INFLUENCE OF COLD-STORAGE TEMPERATURES ON STRAWBERRY LEAK CAUSED BY *RHIZOPUS* SPP.

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ABSTRACT

Strawberry leak is a postharvest disease of cold-stored strawberry fruit. Between October 2007 and March 2009, the effect of temperature on growth and pathogenicity was tested for 39 isolates of *Rhizopus* and *Mucor* spp., the causal agents of strawberry leak. All isolates were grown on potato dextrose agar at target temperatures of 2–7°C. Most isolates grew at the lower temperatures tested, but temperature fluctuations in the incubators were also detected. A selection of the most cold-tolerant *Rhizopus* spp. isolates was used in a growth experiment on potato dextrose agar, sliced and whole fruit stored at 3°C. Fungal growth was monitored daily by removing samples at intervals and exposing them to 20°C for 2 h, before returning them to 3°C. Little difference was detected in overall growth patterns between numbers of interruptions, indicating that cold-storage only needs to be interrupted once for the fungus to be activated. **Keywords:** cold-storage, strawberry leak, temperature, *Rhizopus* spp., *Mucor* spp.

INTRODUCTION

Strawberry leak, a very common fungal postharvest disease in the New Zealand berryfruit industry, is found in the field on ripe fruit as well as on transported and cold-stored fruit (Timudo-Torrevilla et al. 2005; Walter et al. 2007). The disease is primarily caused by *Rhizopus stolonifer* but *Mucor piriformis* can also be a causal agent (Maas 1998). Affected fruit is characterised by distinctive brown spots of soft and watery rot, which cause the fruit to collapse and 'leak' juice. Infection can spread quickly by mycelia, but only wounded tissue can be infected (Maas 1998). To date, there has been little success with fungicide treatment. Timudo-Torrevilla et al. (2005) reported that although captan was the most frequently used fungicide against strawberry leak, its efficacy was not very high. They also showed that this efficacy was not dependent on the number of spray applications, and some concern was raised that inefficient over-spraying was occurring (Timudo-Torrevilla et al. 2005). Since the disease is also managed by keeping fruit stored at low temperatures, the recent detection of cold-tolerant isolates of the causal agents has prompted a call for a change in integrated disease management to improve cold-storage (Walter et al. 2007).

The aim of this work was to study cold-tolerant isolates and to determine their temperature limits for growth and infection of strawberry fruit. Leak development on strawberry fruit continuously incubated at cold temperatures was determined, and, in addition, experiments were carried out to investigate cold-storage interruptions on fruit inoculated with strawberry leak. This information was used to explore how cold-storage interruptions in cooling facilities and subsequent cold-temperature transport to

commercial outlets can influence fungal growth of leak-causing organisms and therefore the shelf life of strawberry fruit.

MATERIALS AND METHODS

Isolates

Selected isolates ($n=39$) of *Rhizopus* or *Mucor* spp. (henceforward referred to as leak isolates) isolated from strawberry fruit during 2007–2008 were removed from storage (3°C) on Potato Dextrose Agar (PDA, Merck), sub-cultured onto PDA plates and incubated at room temperature for 4 days. Preliminary sequence analyses and morphological assessments (Walter et al. 2007) indicated that six of the isolates belonged to the *Mucor* spp., with the remainder being *Rhizopus* spp.

Growth on agar at 0–15°C

Mycelial plugs (5 mm diameter) from 4-day-old PDA cultures were placed in the centres of a PDA plate, mycelium facing the agar. There were two plates per isolate. Plates were individually sealed with cling-film (to prevent mycelium growing outside of the plates), and incubated in the dark at up to five target temperatures ranging over 0–15°C, with 15°C being a control treatment. Incubators were equipped with Squirrel data loggers (Quick Measure Temperature Loggers, HortPlus), and Squirrel data loggers were also placed inside PDA Petri dishes among inoculated plates to check for incubator temperature fluctuations. This experiment was conducted seven times. In the first experiment, the five target temperatures were 3, 5, 7, 9 and 15°C, although temperature fluctuations were $\pm 3.5^\circ\text{C}$. In the subsequent experiments only two incubators were used, and these had $\pm 1^\circ\text{C}$ fluctuations. Target temperatures in experiments 2–7 were: 3 and 5°C; 3 and 15°C; 0 and 5°C; 2 and 6°C; 3 and 6°C; and 4 and 5.5°C. Growth was assessed by measuring mycelial diameter (mm) in two perpendicular directions after 4 and 7 days in experiments 1–6, and after 3, 4, 5, 6 and 7 days in experiment 7.

