Etiology and Management of Grape Sour Rot

Cristina Huber, BSc Hons.

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Department of Biological Sciences, Brock University
St. Catharines, Ontario

DEDICATION

This dissertation is dedicated to my first daughter, Reynard Grietje Vrieswyk.

ABSTRACT

Sour rot is characterized by increased volatile acidity (VA) in ripe grapes. VA is associated with spoilage organisms and wineries may reject grape crops based on their concentration of acetic acid. Our research associated *Hanseniaspora uvarum*, *Gluconobacter oxydans*, and to a lesser extent, *Gluconobacter cerinus* and *Acetobacter malorum* with sour rotted grapes in the Niagara Peninsula, designated viticultural area, Ontario, Canada, and the pathogenicity of these organisms was confirmed by laboratory assays. Only *G. oxydans* was shown to penetrate around the site of pedicel attachment to the grape. The yeasts required further wounding. *Candida zemplinina* was also associated with the sour rot microbial community. This species showed variable pathogenicity by strain and most strains were not highly pathogenic. *C. zemplinina* gained dominance in the microbial population of grapes only after sour rot symptoms were observed, indicating a succession which was studied in laboratory assays. There was a correlation between temperature, moisture, and berry ripeness and the development of sour rot when conditions were monitored in a *Vitis vinifera* cv. Riesling vineyard over four years, and this was confirmed in laboratory assays.

Disease management options are limited since sour rot is caused by a complex of yeasts and bacteria, with symptoms developing just as grapes approach maturity. Post-veraison treatments for sour rot were investigated. Wineries routinely add potassium metabisulphite (KMS) to the surface of fruit in bins and to grape juice to kill spoilage organisms. Replicated field trials were conducted in *V. vinifera* cv. Riesling in 2010 and 2011 to determine the efficacy of KMS at different concentrations and pre-harvest timings as a fruiting-zone spray. Potassium bicarbonate (Milstop) was also evaluated for its efficacy against sour rot. Plots were rated for incidence and severity of sour rot and VA (g acetic acid/L juice). KMS treatments at concentrations above 5 kg/1000L and Milstop sprayed at the label concentration of 5.6 kg/1000L were able to reduce the severity of sour rot compared to untreated control plots which had a

severity above 50% (2011). KMS was able to reduce VA to below the winery rejection threshold of 0.24 g acetic acid/L when sour rot severity reached 12% in untreated plots (2010). When tested in the laboratory in disk diffusion assays conducted on yeast peptone dextrose agar, KMS at a concentration of 10 g/L had the greatest efficacy against *G. oxydans* and *H. uvarum*. Grape incubation assays showed the potential of KMS acidified with tartaric acid to reduce sour rot symptoms. Acidification did not show as much potential in field trials, calling for further research.

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CHAPTER ONE: LITERATURE REVIEW

Sour rot is a late-season bunch rot occurring after the period of veraison in grapes. It was first reported in the Niagara Peninsula, designated viticultural area, Ontario, Canada, in a 2005 *Vitis vinifera* cv. Riesling vineyard, and has since been identified as an economic issue (Ker, 2006). Sour rot has been declared for more than 1.5 million dollars in regional crop loss, when weather conditions promote infection over a growing season, such as rain storms and hail in 2008 (Agrocorp, 2008). Thin-skinned, tightly packed cultivars, such as Riesling, can be particularly susceptible to sour rot (Zoecklein et al., 2000). In the following literature review and research, the organisms responsible for sour rot, their presentation in the grape, community ecology, and responses to environmental conditions will be elucidated. By applying this knowledge, the research aims to develop treatments to reduce sour rot in the vineyard.

THE MICROBIAL ECOLOGY OF WINE GRAPES AND SOUR ROT

1.1 The microbial ecology of wine grapes, *Vitis vinifera*

The study of yeasts and microbes on grape berry surfaces is important to the wine industry as they can be a principal source of spoilage contaminants (Loureiro and Malfeito-Ferreira, 2003). One of the forefathers of microbiology, Louis Pasteur was the first to report wine microbes present on grape surfaces at the turn of the 20^{th} century (Renouf et al., 2005). The yeast cells present on the grape originate from all parts of the vine, the soil, other plants, and animals. Insects are the principal vectors of yeasts (Loureiro and Malfeito-Ferreira, 2003). The yeasts colonize grape surfaces according to ripeness, with more as the grapes mature, up to 10^3-10^5 cells/g on sound grapes. The yeasts are accompanied by moulds and bacteria, including acetic acid bacteria and lactic acid bacteria (Loureiro and Malfeito-Ferreira, 2003). The composition of the mixed population is affected by rainfall, temperature, grape variety, and agrochemicals, and the population tends to localize around sites where juice may escape (Loureiro and Malfeito-Ferreira, 2003). The microbes require a source of carbohydrates and other nutrients, and

adherence is affected by the waxy outer layer of the grape cuticle, while the skin keeps most of the nutrient-rich juice inside (Loureiro and Malfeito-Ferreira, 2003).

The grape population tends to include oxidative yeasts of no oenological interest such as aerobic basidiomycetes in the genera *Sporobolomyces*, *Cryptococcus*, *Rhodotorula*, and the aerobic ascomycete, *Aureobasidium* (Barata et al., 2012a). It can also include anaerobic ascomycetes associated with wine quality, apiculate yeasts in the genus *Hanseniaspora*, and weakly fermentative yeasts in the genera *Candida*, *Pichia*, *Metschnikowia* and *Kluyveromyces*. On the other hand, the strongly fermentative yeast, *Saccharomyces cerevisiae*, is usually absent from grape surfaces (Fleet, 2003; Loureiro and Malfeito-Ferreira, 2003). The ascomycetes are favoured by microfissures and a softening grape cuticle, releasing nutrients from the grape pulp (Barata et al., 2012a). Damage to the grape berry is the main factor influencing the recovery of microbes from grape surfaces, favouring ascomycetes with wine spoilage potential, as well as acetic acid bacteria in the genera *Gluconobacter* and *Acetobacter* (Barata et al., 2012a).

Grape berries can be damaged by a number of factors. These include mechanical damage which can be caused by weather events such as hail, cultural practices, insects such as the vinegar fly (*Drosophila melanogaster* Meigen), bees, wasps, moths, and birds, as well as phytopathogenic moulds such as *Erisiphe necator*, and *Botrytis cinerea* (Loureiro and Malfeito-Ferreira, 2003). Damage can also be caused by an increase in berry volume, caused by wet weather, and particularly affecting varieties with thin skin and tight clusters (Loureiro and Malfeito-Ferreira, 2003).

