = R2F2 = DQ242632; CBS 10261 = R3G-S1 = DQ242633; CBS 10262 = G1B1; CBS 10263 = 1B5 = DQ242631; only 90–97% BLAST similarity with species such as *Tremella giraffa*, *T. mycophaga*, *C. luteolus* and *Cryptococcus* species).

These molecular re-identifications clearly question the accuracy of the traditional identification for these apple-inhabiting yeasts using physiological and morphological characteristics only. Furthermore, they suggest a higher biodiversity of yeasts occurring on the apple surface than previously assumed (Gildemacher *et al.*, 2004).

Effect of fungicide application and yeasts on russet formation

Treatments with captan and dithianon equally reduced russet for both the calyx and stalk sides of the apple fruit compared with the water-treated control (Fig. 2). All inoculations with the yeasts and *A. pullulans* aggravated russet compared with the controls, regardless of the side of the apple (Fig. 3). It is noteworthy that the buffer-treated control did not differ from the non-treated control, thus indicating that spraying had no effect on russet formation. Clearly, not all yeasts had the same russetting effect. *Rhodotorula glutinis*, *S. pararoseus*, *A. pullulans* and the mix of the yeast species increased russet most, whereas *C. victoriae* and *M. pulcherrima* inoculation increased russet formation to a lesser extent (Figs 3 and 4). Matteson Heidenreich *et al.*

(1997) had previously identified *R. glutinis* and *A. pullulans* as russet-inducing species. Here we have demonstrated that both of these species, plus another red yeast, *S. pararoseus*, and the mix of four yeast species stimulate russet formation when artificially inoculated on apple fruits. The application of fungicides usually used in scab-control programmes reduced both the yeast count and the amount of russetting, thus further supporting the hypothesis that yeast growth on the apple surface can stimulate russet development (Gildemacher *et al.*, 2004).

DGGE analysis of treatment effects on apple fruit yeast communities

The effect of the yeast inoculations and the fungicide treatments on the fungal community structure occurring on the apples was studied using DGGE. This method results in a global impression of the community structure present, and may be a valuable addition to cultural approaches. These latter may fail to identify all species present. In particular, when traditional physiological and morphological methods are used (see above), they tend to select for rapidly growing filamentous fungi and yeasts that are well adapted to the culture media used, and, moreover, this approach may result in unmanageable numbers of isolates. For instance, in our study we compared 108 apple samples (see above). Approximately 300 yeast isolates could be obtained per sample (see e.g. Gildemacher *et al.*, 2004), resulting in more than 30 000 yeast isolates. The PCR-based DGGE also suffers from major drawbacks, however. For instance, the resolution obtained depends entirely on the primers used, and the detection limit is highly sensitive to the population sizes of all species involved, implying that the species dominating the population determines the resolution of all species (Prakitchaiwattana *et al.*, 2004).

DGGE applied to the 4-week-old non-inoculated apples showed that the rough parts of the apple skin contained fungal (i.e. yeast) biodiversity, as can be seen by the presence of distinct bands in the gel, whereas the smooth parts of the apple skin resulted in an almost complete absence of bands, except for the lowermost band that contained only plant DNA (Fig. 5). This suggests that the fungal population, which is dominated by yeasts, differs quantitatively between smooth and russeted parts of the skin in very young apples. Importantly, this is the state when they are most vulnerable to russet formation.

DGGE applied to the pure cultures of the six reference strains separated the amplicons of the various species (data not shown), but some were so close to each other that in our field samples not all species could be separated with a sufficient level of confidence. Apparently, the 18S rRNA gene region targeted in our analysis did not contain the

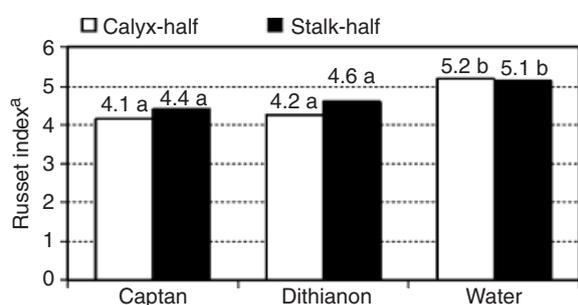


Fig. 2. Russet index of captan-, dithianon-, and water-treated apples. Russet index = (no. apples smooth \times 1 + no. apples slight russet \times 3 + no. apples moderate russet \times 5 + no. apples strong russet \times 7 + no. apples very strong russet \times 9) / total number of apples. Bars with the same letters do not differ significantly.