Growth on fruit at 3°C

Ten of the 39 original leak isolates that had demonstrated good growth on PDA at 2–4°C (isolates 137, 140, 142, 148, 149, 152, 153, 157, 158 and 161, all of the *Rhizopus*-type) were selected for an experiment on strawberry fruit at the end of the fruit growing season in March 2008. Another two isolates that did not show growth at those temperatures (isolates 165 and 167, also of the *Rhizopus*-type), were also chosen. The 12 isolates were grown on PDA in the dark for 48 h at 15°C to encourage mycelial growth.

Fresh strawberries were picked into sterile trays with individual compartments from a commercial, unsprayed strawberry field in the Christchurch area and pre-cooled in a refrigerator (3°C) for approximately 2 h. Fruit temperatures were monitored with a Testo 106 temperature probe (Eurotech Instrumentation) and when fruit were at 3°C they were injured with a sterile needle and the wound inoculated with a mycelial tuft. Control fruit were injured, but not inoculated. There was an additional control treatment (no injury) for each isolate. There were three inoculated fruit per replicate and there were three replicates for each isolate. The aforementioned trays with fruit were incubated in the dark at $3\pm 1^\circ\text{C}$ for 7 days, after which the number of fruit showing leak symptoms was assessed.

Interrupted cold-storage experiments

Six isolates from the above experiment (140, 142, 148, 149, 153 and 165) were selected for interrupted cold-storage experiments. They were inoculated onto PDA and sliced strawberry fruit (5 mm thick) using 5 mm mycelial plugs from 4-day-old PDA cultures. There were two replicates for each substrate and isolate treatment combination, with treatments indicating different numbers of interruptions. Each day, an additional set of plates was removed resulting in continuous cold incubation and 1–6 interruptions: treatment 1 with six interruptions total, treatment 2 with five interruptions total and so forth; thus treatment 6 was interrupted on the last day only. Each set of plates contained a mixture of substrates and isolates. All substrates were incubated at $4\pm 0.5^\circ\text{C}$ for 7 days, to simulate a 'cold chain' during storage and/or transport. Control treatments for all isolates and both substrates were incubated at 20°C for 7 days. The cold incubation over 7 days was interrupted at daily intervals by exposing the plates to room temperature

(approximately 20°C) for 2 h per day followed by cooling in a refrigerator (4±3°C) for 2 h before returning them to cold incubation at 4°C (±0.5°C). Fungal growth on agar plates and fruit slices (mycelial extension, mm) was measured daily (two perpendicular directions) during the '2 h at 20°C cycle', with plates being evenly spread in a single layer, without touching, on the laboratory bench. As before, incubator temperatures were monitored with a data logger suspended in free air and a data logger lodged on top of a PDA plate in the tray containing treatment 6. The entire experiment was carried out on two separate occasions and assessed daily as described above.