Microbial populations are not consistent year to year or from vineyard to vineyard. *Hanseniaspora uvarum* (Niehaus) Shehata, Mrak & Phaff ex M.T. Sm is known to be one of the most prevalent grape-associated microorganisms (Gravot et al., 2001). The name *Kloeckera apiculata* was formally applied to the imperfect form of this apiculate yeast, which does not sporulate (Villa and Longo, 1996). The perfect form, *H. uvarum*, which sporulates sexually, has been identified from grape juice in the Niagara Peninsula, Canada (Holloway et al., 1990).

Kloeckera was the most dominant yeast over three years in three South African vineyards. When *K. apiculata* had reduced numbers, *Candida stellata* (Kroemer & Krumbholz) S.A. Mey. & Yarrow tended to dominate (Jolly et al., 2003). *C. stellata* was updated taxonomically, and grape-associated isolates were identified as *Candida zemplinina* Sipiczki (Csoma and Sipiczki, 2008), now synonymized with *Starmerella bacillaris* (Duarte et al., 2012). Similar results were found in Spain in six wineries over two years. *K. apiculata*, *Candida pulcherrima*, and *Pichia membranifaciens* were amongst the most commonly recovered species. *C. stellata* was only recovered in the wettest vintage (Longo et al., 1991).

In Argentina, *K. apiculata* constituted up to 50% of the population in two out of three grape samples, and *Metschnikowia pulcherrima* dominated the third sample along with *C. stellata*. Due to the waxy cuticle of the grape, it was found that disruptive pre-isolation treatments increased microbial yields. The microbial population increased with grape maturity, and rainfall was found to increase the population, leading to berry swelling and increased sugar on the grape surface (Combina et al., 2005). In China, three grape varieties were studied in four regions. *H. uvarum* and members of the *Cryptococcus* genus were most abundant in samples. *Pichia*, *Candida*, *Zygosaccharomyces*, *Metschnikowia*, and others were also present. All species were recovered with differences in population size. *H. uvarum* numbers varied more by region than by grape cultivar, fewer being found in regions with cool and dry conditions (Li et al., 2010). One study did indicate some population changes by grape cultivar. *H. uvarum*, *Aureobasidium pullulans*, *Rhodotorula*, *Cryptococcus*, *Metschnikowia*, *Pichia*, and three other genera were reported in a study performed in Slovenia (Raspor et al., 2006). *H. uvarum, A. pullulans*, or *Rhodotorula glutinis* tended to dominate samples, with higher recovery than their competitors. It was found that *A. pullulans* was isolated more frequently from the red cultivars than from the white (Raspor et al., 2006).

In Bordeaux, France, Renouf et al. (2005) found that Merlot berries harboured a greater yeast population than Cabernet Sauvignon; however, variation in the yeast population was

principally attributable to berry development stage. *A. pullulans* was recovered mainly at berry set and was not found at harvest, when fermentative yeasts tended to dominate and the acetic acid bacterium, *Gluconobacter oxydans* Henneberg could be found. The population at harvest was dominated by *Pichia* and *Candida*, and *H. uvarum* was recovered only after veraison (Renouf et al., 2005). Harvest may host the greatest number of microbes due to the elevated sugar concentration, the greatest grape surface area and the least frequent application of agrochemicals (Renouf et al., 2005).

As well, population differences were noted in spatial relationship to the berry cluster. The region closest to the peduncle hosted ten to one hundred times more yeast cells than the lower and central bunch regions. Yeast numbers were highest around the berry pedicel and in gummy secretions there, seen though scanning electron microscopy (Rosini et al., 1982). Apiculate yeasts dominated, representing the largest numbers in the samples. The population of *K. apiculata* increased from three weeks pre-harvest until harvest. No yeasts were found before three weeks pre-harvest. Insects, particularly *Drosophila melanogaster*, were regarded as primarily responsible for dissemination (Rosini et al., 1982). In a recent study, more bacteria were recovered in late summer, while yeasts dominated counts in the autumn before harvest in British Columbia vineyards (Sholberg et al., 2006).

Agrochemicals may also affect populations of epiphytic organisms. Treatments with fungicides such as myclobutanil (Nova 40W), sulphur (Kumulus), iprodione (Rovral 50W), captan (Maestro 75DF), and mancozeb (Dithane) reduced yeast numbers compared to untreated grapes (Sholberg et al., 2006). When sulphur and copper sulfate were applied four times, the population of *Aureobasidium* and *Cryptococcus* increased while fermenting yeasts, especially *H. uvarum*, were reduced in number (Comitini and Ciani, 2008).

The effect of fungal rot on microbial populations has been of interest in wine spoilage. The diversity of yeasts, especially fermentative and spoilage species, increased with *B. cinerea* infection. Although *H. uvarum* was dominant in all samples, healthy berries included *A.*

pullulans, which was not found on *B. cinerea*-infected grapes (Nisiotou and Nychas, 2007). The same research group expected to find increased populations of acetic acid bacteria in *B. cinerea*-infected grapes, but concluded the dominant bacterium, *Klebsiella oxytoca*, may have masked more fastidious acetic acid bacteria populations, out-competing them in the artificial growth media. The acetic acid bacteria did not grow in large enough quantities to be counted from diluted cultures (Nisiotou et al., 2011). Acetic acid bacteria can shrink in size and enter a metabolically inactive state when under stress (Du Toit and Pretorius, 2002). *S. cerevisiae, H. uvarum, Pichia kluyveri, M. pulcherrima*, and *C. zemplinina* were recovered from wines made from *B. cinerea*-infected grapes (Mills et al., 2002). Direct counts using molecular techniques such as the reverse-transcriptase polymerase chain reaction (RT-PCR) of 26S rRNA yielded higher counts, for example a viable but not culturable (VBNC) population of *Candida* (Mills et al., 2002).