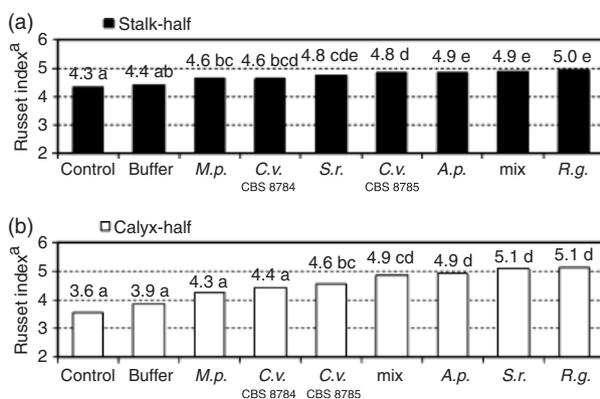


Fig. 3. Russet index (see Fig. 2) of apples inoculated with various yeast species and control treatments; bars in the same graph with the same letters do not differ significantly ($\alpha = 0.05$). (a) Stalk-end, $LSD_{0.05} = 0.23$, F prob < 0.001 ; (b) calyx-end, $LSD_{0.05} = 0.31$, F prob < 0.001 . *M.p.*, *Metschnikowia pulcherrima*; *C.v.*, *Cryptococcus victoriae*; *S.p.*, *Sporidiobolus pararoseus*; *A.p.*, *Aureobasidium pullulans*; *R.g.*, *Rhodotorula glutinis*; mix, *M.p.* + *C.v.* + *S.p.* + *R.g.*; Buffer, buffer used for yeast inoculation; Control, unsprayed. LSD, least significant difference.



Fig. 4. The effect of inoculations with various yeast species on russet formation. (a) Immature Elstar apples showing russeting after treatment with *Aureobasidium pullulans*; (b) almost complete absence of russeting after inoculation with *Cryptococcus victoriae*.

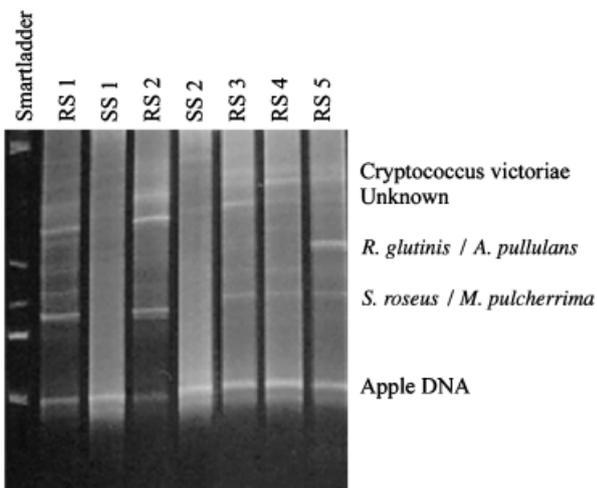


Fig. 5. DGGE gel showing the presence of fungal amplicons in rough parts of four-week-old non-inoculated apples. RS, sampled from russeted skin; SS, sampled from smooth skin. Numbers indicate different sample apples.

necessary variation. Consequently, three distinct mobility groups were distinguished. The first DNA band (group 1) migrated to the higher part of the gel and represented *C. victoriae*. The amplicons of *R. glutinis* and *A. pullulans* migrated to the middle part of the gel (group 2), and those of *S. paraseus* and *M. pulcherrima* migrated to the lower part of the gel (group 3). It may well be possible to improve the resolution of the method by using primers derived from less conserved parts of the rRNA gene, such as the D1/D2 domains of the 26S rRNA gene or the ITS (Kowalchuk, 1998; Prakitchaiwattana *et al.*, 2004). Nevertheless, some gross changes in fungal community structure were observed in our 18S rRNA-gene-based DGGE approach as a result of the inoculation of the apples with selected yeast species and/or treatment with fungicides (Fig. 6).