A third experiment using only whole strawberry fruit was set up with 10 strawberry fruit in sterile trays with individual compartments. Fruit was picked and pre-cooled (3 h at 4°C) as above, at the end of the growing season in February 2009. For each of the six isolates described above, eight fruit per tray were inoculated as before with a mycelial tuft and two fruit served as non-inoculated controls. Each set contained six trays (one per isolate), and cold incubation was carried out as before. Each day, an additional set of trays was removed resulting in continuous cold incubation and 1-4 interruptions, with treatment 1 having four interruptions in total, and treatment 4 having just one interruption, on the last day. Control treatments for all isolates were incubated at 20°C for 6 days. As before, incubator temperatures were monitored with a data logger suspended in free air and a data logger lodged on top of a tray in treatment 4. Disease monitoring was conducted as described above during the 2 h interruptions after 1, 4, 5 and 6 days incubation. Rot was assessed each day on a fruit score, where 0 = no symptoms; 1 = small sunken lesion; 2 = large sunken lesion; 3 = sunken lesion with juice leaking; 4 = fruit covered in mycelium. After the last day of incubation and rot assessment, all cold-storage trays were exposed to 20°C for an additional period of 48 h to check for delayed onset of disease symptoms.

Grower survey

In the Auckland region, clean, ripe fruit were collected from 6-8 grower properties after grading and packing into strawberry clamshells (250 g lidded plastic punnets) on 4 November and 10 December 2008. For each grower and each collection date, an additional fruit sample was obtained from a local distribution centre. Each fruit sample consisted of 12 clamshells.

On arrival at the Plant & Food Research laboratory, Mt Albert, Auckland, the fruit was kept at 4°C overnight and then incubated in the clamshells at 3, 4, 5, and 6°C for up to 12 days in large, walk-in, postharvest cold chambers. At each collection time and sampling site, 12 clamshells were collected resulting in triplicate samples at each temperature. A Squirrel data logger (Eltek) was placed in each incubator and fruit temperature was also measured for one fruit/clamshell after 7 days incubation with a Testo 106 temperature probe (Eurotech Instrumentation). Appearance of fruit and presence of postharvest rots were determined after 5, 7, and 9 days incubation using a clamshell scale of 0 = no blemish; 1 = 1 fruit with blemish (water soaked lesion, bruise, discoloration, sunken lesion excluding malformed fruit or not fully red fruit); 2 = 2 or more fruit with blemishes; 3 = fruit clearly damaged; 4 = rots clearly visible with mycelial growth present. For the assessment, fruit were carefully unpacked as needed and strawberries showing mycelial growth were removed and further incubated for 2-4 days at 20°C for rots to be identified.

Statistical analyses

Unless otherwise stated, descriptive analyses are given. Due to target temperatures not always being met accurately and due to the temperature fluctuation in incubators the actual median temperatures as determined by data loggers were used as factors in analysis of variance (ANOVA), using general linear model (GLM) for mycelial growth on agar and fruit slices and whole fruit experiments. For the growth on agar and fruit slice experiments, average mycelial diameter was calculated and ln-transformed. Log_e-back-transformed means are presented.

RESULTS

Growth on agar at 0-15°C

The ability of leak isolates to grow on PDA at cold temperature incubation was highly dependent on isolate ($P<0.001$), with no interactions among isolates, temperatures and experiments ($P>0.05$). Experimental differences ($P<0.001$) in mycelial extension are attributed to the slight differences in temperatures assessed and incubators used in the repeat experiments. All isolates showed mycelial growth at actual median temperatures $>3^{\circ}\text{C}$. A strong linear relationship was observed for actual median temperature and growth (Fig. 1). At the actual median temperatures of 1.1 and -0.5°C (target temperatures 2 and 0°C), four and ten isolates respectively did not grow. Temperatures above 10°C were excluded from the analysis and presentation (Fig. 1) as many isolates reached the margin (or 80 mm diameter) of the Petri dishes during the incubation period. Assessments of daily mycelial extension (Experiment 7 only), showed that diameter increased linearly at an average rate of 10.4 mm/day ($y=10.4x+7.8$; $R^2=0.967$) when data were pooled for all leak isolates and the actual median temperatures of 4 and 5.5°C .

FIGURE 1: Mycelial growth of leak isolates on potato dextrose agar after 7 days incubation at a range of temperatures. Values are the log_e-back-transformed means from 35 isolates in seven consecutive experiments. Bars show the 95% confidence intervals. Minimum diameter is 5 mm for the mycelial plug used in inoculation.