In studies looking into the distribution of acetic acid bacteria, *Gluconobacter* tended to thrive in fresh must and high-sugar environments, while *Acetobacter* tended to prefer ethanol as a carbon source, surviving during the course of fermentation (Du Toit and Lambrechts, 2002). As a result, spoiled grapes often contained higher populations of *Acetobacter*, while healthy grapes carried more *Gluconobacter* (Prieto et al., 2007). In a Chilean survey, *Acetobacter cerevisiae* Cleenwerck, Vandemeulebroecke, Janssens & Swings, which is genetically and phenotypically very similar to *Acetobacter malorum*, was found in the north of the country and *G. oxydans* was recovered in the south. Grape cultivar was found not to be a significant factor influencing bacterial populations (Prieto et al., 2007). In Spain, a survey of acetic acid bacteria found that rainfall one day before harvest reduced acetic acid bacterial populations, perhaps due to a washing effect (Gonzalez et al., 2005).

In Ontario, microbial sampling was performed using cultural methods from Riesling must. *C. zemplinina*, *H. uvarum*, *A. pullulans*, and *P. kluyveri* dominated samples, similar to results found in other wine growing regions around the world (Holloway et al., 1990). Sampling

was conducted in icewine musts of Ontario. *A. pullulans* dominated these counts. *Cryptococcus* and *Rhodotorula* were second most dominant, and *H. uvarum* comprised less than 6% of the population after the grapes were frozen. Bacteria, including *Pseudomonas* and members of Enterobacteriaceae, were less than 3% of the population (Subden et al., 2003). Bacteria known to associate epiphytically with wine grapes include species in the family Enterobacteriaceae, and species of *Bacillus*, *Serratia*, *Staphylococcus*, and *Pseudomonas* (Barata et al., 2012a).

1.2 Factors that lead to sour rot

Sour rot develops as grapes accumulate sugar in the post-veraison period. Rot begins at the site where the berry attaches to the pedicel or at an injury site (Gravot et al., 2001) and can become extensive in a susceptible vineyard as the grapes reach maturity, when sugar becomes available to the microbial population (Gravot et al., 2001; Marchetti et al., 1984). Under water stress, berries can decrease in size, and when water becomes available, the increase in size can lead to microfissures that serve as potential entrance points for the sour rot microbes (Barata et al., 2012a; Gravot et al., 2001). This may be why varieties with thin skins and tight clusters are particularly susceptible to sour rot (Zoecklein et al., 2000). Abiotic factors such as rain and hail (Barata et al., 2012a) as well as mechanical injury during viticultural practice can introduce wounds to the grape, providing entry points for the sour rot causing organisms.

Other diseases may be implicated in causing grape injury, for example grey rot (Nisiotou and Nychas, 2007) and powdery mildew (Gadoury et al., 2007). Powdery mildew can cause diffuse infections even as ontogenic resistance develops in the grapes when lenticels harden, about three weeks post-bloom. These infections are not easily detected by the eye, and can cause weak points for the entry of spoilage microbes (Gadoury et al., 2007). Yellow jackets feed on infected berries (Gadoury et al., 2007), and grape berry moths also causes injury to berries (Bisiach et al., 1986).

Drosophila melanogaster is always associated with sour rot (Gravot et al., 2001; Guerzoni and Marchetti, 1987; Marchetti et al., 1984) and is an important vector of the disease (Bisiach et

al., 1986; Duncan et al., 1995; Oliva et al., 1999). Control grapes were exposed to *D. melanogaster* flies, which were captured under field conditions, then incubated under *in vitro* conditions in a jar. The fly introduced sour rot to these grapes (Duncan et al., 1995). When sour rotten grapes were netted in the field, all clusters invariably showed the presence of *D. melanogaster* eggs and development of larvae. When healthy grapes were wounded in the field with a sterile scalpel and netted with pores small enough to exclude *D. melanogaster*, these artificial wounds were able to heal without the development of sour rot, proving that the wounds had to be contaminated. *D. melanogaster* was concluded to vector the microbes involved with sour rot (Barata et al., 2012c). *D. melanogaster* can also inhabit wineries, contaminating musts with yeasts and bacteria, which may reduce wine quality outside of the influence of sour rot in the fields (Loureiro and Malfeito-Ferreira, 2003).

1.3 Symptoms of sour rot

Sour rot is characterized by a discolouration of berry flesh, from pink to brown in white grapes and purple to red in black grapes (Gravot et al., 2001). This discolouration usually begins at an injury site or where the berry attaches to the pedicel (Bisiach et al., 1986). The berry disaggregates, while the skin becomes thin and the cluster disintegrates. The berry detaches from the pedicel and juice containing the pathogens oozes out (Bisiach et al., 1986; Guerzoni and Marchetti, 1987; Loureiro and Malfeito-Ferreira, 2003; Zoecklein et al., 2000) (Figure 1.1). These symptoms are associated with alterations to grape pulp, caused by the fermentative activity of microbes but not the specific enzymatic breakdown of the plant's tissues (Vercesi et al., 1986). For example tissue disaggregation can be attributed to the pressure caused by the production of CO_2 as microbes infesting the grapes convert sugar into alcohol (Guerzoni and Marchetti, 1987). Grey rot caused by *B. cinerea* produces slip skins, where the skin easily pulls away from the flesh. In contrast, sour rot produces extremely fragile skins filled with juice rather than fleshy tissue (Zoecklein et al., 2000).

The fermentative breakdown of the grapes produces several undesirable compounds from a winemaking perspective. In the uncontrolled conditions of the rotten grape, acetic acid and ethyl acetate accumulate so much that they can be smelled in the vineyard. As well, the organisms associated with sour rot produce ethanol, acetaldehyde, galacturonic acid, and glucuronic acid (Barata et al., 2011a; Zoecklein et al., 2000). Berry weight and fruit volume decrease due to dehydration as the juices leak (Zoecklein et al., 2000), and also due to feeding by secondary invaders such as yellow jackets (Gadoury et al., 2007). This is accompanied by an increase in sugar content in musts and wines made from sour rotted grapes (Barata et al., 2011b), which increases acetic acid production by microbes (Marchetti et al., 1984).

Figure 1.1 Symptoms of sour rot pictured in *Vitis vinifera* cv. Riesling. Grapes were harvested from the Niagara Peninsula, designated viticultural area, Ontario, Canada, in October 2009.

1.4 Microbial ecology of sour rotted grapes

Where there may be 10^3-10^6 microbial cells/g in healthy berries, sour rotted grapes can hold up to 10^8 microbial cells/g (Barata et al., 2008b). Berry damage is associated with a sudden increase in the yeast population (Barata et al., 2008b), just before harvest (Duncan et al., 1995). The recovery of organisms associated with sour rot can vary over different seasons, and

regionally (Zoecklein et al., 2000). As well, there are eco-pathological differences in strains from the same species (Vercesi et al., 1986), especially in the formation of secondary metabolites, which makes sour rot a heterogeneous disease.