DGGE analysis of the non-inoculated and the buffer-treated six-week-old apple samples (Figs 6a, b) resulted in four bands (numbered 1–4) of weak intensity. All three

mobility groups were visible in the captan- and in the water-treated apples, as well as a fourth unknown band 3 in Fig. 6. In the dithianon-treated apples (Figs 6a, b, dithianon), the DNA bands corresponding to the *R. glutinis/A. pullulans*

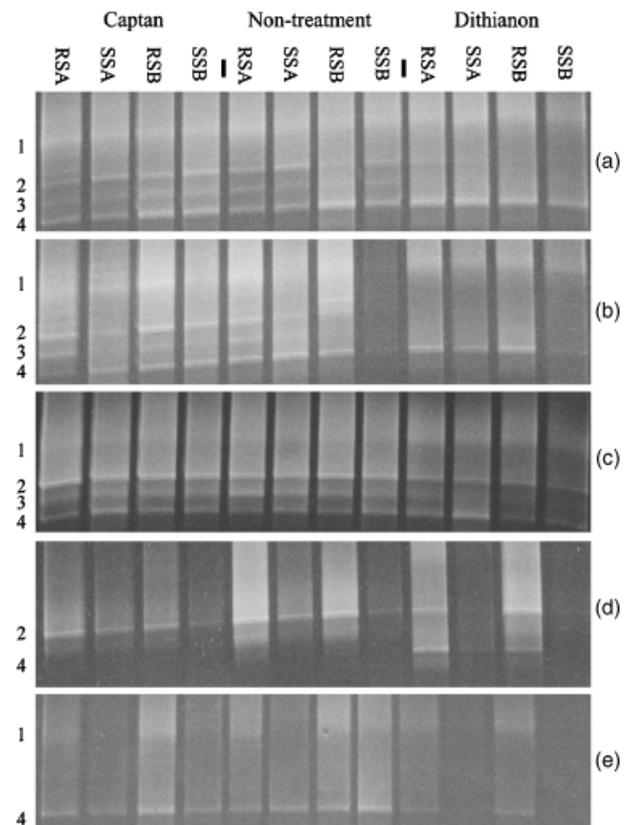


Fig. 6. DGGE from 6-week-old apple samples subjected to different fungicide regimes and yeast inoculations. Inoculations: (a) none; (b) buffer solution; (c) a mix of yeast species; (d) *Rhodotorula glutinis*; (e) *Sporidiobolus paraseus*. DGGE mobility groups are 1, *Cryptococcus victoriae*; 2, *Aureobasidium pullulans/R. glutinis*; 3, band of unknown origin; 4, *Metschnikowia pulcherrima/S. paraseus*. Sample codes are SS, smooth skin; RS, russeted skin; followed by A, composite sample from field replications 1–5; followed by B, replications 6–10.

mobility group (band 2) and the unknown species (band 3) were not visible, indicating that the fungicide reduced the population size of the species involved effectively. Incidentally, a further unknown DNA band, different from the consistently present unknown band 3, was present above band 2 (e.g. Figs 6a, Captan RSA and 6b, Captan RSA non-treatment RSB), indicating that other fungal species occurred on these apples as well.

The inoculation of the apples with the mix of four yeast species modified the picture of DGGE bands (Fig. 6c) compared with the controls. The same four DNA bands were observed, but the amplicons showed a higher intensity. This was particularly evident for band 2 belonging to the *R. glutinis*/*A. pullulans* mobility group. Furthermore, the three DNA bands (1, 2 and 4) were also present in the dithionon-treated apples, indicating that inoculation could overcome the effect of the fungicide shown in the control treatments. Inoculations with *C. victoriae*, *A. pullulans* and *M. pulcherrima* resulted in DGGE banding patterns similar to those for the controls (data not shown). Inoculation with *R. glutinis* (Fig. 6d) and *S. pararoseus* (Fig. 6e) resulted in a considerable effect on the structure of the fungal communities present. The DNA bands corresponding to the inoculated species dominated (band 2 in Fig. 6d and band 4 in Fig. 6e), indicating suppression of the other species inhabiting the apple surface in the control treatments. The DNA band corresponding to *C. victoriae* was not present after inoculation with *R. glutinis* (Fig. 6d), while it was just visible after inoculation with *S. pararoseus* (Fig. 6e), suggesting differences in the competitive capabilities of the two red yeasts. After inoculation with *R. glutinis*, the DNA band corresponding to the *R. glutinis*/*A. pullulans* group (band 2) became visible in the dithionon treatments (Fig. 6d – dithionon), while it was not visible without inoculation (Fig. 6a dithionon and 6b dithionon), indicating that inoculation could partly overcome the effect of the fungicide. In Fig. 6d, the russeted apple skin showed stronger band intensities than the smooth skin, indicating a differential presence of red yeasts and/or *A. pullulans* for russeted apple skin. This may indicate the involvement of yeasts in russet formation, but could also be a result of differences in growth conditions for the yeast species between smooth and russeted skin.