Growth on fruit at 3°C

All isolates caused leak rot when incubated at room temperature. At 3°C , no leak rot symptoms could be observed for the two control treatments (injury only and nil control) and for isolates 137, 165 and 167. For the remaining isolates, leak symptoms after 7 days incubation at 3°C were caused on one fruit each from the total of nine fruit (by isolates 140 and 157), on three fruit (by isolates 142, 152, 158 and 161), on four fruit (by isolates 148 and 149) and on six fruit (by isolate 153). This equates to approximately 31% of fruit becoming infected when inoculated with cold-tolerant leak isolates (i.e. growing on PDA at $2-4^{\circ}\text{C}$) and held at 3°C for 7 days.

Interrupted cold-storage experiments

The differences in mycelial growth on agar and fruit slices under continuous incubation at 20°C and at an actual median temperature of 4°C , with 1-6 2-hourly interruptions of exposure to room temperatures, are illustrated in Figure 2. No difference between isolates was detected. Potato dextrose agar supported faster growth ($P<0.001$) than strawberry fruit slices. All leak isolates grew on both substrates at 4°C incubation with 1-6 interruptions, which is shown in more detail in Figure 3. In reading the graph, it should be noted that, depending on treatment, mycelial diameter was measured for the first interruption after

1, 2, 3, 4, 5 or 6 days of continuous incubation at 4°C. Based on observation, growth increased during continuous cold-incubation (also illustrated in Figure 1). Nonetheless, each interruption increased mycelial extension (Fig. 3).

FIGURE 2: Mycelial diameter (mm) of leak isolates incubated on potato dextrose agar and on strawberry fruit slices for 7 days. Values are the log_e-back-transformed means from two experiments and include data from six isolates. Incubation at 4°C was interrupted 1-6 times for 2 h exposures at room temperature. Under continuous incubation at 20°C isolates reached the margin of the Petri dish (80 mm) and fruit slice (32 mm) after 3-4 days of incubation. Minimum diameter is 5 mm for the mycelial plug used in inoculation.

FIGURE 3: Mycelial diameter (mm) of leak isolates on potato dextrose agar and strawberry fruit slices measured after the 2 h interruption at 20°C. Values are the log_e back-transformed means from two experiments and six isolates. Minimum diameter is 5 mm for the mycelial plug used in inoculation. Pooled standard error SE=3.09 and 1.74 for agar and fruit slice data, respectively.

Experiments on whole fruit confirmed that increasing the number of interruptions in cold-storage also increased ($P<0.001$) leak development. Again, there was no difference between the isolates. At 6 days the average leak score for 20°C was 3.8 (pooled standard error SE=0.131) relative to the leak score for 4°C, which was 0.4-1.7 depending on the number of interruptions (pooled standard error SE=0.12). All fruit, except non-inoculated controls, showed leak symptoms (average score of 3.6, SE=0.12) when incubated at 20°C for 48 h at the conclusion of the interrupted 4°C incubation period.

Grower survey

As determined by the data loggers, the actual median incubator temperatures were 2.9, 4, 4.8 and 5.9°C with a minimum and maximum temperature recorded of 2.8-3.05, 3.95-4.1, 4.75-5.1 and 5.7-6.0, respectively. This resulted in average fruit temperatures of 3.5, 4.5, 5.5 and 6.4°C (with a pooled standard error SE=0.43) in the 3, 4, 5 and 6°C incubators, respectively.

From a total of 2214 fruit incubated at the four temperatures without any cold-storage disruption, only one fruit from the 5.5°C incubator showed *Botrytis cinerea* symptoms after 5 days incubation. After 7 days, *B. cinerea* developed on an additional two fruit from the 5.5 and 6.4°C incubators, and one fruit from the 6.4°C incubator was colonised by *Rhizopus* spp. After 9 days continuous cool storage, rots started to develop, with 161 (7.3%) *B. cinerea* fruit infection, 85 (3.8%) leak infections and four other fruit rots (two *Cladosporium* spp. and two *Phomopsis* spp.). After 12 days of incubation a total of 423 (19.1%) *B. cinerea*, 141 (6.4%) leak and 132 (6%) other fruit rots had developed. The other rots consisted of *Phomopsis* spp. (64%), *Colletotrichum acutatum* (12%), *Penicillium* spp. (9%), *Cladosporium* spp. (7%), bacterial soft rot (3%) and a mixture of other non-identified organisms (5%). The type and level of postharvest rots observed after 9 and 12 days were not dependent on incubation temperatures ($P>0.1$).