Yeasts recovered from sour rotted grapes include the apiculate yeast *K. apiculata / H. uvarum*, and other ascomycete yeasts such as *Candida*, *Metschnikowia*, and *Pichia* (Barata et al., 2012b; Gadoury et al., 2007; Gravot et al., 2001; Guerzoni and Marchetti, 1987; Zoecklein et al., 2000). Occasionally, spoilage species such as *Brettanomyces* are recovered (Gadoury et al., 2007). As well, yeasts which are more commonly regarded as grape epiphytes, and not able to survive in must, are occasionally recovered. This includes *A. pullulans* and *Rhodotorula* (Gadoury et al., 2007).

Acetic acid bacteria, including *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter*, are most often recovered from sour rotted grapes (Barata et al., 2012b; Gadoury et al., 2007; Gravot et al., 2001; Zoecklein et al., 2000).

In vitro inoculations with the yeasts *K. apiculata / H. uvarum* and *C. zemplinina* (Gravot et al., 2001; Guerzoni and Marchetti, 1987; Marchetti et al., 1984), as well as *Metschnikowia* and *Pichia* (Guerzoni and Marchetti, 1987), produced active symptoms of sour rot. Yeasts and bacteria have not been shown to penetrate grape berry skin but have required direct contact with the pulp through wounding to infect the grape (Bisiach et al., 1986).

Acetic acid bacteria were found mainly responsible for sour rot symptoms (Barata et al., 2012a). *Gluconobacter* isolates caused sour rot symptoms when inoculated onto injured berries (Gravot et al., 2001). *Gluconobacter* tended to dominate the microbial population in earlier stages of rot and even colonized sound berries, while *Acetobacter* dominated in later stages of sour rot (Barata et al., 2012a; Du Toit and Pretorius, 2002), preferring ethanol to sugar as a substrate (Bartowsky and Henschke, 2008). Although yeasts such as *H. uvarum*, *C. zemplinina* and *M. pulcherrima* were able to produce symptoms of sour rot in grape pulp, these symptoms were considered minor in the absence of acetic acid bacteria (Barata et al., 2012a).

D. melanogaster flies have been recovered from rotting fruit carrying identical yeasts on their bodies as compared to their substrate (Barata et al., 2012c). *H. uvarum*, *C. zemplinina*, *Gluconobacter oxydans*, and *Acetobacter malorum* were recovered from the bodies of laboratory-reared *D. melanogaster* (Barata et al., 2012c). *A. pullulans* and fermentative yeasts grew from the bodies of surface-sterilized *D. melanogaster* associated with sour rot (Duncan et al., 1995). *D. melanogaster* larvae feed on yeast, so it is of competitive advantage for adults to carry palatable species to the substrates used for breeding, while excluding yeasts vectored by competitive animals such as other flies, beetles, mites, and nematodes (Stamps et al., 2012). *Candida* and *Pichia* were found to be most palatable to larvae, which fed on these species until satiation within an hour, measured by a lack of locomotion (Stamps et al., 2012). On a banana host, higher yeast densities were observed when the fruit was exposed to *D. melanogaster* (Stamps et al., 2012). There were more isolates of *Candida* in the presence of flies and larvae, while the presence of *D. melanogaster* reduced the number of basidiomycetes (Stamps et al., 2012). Also, acetic acid bacteria were found to be a component of the natural gut microflora of *D. melanogaster* (Roh et al., 2008).

1.5 Properties of sour rot

1.5.1 Virulence factors

The yeasts associated with sour rot have not been often thought of as phytopathogens (Guerzoni and Marchetti, 1987), with biochemistries evolved specifically to attack the host. Rather, these yeasts are members of the typical wine grape consortium, microbes able to survive in the sugar-rich grape environment under fermentative conditions (Barata and Loureiro, 2012a). For example, *H. uvarum* is the most common grape-associated microbe even in the absence of sour rot symptoms (Barata et al., 2012a). The yeasts and bacteria associated with sour rot cannot penetrate intact skin and must come in contact with the pulp through wounds (Bisiach et al., 1986). Lipolytic, pectinolytic, and proteolytic enzymes help sour rot-associated yeasts to colonize the internal regions of grapes and may be considered virulence factors (Marchetti et al.,

1984; Villa and Longo, 1996). Once yeasts proliferate in contact with the sugary pulp, these virulence factors may expand wound sites.

H. uvarum contains polygalacturonase, pectin esterase, and pectin lyase enzymes, and can clear a zone on pectin-containing media (Masoud and Jespersen, 2006), facilitating its ability to break down grape tissues. *Candida* and *Pichia* have been known to produce polyglacturonase and pectin methylesterase (Carrascosa et al., 2011). *H. uvarum* contains proteases, releasing nutrients for other grape inhabitants (Capece et al., 2005; Carrascosa et al., 2011). These can reduce protein levels in grape juice by one third after seven days of growth (Dizy and Bisson, 2000). In a study using several wild yeast strains, two of 12 strains of *H. uvarum* produced high levels of pectinase, and three of 12 produced high levels of protease. Of 13 strains of *C. zemplinina*, two produced high levels of pectinase and five produced high levels of protease (Strauss et al., 2001).

A. pullulans produces pullulan, a sticky polymer that may facilitate microbial adherence to the grape berries, and that can create an oxygen-impermeable film, encouraging fermenting species. It also produces two pectinolytic enzymes (Parini et al., 1988; Renouf et al., 2005), consistent with the virulence factors listed.

Acetic acid bacteria are not noted for their production of proteolytic, lipolytic, or pectinolytic enzymes. Their growth is stimulated in the presence of yeast extracts and the acetic acid they produce inhibits yeast proliferation (Drysdale and Fleet, 1988). *Gluconobacter* prefers glucose as a metabolic substrate but not all strains of *Acetobacter* can utilize glucose. These *Acetobacter* strains use ethanol as a substrate (Drysdale and Fleet, 1988; Du Toit and Pretorius, 2002), which may be produced by other microbes in the sour rot complex. The majority of acetic acid bacteria are motile by flagella (Carrascosa et al., 2011), perhaps facilitating entrance into the grape. By strain, some produce a film that makes them resistant to chemical treatments and environmental stressors (Du Toit and Pretorius, 2002).