Captan and dithionon diminished russetting of the mature apples to a similar extent (Gildemacher *et al.*, 2004 and this work), but no effect of captan was visible in our DGGE analysis of six-week-old apples. This seems to be in conflict with observed reductions in yeast counts on apple leaves after spraying with captan (Hislop & Cox, 1969). Previous *in vitro* fungicide susceptibility tests (Gildemacher *et al.*, 2004) had shown that captan was active at low dosages against *A. pullulans*, an unknown anamorphic *Tremella* species (cited as *C. albidus* in Gildemacher *et al.*, 2004), *C. victoriae*

(cited as *C. laurentii* in Gildemacher *et al.*, 2004), and *M. pulcherrima*. However, both red yeasts *S. pararoseus* and *R. glutinis* were insensitive, and this could have masked the effect of the fungicide on the more susceptible species in our DGGE analyses. As the detection levels of DGGE analyses seem to depend on the population sizes of dominating species (Prakitchaiwattana *et al.*, 2004), the dominance of the red yeasts could have obscured the effect of captan on sensitive species belonging to the same mobility group as well.

General discussion

The presence of antagonistic interactions between mycobiota occurring on the apple skin has been demonstrated by a number of authors (Janisiewicz, 1987; Lima *et al.*, 1998). Filonow (1998) showed that competition for sugars was one of the mechanisms behind the antagonism between yeasts and grey mold (*Botrytis cinerea*), and he showed that *Sporobolomyces roseus* had a stronger antagonistic capacity than *C. laurentii*. The antagonism may also be the result of the production of killer toxins (Golubev, 1998) by some of the inoculated species. Lima *et al.* (1998) observed that *R. glutinis* was better able to colonize intact apples, while *C. laurentii* grew better in wounded apple tissue. This capacity of *R. glutinis* to colonize healthy apples may account for the observed dominance of this yeast species after inoculation on young apples. It has already been demonstrated that the population sizes of this species on the apple surface may vary considerably, as the percentage of isolates of this species was found to be 8.7 and 18.2% on 6- and 8-week-old apples, respectively (Gildemacher *et al.*, 2004). Fluctuations in population sizes, although to a lesser extent, have also been observed for the other russet-augmenting yeast *S. pararoseus* (cited as *S. roseus* in Gildemacher *et al.*, 2004).

Cryptococcus victoriae (cited as *C. laurentii* in Gildemacher *et al.*, 2004), which appeared to be the dominant yeast inhabitant of the apple surface as it comprised up to one-third of the yeast CFUs (Gildemacher *et al.*, 2004), seems to contribute less to russet formation (Fig. 4b). It remains to be discovered whether this is related to the noted absence of protease activity (Gildemacher *et al.*, 2004) or to other factors.

A general consensus exists that the development of russet is related to the early stages of the development of the apple skin. Eccher (1978) attributes the development of russet in susceptible varieties to the irregular arrangement of epidermal cells, resulting in an irregular cuticula prone to cracking under adverse weather conditions, thus exposing the underlying tissue. The death of epidermal cells through this exposure leads to the development of cork tissue, which appears as russet. Russetting depends partly on genetically

determined physiological characteristics of the susceptible apple cultivar (Eccher, 1978). Where in this process yeasts and dimorphic ascomycetes play a facilitating role remains to be identified. Matteson Heidenreich *et al.* (1997) suggested that enzymes produced by yeasts occurring on the apple fruit surface play a role in russet formation, and Gildemacher *et al.* (2004) found that all yeast species isolated from the russeted apple surface produced cutinolytic, lipolytic and proteolytic enzymes that may be harmful to the apple skin. It seems quite possible that the apple-inhabiting yeasts and dimorphic ascomycetes, such as *R. glutinis*, *S. pararoseus* and *A. pullulans*, actively degrade the apple fruit cuticula during the early stages of fruit development, and, consequently, expose the epidermal cells to the influence of harmful weather conditions that may result in apple russetting.