Total postharvest fruit rots were higher ($P<0.001$) in the December (10.3%) than the November (5%) samples. There were also differences in rot levels between the sampling sites ($P<0.001$), with, for example, *B. cinerea* levels ranging from 1.4-18.4% and leak infections from 0.4-5.1%. Cultivar ('Camarosa', 'Ventana' and 'Gaviota') and harvest weather (wet or fine) affected total postharvest rots ($P<0.001$). However, *Botrytis* rots were only affected by cultivar ($P<0.001$) with 'Gaviota' (18.4%) showing more rots than 'Camarosa' (4.1%) and 'Ventana' (3.7%). In contrast, leak rots were only affected by harvest weather ($P<0.001$) with less disease found when fruit was picked during dry (0.7%) than wet conditions (3.8%).

The gradual increase of postharvest storage rots over storage time was also noted in the clamshell scale analysis ($P<0.001$). From the 192 clamshells incubated, only one showed visible mycelial growth (clamshell scale of 4) after 5 days, increasing to two (1%), 71 (37%) and 145 clamshells (76%) after 7, 9 and 12 days incubation, respectively. The median scale for clamshells after 5, 7, 9 and 12 days incubation was 0, 1, 2 and 4, respectively.

DISCUSSION

It was evident that several leak-causing isolates showed cold tolerance when grown on PDA at 2-7°C. Those same isolates also infected strawberry fruit when incubated at similar temperatures. This series of experiments has conclusively demonstrated that mycelium of some *Rhizopus* spp. isolates can grow and infect wounded strawberry fruit at about 3°C. Until now, evidence that *Rhizopus* spp. can grow at low temperatures and produce viable mycelium to infect produce has only been documented for slightly higher temperatures. For instance, minimum temperatures of 5 and 4.4°C for *R. stolonifer* to grow and infect squash (Kwon et al. 2000) and stonefruit (Ogawa et al. 1995), respectively, have been reported. According to Maas (1998) the minimum temperature for spore germination and growth of *R. stolonifer* is about 6°C but *M. piriformis* has the ability to grow and infect strawberry fruit at a minimum of 0°C. Sommer et al. (2002) stated that *Mucor* spp. can rot stonefruit at an even lower minimum of -1°C.

From the present interrupted cold-storage experiments it has been demonstrated that the greater the number of interruptions or hours exposure to 20°C, the greater the growth rate of the leak isolates ($P < 0.001$) on PDA and fruit slices. The implications are that more cold-storage interruptions mean a greater fungal growth explosion, but any interruption in cold temperatures will activate fungal growth regardless of timing.

In contrast to the interrupted cold-storage experiment, fruit from the grower survey were incubated under continuous cold-storage at temperatures of 3.5-6.4°C. Leak rots started to emerge after 7 days, with 6.4% of fruit infected with leak after 12 days incubation. This confirms the laboratory research, where fruit were wounded and artificially inoculated with *Rhizopus* spp., and demonstrates that natural leak infections also develop and continue to grow at cold-temperature incubation. The maximum strawberry shelf life observed was 5-7 days with 1% of strawberry clamshells featuring postharvest rots after 7 days continuous cold storage.

The work presented here concurs with preliminary evidence that cold-tolerant strains of *Rhizopus* isolates exist (Walter et al. 2007) and that temperature management is an important factor in the development of postharvest leak (Sommer et al. 2002). The cold-tolerant *Rhizopus* spp. used here needed only one cold-storage interruption to be activated.

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