1.5.2 Fermentative properties

The organisms mainly associated with sour rot symptoms, *H. uvarum*, *C. zemplinina*, *Gluconobacter*, and *Acetobacter*, are all able to survive and grow in must as a part of the wine microbial consortium (Barata et al., 2012a), while normally producing oxidation faults in the wine, in the form of acetic acid, ethyl acetate, and acetaldehyde (Clemente-Jimenez et al., 2004; Du Toit and Pretorius, 2002; Garde-Cerdan and Ancin-Azpilicueta, 2006). This is why the oenological consequences of sour rot are considered much more severe than *B. cinerea* grey rot (Bisiach et al., 1986). The major chemical markers of sour rot are an elevated acetic acid content, from 0.3-12.4 g/L in juice from sour rotted berries, accompanied by an increase in ethyl acetate (Guerzoni and Marchetti, 1987; Marchetti et al., 1984). In Ontario, grape juice may be rejected at wineries if acetic acid levels exceed 0.24 g/L (Grape Growers of Ontario, 2010). This threshold is well below the legal limit in table wines, which is 1.3 g/L according to the Vintner's Quality Alliance Ontario regulations (Cliff and Pickering, 2006). However, even low levels of acetic acid at harvest indicate the presence of spoilage organisms which may multiply during fermentation (Barata et al., 2008a).

In wild yeast-inoculated fermentations, *S. cerevisiae* and *C. zemplinina* produced from 0.40-0.49 g/L and 0.80-1.0 g/L acetic acid, respectively (Andorra et al., 2010; Soden et al., 2000), while *H. uvarum* produced 37.50 g/L, not completing the fermentation, producing only 4% ethanol (Andorra et al., 2010). In another experiment, wild fermentations containing these yeasts took longer to complete compared to fermentations using just *S. cerevisiae*, and resulted in higher levels of acetic acid up to 0.405 g/L and acetaldehyde up to 15.5 µg/L (Garde-Cerdan and Ancin-Azpilicueta, 2006). *H. uvarum* was found to be more active in fermentations at temperatures below 20°C, surviving longer and producing more acetic acid, ethyl acetate, and acetaldehyde at 10°C compared to fermentations at warmer temperatures (Erten, 2002). *H. uvarum* was shown to vary by strain, with some producing up to 5 g/L acetic acid, while others produced less than 1 g/L (Romano et al., 2003).

The sensory threshold for ethyl acetate is 60 mg/L in white musts and 115 mg/L in red musts (Corison et al., 1978). In isolated fermentations, *C. zemplinina* produced 74.89 mg/L ethyl acetate and fermented to dryness, while *H. uvarum* produced 69.65 mg/L ethyl acetate but did not complete the fermentation (Clemente-Jimenez et al., 2004). *H. uvarum* strains can produce up to 250 mg/L ethyl acetate (Moreira et al., 2008). *Pichia anomala* and *M. pulcherrima* can also produce increased levels of ethyl acetate and acetic acid in the early stages of fermentation compared to *S. cerevisiae* (Loureiro and Malfeito-Ferreira, 2003; Rojas et al., 2003). As the yeasts associated with sour rot produce acetic acid, ethyl acetate, and acetaldehyde (Barata et al., 2011a; Zoecklein et al., 2000), chemicals associated with oxidation faults in finished wines, there is a risk of producing an unacceptably faulted wine when using sour rotted grapes.

Wines made with sour rotted grapes contained higher residual sugars, higher values of alcohol, increased volatile acidity (Barata et al., 2011a), decreased colour stability (Barata et al., 2011b), as well as honey-like notes caused by the chemicals ethyl phenylacetate (EPA), and phenylacetic acid (PAA) (Barata et al., 2011a). EPA is a volatile phenol described as "over sweet" (Tat et al., 2007), with a consumer rejection threshold of 140 µg/L. Sour rotted grapes can introduce levels of EPA up to 304 µg/L (Campo et al., 2012). PAA has a rejection threshold of 700 µg/L, and sour rot infection can lead to levels of up to 1668 µg/L (Campo et al., 2012). The current theory is that EPA, formed from PAA, is produced by the grape as a plant defense mechanism in response to alterations of the berry surface (Campo et al., 2012). The compound may mediate the healing of wounds (Barata et al., 2012c). Such volatile organic compounds could attract insects (Barata et al., 2012a), which can spread the disease.

Some sour rot-associated yeasts are film-forming yeasts, such as *Candida* and *Pichia*. They prefer an aerobic environment, growing quickly on the surface of fermenting wines if conditions are not maintained anaerobically, for example by tank topping or the use of inert gases (Malfeito-Ferreira, 2011). The film-forming yeasts can produce acetaldehyde. They are also a problem in wine storage when sulfur dioxide is not adequately applied, forming a ring of

film around the bottleneck if oxygen diffuses through the cork (Malfeito-Ferreira, 2011). Strains of *C. zemplinina* are recognized as osmotolerant, surviving in high sugar musts, and psychrotolerant, surviving at cool temperatures, as well as in high levels of ethanol (Sipiczki, 2003; Sipiczki, 2004). *C. zemplinina* is considered an acetogenic (Tofalo et al., 2012), and fructophilic yeast (Andorra et al., 2010).

Yeasts growing in grape juice can strip nutrients, limiting their own growth and the growth of other organisms. As species ferment, they form CO_2, which excludes aerobic organisms (Fleet, 2003). However, as yeasts undergo their metabolism, some enzymes such as proteolytic enzymes may release growth substrates into the juice. As the yeasts increase in biomass and undergo autolysis at the end of their growth cycle, amino acids and vitamins become released into the juice which can lead to a succession of organisms. The biomass released by autolysis may bioadsorb compounds such as metal ions or phenols (Fleet, 2003). Some yeasts are able to compete with each other. For example, *M. pulcherrima* may inhibit *S. cerevisiae* and other yeasts (Fleet, 2003). The early activity of yeasts in juice can determine the course of fermentation. Elevated levels of non-desirable organisms can inhibit *S. cerevisiae*, causing a sluggish or stuck fermentation (Fleet, 2008).