The fungicides used were able to reduce russet formation, but it remains doubtful whether chemical measures, i.e. spraying with fungicides, can eradicate the problem. In addition, one has to be cautious in applying a chemical solution for the russetting problem, as fungicide-resistant yeast strains exist (Smolka, 1993; Gildemacher *et al.*, 2004). Moreover, yeasts can function as antagonists against various post-harvest diseases (Andrews *et al.*, 1983; Filonow, 1999), and possibly also against scab (*Venturia inaequalis*) (Mercier & Wilson, 1994; Ouimet *et al.*, 1997).

The noted differences between yeast species in russet stimulation, the probable antagonistic interactions between apple-inhabiting yeast species, and the possibility of manipulating the yeast species composition in an orchard suggest that it may be possible to modulate the microbial community structure in order to reduce the level of russetting. Knowledge about the limited period of high susceptibility to russet initiation during the early development of the young apple fruits (Creasy, 1980; Creasy & Swartz, 1981; Gildemacher, 2000) can facilitate the implementation of interventions to reduce the russet hazard.

An interesting avenue of further investigation would be the potential of biological control measures aimed at occupying the very young apple fruit with yeasts that prevent colonization of these fruits by more harmful yeasts that can break down the apple cuticle, as shown by Goffinet *et al.* (2002). It must, however, be stressed that in this research all yeasts increased russet when inoculated on apple fruits, although at different magnitudes. Moreover, yeasts are probably not the only cause of russet, and smooth fruits are not guaranteed in the absence of yeasts on the fruit surface.

When the role of yeasts during the development of russetting, the effect of the population dynamics of yeast species, their ecological interactions and the interactions with weather parameters are better understood, more effective strategies may be formulated to prevent apple fruit russet.

References

- Andrews JH, Berbee FM & Nordheim EV (1983) Microbial antagonism to the imperfect stage of the apple scab pathogen, *Venturia inaequalis*. *Phytopathology* **73**: 228–234.
- Batzer U, Berling R & Helm HU (2000) Experiences with the scab simulation programme Schorfexpert. *Erwerbobstbau* **42**: 1–7.
- Bremer H & Bünemann G (1982) Nebenwirkungen organischer Schorffungizide. II. Ertrag und Fruchtqualität bei 'Golden Delicious'. *Gartenbauwissenschaft* **47**: 204–211.
- Catzefflis J (1979) Response of apple trees to water stress. *Acta Horticult* **89**: 83–87.
- Creasy LL (1980) The correlation of weather parameters with russet of Golden Delicious apples under orchard conditions. *J Am Soc Hortic Sci* **105**: 735–738.
- Creasy LL & Swartz HJ (1981) Agents influencing russet on Golden Delicious apple fruits. *J Am Soc Hortic Sci* **106**: 203–206.
- Eccher T (1978) Russetting of Golden Delicious apples as related to endogenous and exogenous gibberellins. *Acta Horticult* **80**: 381–385.
- EPPO (1989) Crop growth stage keys – Apple and Pear. *Bulletin OEPP/EPPO Bulletin* **19**: 373–384.
- Filonow AB (1998) Role of competition for sugars by yeasts in the biocontrol of grey mold of apple. *Biocontrol Sci Tech* **8**: 243–256.
- Filonow AB (1999) Yeasts reduce the stimulatory effect of acetate esters from apple on the germination of *Botrytis cinerea* conidia. *J Chem Ecol* **25**: 1555–1565.
- Gildemacher PR (2000) Duizend-en-één factoren van invloed op verruwing. *Fruiteelt* **90**: 18–19.
- Gildemacher PR, Heijne B, Houbraken J, Vromans T, Hoekstra ES & Boekhout T (2004) Can phyllosphere yeasts explain the effect of scab fungicides on russetting of Elstar apples? *Eur J Plant Path* **110**: 929–937.
- Goffinet MC, Burr TJ & Heidenreich MC (2002) Anatomy of apple russet caused by *Aureobasidium pullulans*. *New York Fruit Quarterly* **10**: 3–6.
- Golubev WI (1998) Mycocins (killer toxins). *The Yeasts, a Taxonomic Study*, 4th edn (Kurtzman CP & Fell JW, eds), pp. 55–62. Elsevier, Amsterdam.
- Gupta AK, Boekhout T, Theelen B, Summerbell R & Batra R (2004) Identification and typing of *Malassezia* species by amplified fragment length polymorphism (AFLP) and sequence analysis of the internal transcribed spacer (ITS) and large subunit (LSU) regions of ribosomal DNA. *J Clin Microbiol* **42**: 4253–4260.
- Hatch AH (1975) The influence of mineral nutrition and fungicides on russetting of 'Goldspur' apple fruits. *J Am Soc Hortic Sci* **100**: 52–55.
- Hislop EC & Cox TW (1969) Effects of captan on the non-parasitic microflora of apple leaves. *Trans Br Mycol Soc* **52**: 223–235.
- Janisiewicz WJ (1987) Postharvest biological control of blue mold on apples. *Phytopathology* **77**: 481–485.