Wild yeasts tend to become inactive and die off in succession as the ethanol content of juice increases during fermentation. Most strains of *Hanseniaspora*, *Candida*, *Pichia*, and *Metschnikowia* are not tolerant past ethanol concentrations of 5-7%. This allows *S. cerevisiae* to ferment the juice to completion, leaving less than 2 g/L residual sugar (Fleet, 2003). In fermented products such as wine, it can be hard to distinguish beneficial fermentative activity from spoilage activity (Loureiro and Malfeito-Ferreira, 2003). For example, ethyl acetate in small amounts, under 50 mg/L, can be considered to add complexity (Garde-Cerdan and Ancin-Azpilicueta, 2006).

There have recently been some positive qualities attributed to the wild wine-associated yeasts such as *Hanseniaspora*, *Candida*, and *Pichia*, especially as it has become recognized that

S. cerevisiae-inoculated wines can be standardized and ordinary, lacking in complexity (Fleet, 2008). Spontaneous fermentations incorporating region-specific yeasts and strains can add quality to wines by imparting a unique regional character and enhancing the concept of terroir, which can add commercial value (Fleet, 2008). *C. zemplinina* is highly fructophilic and by reducing fructose levels without depleting glucose, it can help *S. cerevisiae* in sluggish fermentations (Ciani and Ferraro, 1998).

The yeasts associated with sour rot can increase the monoterpene content of fermenting wines by hydrolyzing glycosides (Zoecklein et al., 2000). Monoterpene alcohols can impart flavour complexity in wines. For example, citronellol, geraniol, linalool, and nerol impart fruity, estery, spicy and vegetative aromas (Fleet, 2008). Grape terpenes are linked covalently to glucose and disaccharides, requiring microbial glycosidases to break them down. Wild yeasts such as *H. uvarum* have more glycosidase activity than inoculated yeasts (Fleet, 2008). *H. uvarum* is a great ester producer, although higher esters are in balance with the production of ethyl acetate, which is worrisome from a spoilage perspective (Andorra et al., 2010; Plata et al., 2003). While producing 250 mg/L ethyl acetate, well past the sensory threshold, *H. uvarum* has produced high levels of isoamyl acetate and hexyl acetate, associated with banana and perfume scents (Moreira et al., 2008). Of studied yeasts, it produces the highest levels of 2-phenylethyl acetate, a desirable flowery compound (Garde-Cerdan and Ancin-Azpilicueta, 2006).

C. zemplinina produces high levels of isobutyric acid, which is very unpleasant, but also high levels of 2-methyl-1-propanol, isoamyl alcohol, and 2-phenylethanol, which are sweet, fruity, and flowery (Andorra et al., 2010). Some consider *C. zemplinina* to produce low levels of acetic acid, while producing high levels of the desired compound, glycerol (Ciani and Maccarelli, 1998; Tofalo et al., 2012). *C. zemplinina* produced 0.2 g/L acetic acid, while *S. cerevisiae* produced 0.45 g/L during fermentation. *C. zemplinina* also produced less ethyl acetate, but more acetaldehyde than *S. cerevisiae*. In a co-fermentation of the two, the level of acetaldehyde dropped compared to *S. cerevisiae* alone (Ciani and Ferraro, 1998). Due to some of

the desired compounds that non-*Saccharomyces* yeasts, such as *C. zemplinina*, can release into wine, efforts have been initiated to study the co-inoculation or step-wise inoculation of non-*Saccharomyces* yeasts with *S. cerevisiae* during winemaking. Depending on strain, non-*Saccharomyces* yeasts may add complexity to the wine without increasing the concentration of spoilage compounds (Ciani and Ferraro, 1998).

Acetic acid bacteria are ubiqitous organisms primarily responsible for microbial spoilage of wines (Bartowsky and Henschke, 2008; Drysdale and Fleet, 1988; Loureiro and Malfeito-Ferreira, 2003). Acetic acid, produced by these bacteria, is inhibitory to many yeasts (Fleet, 2003). The majority of acetic acid bacteria are gram negative, catalase positive, oxidase negative rods and motile by flagella. These parameters may vary, by species and by strain (Carrascosa et al., 2011; Du Toit and Pretorius, 2002; Drysdale and Fleet, 1988). Some strains produce a film that makes them resistant to chemical treatments and environmental stressors (Du Toit and Pretorius, 2002) and some produce water-soluble brown pigments (Drysdale and Fleet, 1988). *Acetobacter* and *Gluconobacter* can both use inorganic ammonia as a sole source of nitrogen, with no requirement for essential amino acids. *Acetobacter* requires no vitamins for growth. However, *Gluconobacter* requires pantothenic acid, p-aminobenzoic acid, niacin, and thiamin (Bartowsky and Henschke, 2008; Drysdale and Fleet, 1988).

The metabolic activities that make acetic acid bacteria most interesting in winemaking are the oxidation of ethanol to acetaldehyde using alcohol dehydrogenase, and the oxidation of acetaldehyde to acetic acid by aldehyde dehydrogenase. These enzymes are membrane-bound with active sites on the outer surface of the cytoplasmic membrane. They are $NAD(P)^+$ independent, and work optimally at a pH of 4, down to 2 (Bartowsky and Henschke, 2008; Drysdale and Fleet, 1988; Du Toit and Pretorius, 2002). There are cytoplasmic forms of these bacterial enzymes which work at a higher pH of 6-8, which are $NAD(P)^+$ dependent (Du Toit and Pretorius, 2002). Acetic acid bacteria can produce acetaldehyde up to levels of 250 mg/L, and acetic acid up to 150 g/L in vinegar production. In wine, acetaldehyde has a sensory threshold of

100-125 mg/L and thresholds for acetic acid can be as low as 0.7-1.2 g/L (Du Toit and Pretorius, 2002). Acetic acid is esterified with ethanol to produce ethyl acetate, a process favoured by anaerobic conditions (Drysdale and Fleet, 1988).

Acetobacter can oxidize completely acetic acid to water and CO_2 through the TCA cycle, producing the most acetic acid during stationary and death phases. However, this oxidation is inhibited in the presence of ethanol (Drysdale and Fleet, 1988). *Gluconobacter* has a nonfunctional α-ketoglutarate dehydrogenase and succinate dehydrogenase. Lacking a TCA cycle, *Gluconobacter* produces acetic acid throughout its lifecycle (Carrascosa et al., 2011; Du Toit and Pretorius, 2002). Under high-ethanol conditions, above 10%, aldehyde dehydrogenase becomes less stable, so acetaldehyde tends to accumulate in finished wines (Drysdale and Fleet, 1988).

Gluconobacter prefers glucose as a substrate, while not all strains of *Acetobacter* can utilize glucose. Lacking a functional Embden-Meyerhof Parnas pathway to perform glycolysis, *Acetobacter* strains use ethanol as a substrate (Drysdale and Fleet, 1988; Du Toit and Pretorius, 2002). *Gluconobacter* oxidizes glycerol to dihydroxyacetone, reducing levels of this favoured compound in wine. Glycerol levels in table wines can range between 1.86 to 9.94 g/L, imparting sweetness and viscosity. The statistically detectable level of glycerol needed to impart sweetness to a dry white wine was found to be 5.2 g/L (Noble and Bursick, 1984). With a taste detection threshold as low as 4-5 g/L, *Gluconobacter* can reduce levels by more than half (Du Toit and Pretorius, 2002).

Acetic acid bacterial growth can be stimulated by yeast extracts and the products of yeast autolysis, and can also proliferate enough to limit the growth of yeasts and result in stuck fermentations. Acetic acid is a well-known inhibitor of yeasts. Acetic acid bacteria can kill *S. cerevisiae* when the bacterial population exceeds 10^5 cells/mL (Drysdale and Fleet, 1988). Juice from healthy grapes contains approximately 10^3-10^4 acetic acid bacterial cells/mL (Fleet, 2003).

When acetic acid bacteria grow in concert with yeasts during fermentation, ethanol levels drop while levels of acetic acid and ethyl acetate increase (Drysdale and Fleet, 1989).

Many wine yeasts and bacteria have heterogeneous phenotypes (Barata et al., 2012a; Tofalo et al., 2012). *H. uvarum* has shown separation into genetic groups, with PA1 and PA2 being high producers of acetic acid, and PB1 and PB2 being low producers (Capece et al., 2005). *Candida* has even more genetic variability, for example, with six genetic groups recovered from 20 isolates (Romano et al., 1997). In the same study, *K. apiculata* (teleomorph *H. uvarum*) also had genetic variability with two distinct groups from 30 isolates (Romano et al., 1997). The metabolic properties of wine yeasts are strain-specific (Fleet, 2008; Strauss et al., 2001), as are the properties of acetic acid bacteria (Drysdale and Fleet, 1988).

1.6 Treatments to limit sour rot spoilage

1.6.1 Pre-harvest

Symptoms of sour rot occur after veraison, often beginning just as harvest approaches, which limits available treatments (Oliva et al., 1999). Studies indicate that there is no single way to control sour rot with numerous yeasts and bacteria involved. Yeasts such as *H. uvarum*, *C. zemplinina*, and *M. pulcherrima* produce symptoms of sour rot when introduced to grape pulp, yet these symptoms are considered minor in the absence of acetic acid bacteria such as *Gluconobacter* and *Acetobacter* (Barata et al., 2012a). Grape damage favours the sour rot organisms in the field, and from the infected grape juice, they can contaminate the winery and lead to wine spoilage (Barata et al., 2012a). Integrated measures have provided the best results in limiting sour rot symptoms at harvest, for example by managing berry wounds, suppressing vectors such as *D. melanogaster*, and pests responsible for wounding, such as grape berry moths, *B. cinerea* and *E. necator* (Bisiach et al., 1986; Gadoury et al., 2007; Oliva et al., 1999). Control of powdery mildew, caused by *E. necator*, reduced sour rot-causing organisms such as *H. uvarum* by more than 50% (Gadoury et al., 2007). Indirect controls work to minimize wounds, promote thick skins, and change cluster morphology by reducing bunch compactness. This

includes the management of excessive vigour and leaf removal (Duncan et al., 1995). This approach is known as integrated pest management.

Deltamethrin is an insecticide active against *D. melanogaster*, which reduced sour rot by 50%, but was not as effective in wet years where water was a disseminating agent (Bisiach et al., 1986). Very few registered products for grape pest management directly impact spoilage yeast populations (Cadez et al., 2010). Fermentative ascomycetes such as *Candida* and *Metschnikowia* can be inhibited directly by pre-1970s fungicides, but not by modern fungicides (Guerzoni and Marchetti, 1987). Captan, registered before 1970, can reduce the symptoms of sour rot by 73% through its toxicity to yeasts, while vinclozin, registered after 1970, reduced sour rot by 50% by reducing wounds caused by *B. cinerea* (Bisiach et al., 1986). Halo assays using wild yeasts were performed using modern fungicides registered to control *B. cinerea*. Mythos (pyrimethanil) and Switch (fludioxonil + cyprodinil) were registered according to the policy that they must not affect fermentative yeasts (Cadez et al., 2010). As expected, fermentative ascomycetes survived treatment in the halo assays, while basidiomycetes were suppressed (Cadez et al., 2010). Since sour rot is mainly caused by fermentative ascomycetes (Barata et al., 2012a), certain fungicides could have the potential improve the competitive advantage of sour rot microbes. However, in the field, fermentative ascomycetes dominate with and without fungicide treatment (Cadez et al., 2010). The yeasts isolated from plots where fungicide treatments were applied did not differ from an untreated control, with weather and mode of berry sampling playing a much larger role in the population dynamics (Cadez et al., 2010).

When salts were applied in the field to suppress grey rot and sour rot, there was some success when they were sprayed at least 21 days before harvest. The salts contained carbonates and bicarbonates, reported to inhibit spore germination, the elongation of the germ tube, and the production of pectinases in fungi (Nigro et al., 2006). With two salt applications 90 and 30 days pre-harvest, sour rot was reduced by approximately 50% in plots using either calcium carbonate, potassium carbonate, sodium bicarbonate, or sodium carbonate (Nigro et al., 2006). Further

substantiation for the use of calcium is its ability to penetrate grape skins, providing a higher level of protection, perhaps by stabilizing ionic bridges within pectic polysaccarides (Nigro et al., 2006).

1.6.2 Postharvest

The wild microbial population of harvested grape musts is stabilized by racking, fining, filtering, and sulfur dioxide addition (Renouf et al., 2005). The ethanol produced by *S. cerevisiae* is the foremost agent limiting the growth of wild yeasts (Fleet, 2008). Good manufacturing practices and sulfur dioxide can limit microbial spoilage of wines, but damaged berries increase the initial numbers of spoilage microbes leading to more growth at the beginning of fermentation (Loureiro and Malfeito-Ferreira, 2003). To exclude spoilage microbes from grape must, good manufacturing practices include limiting damage in the vineyard, grape selection at the winery, low temperatures during fermentation and the sanitization of winery surfaces (Malfeito-Ferreira, 2011). There is a caveat to low temperature fermentations of less than 15-20°C. *Hanseniaspora* and *Candida* may show more character, as the wild yeasts are more tolerant to ethanol at lower temperatures (Erten, 2002; Fleet, 2008).