- Jones KM, Bound SA, Oakford MJ & Wilson D (1994) A strategy for reducing russet in Red Fuji apples while maintaining control of black spot (*Venturia inaequalis*). *Austr J Exp Agric* **34**: 127–130.
- Kowalchuk GA (1998) Fungal community analysis using Denaturing Gradient Gel Electrophoresis (DGGE). *Molecular Microbial Ecology Manual* 3.4.6. Kluwer Academic Publishers, Dordrecht, pp. 1–16.
- Lima G, de Curtis F, Castoria R & de Cicco V (1998) Activity of the yeasts *Cryptococcus laurentii* and *Rhodotorula glutinis* against postharvest rots on different fruits. *Biocontrol Sci Tech* **8**: 257–267.
- Matteson Heidenreich MC, Corral-Garcia MR, Momol EA & Burr TJ (1997) Russet of apple fruit caused by *Aureobasidium pullulans* and *Rhodotorula glutinis*. *Plant Dis* **81**: 337–342.
- Meador DB & Taylor BH (1987) Effect of early season foliar sprays of GA4+7 on russetting and return bloom of Golden Delicious apple. *HortScience* **22**: 412–417.
- Mercier J & Wilson CL (1994) Colonization of apple wounds by naturally occurring microflora and introduced *Candida oleophila* and their effect on infection by *Botrytis cinerea* during storage. *Biol Control* **4**: 138–144.
- Miller RH (1982) Apple fruit cuticles and the occurrence of pores and transcuticular canals. *Ann Bot* **50**: 355–3710.
- O'Donnell K, Cigelnik E, Weber NS & Trappe JM (1997) Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. *Mycologia* **89**: 48–65.
- Ouimet A, Carisse O & Neumann P (1997) Evaluation of fungal isolates for the inhibition of vegetative growth of *Venturia inaequalis*. *Can J Bot* **75**: 626–631.
- Prakitchaiwattana CJ, Fleet GH & Heard GM (2004) Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Res* **4**: 865–877.
- Smith CB, Morrow CT & Greene GM (1987) Corking of 'Delicious' apples (*Malus domestica* Borkh) on four rootstocks as affected by calcium and boron supplied through trickle irrigation. *J Plant Nutr* **10**: 1917–1924.
- Smolka SE (1993) Assessment of pesticide side effects on beneficial microorganisms from the phylloplane. *IOBC WPRS Bull* **16**: 155–160.
- Spotts RA & Cervantes (2002) Involvement of *Aureobasidium pullulans* and *Rhodotorula glutinis* in russet of d'Anjou pear fruit. *Plant Dis* **86**: 625–628.
- Taylor BL (1978) Effects of gibberellin sprays on fruit russet and tree performance of Golden Delicious apple. *J Hort Sci* **53**: 167–169.
- Tromp J, Jonkers H & Wertheim SJ (eds) (1976) *Grondslagen van de Fruitteelt*. Staatsuitgeverij's, Gravenhage.
- Vogl M, Streitberg H & Hanke V (1985) Fruchtberostung bei der Apfelsorte 'Gelber Kostlicher'. *Arch Gartenb* **33**: 371–386.
- Walter TE (1967) Russetting and cracking in apple, a review of world literature. *Ann Rep East Malling Research. Station for 1966*, pp. 83–95.
- Wertheim SJ (1982) Fruit russetting as affected by various gibberellins. *J Hort Sci* **57**: 283–288.