The addition of sulfur dioxide to grape juice and wine eliminates the growth of oxidative species of yeast, while fermenting species are more likely to survive (Villa and Longo, 1996). Sulfur dioxide equilibrates into three different forms in aqueous solution: the fully protonated form known as sulfurous acid (H_2SO_3), and the deprotonated forms known as bisulfite (HSO_3^{-1}) and sulfite (SO_3^{-2}). At juice or wine pH below four, only approximately 5% of added sulfur dioxide is in the fully protonated form, which is also known as the molecular form, and is the active antimicrobial form. Bisulfite is the dominant form representing 90-99% and sulfite represents less than 1% (Fugelsang and Edwards, 2007). The bisulfite and sulfite forms are antioxidants (Du Toit and Pretorius, 2002). Molecular sulfur dioxide can enter susceptible yeast or bacterial cells, deprotonate at the higher pH inside the cell to bisulfite and sulfite, and these forms react with intracellular components to halt their metabolism (Margalit, 1997). Also, once

inside *S. cerevisiae*, bisulfite and sulfite, in small amounts, can be converted to sulfide through the sulfate reduction pathway and enter cysteine metabolism, or form hydrogen sulfide, a spoilage compound (Swiegers et al., 2005).

In the composite solution of grape juice or wine, sulfur dioxide can be present in both a bound and free form. The bisulfite form, the most dominant form at juice and wine pH, binds to compounds in juice and wine that have free carbonyl groups, forming sulfonates which do not further react. Carbonyl compounds can originate from grape pulp, for example glucose, 5-oxofructose, and δ-gluconolactone, or from yeasts such as acetaldehyde, pyruvic acid and 2-oxoglutaric acid. Acetic acid bacteria also produce sulfite-binding molecules including gluconic acid, 5-oxofructose, and dihydroxyacetone (Barbe et al., 2001; Carrascosa et al., 2011; Du Toit and Pretorius, 2002). The remaining unbound sulfites will equilibrate between the three forms of sulfurous acid (molecular sulfur dioxide), bisulfite and sulfite, with the concentration of each form being dependent on the pH of the juice or wine (Fugelsang and Edwards, 2007). A concentration of at least 25-40 mg/L unbound sulfur dioxide at pH 3.5 is required to have a sufficient concentration in the free molecular form with antimicrobial properties (Margalit, 1997).

In wine fermentations with no sulfur dioxide added in the beginning to the must, *H. uvarum* was able to survive amongst the dominant *S. cerevisiae* cells as a small portion of the population, even to the end of the fermentation. However, with 50 mg/L sulfur dioxide, non-*Saccharomyces* populations did not persist. Inoculation with a commercial strain of *Saccharomyces* had a similar effect, out-competing the wild yeasts (Henick-King et al., 1998). Species of *Hanseniaspora* could survive up to 60-70 mg/L sulfur dioxide in the must of white grapes, and 100-150 mg/L in red (Longo et al., 1991). *Pichia* required 350 mg/L sulfur dioxide to limit growth in wine (Malfeito-Ferreira, 2011).

Acetic acid bacteria are easily controlled through good manufacturing practices such as sanitation and tank topping (Barata et al., 2012a; Malfeito-Ferreira, 2011), due to their aerobic nature (Du Toit and Pretorius, 2002) which is recognized taxonomically (Bartowsky and Henschke, 2008). Yet there are experiments to show they can prefer low levels of oxygen (Carrascosa et al., 2011), and that they can survive under semi-anaerobic conditions (Drysdale and Fleet, 1988). *Acetobacter* can grow with *S. cerevisiae*, remaining viable in anaerobic wine with 10-14% ethanol (Du Toit and Pretorius, 2002), yet *Acetobacter* shows an increased sensitivity to anaerobic conditions and ethanol when pH is below 3.3 (Du Toit and Pretorius, 2002).

Acetic acid bacteria have a wide range of temperature tolerance, with weak growth as low as 8-10°C, and thermotolerant strains up to 37-40°C (Du Toit and Pretorius, 2002), preferring 25-30°C (Carrascosa et al., 2011). No chemical controls were able to eliminate acetic acid bacteria from wooden staves, not sulfur dioxide, potassium carbonate, chlorine, nor sorbic acid. Only rinsing with hot water of 85-88°C could remove the bacteria (Du Toit and Pretorius, 2002). Acetic acid species in wine were observed to survive even in the presence of sulfur dioxide. At levels above 0.8 mg/L free molecular sulfur dioxide (sulfurous acid), *Acetobacter pasteurianus* lost viability in stored red wine, but could still be detected through epifluorescence after 70 days (Du Toit et al., 2005).

Acetic acid bacteria can produce metabolites that bind to sulfur dioxide, reducing the level of free sulfur dioxide in wine must available to serve antimicrobial and antioxidant roles. This necessitates an increase in total sulfur dioxide addition to maintain a sufficient concentration of free molecular sulfur dioxide for ongoing antimicrobial activity. For example, *Gluconobacter* oxidizes glucose to gluconic acid, fructose to 5-oxofructose, and glycerol to dihydroxyacetone, all which bind sulfur dioxide (Barbe et al., 2001; Carrascosa et al., 2011; Du Toit and Pretorius, 2002). In a fermentation experiment comparing the effect of 60 mg/L sulfur dioxide compared with an *S. cerevisiae* starter culture on acetic acid bacteria, it was found that

sulfur dioxide better reduced acetic acid bacteria by the end of fermentation, but the starter culture reduced acetic acid bacteria earlier in the fermentation (Gonzalez et al., 2005).

After racking, sulfur dioxide addition, fermentation and malolactic fermentation, the yeast and acetic acid microbiota found in wines produced from sour rotted grapes was similar to that found in wines from sound grapes (Barata et al., 2012b). In fermentations of sour rotted grapes there was no more increase in volatile acidity during the alcoholic fermentation than in fermentations using healthy grapes, using standard practices and starter cultures. Sour rot fermentations had a greater final volatile acidity, but this was only due to a greater initial value (Barata et al., 2011a). The further study of sour rot over the course of wine fermentation is warranted.

1.7 Research aims

The following research seeks to identify the yeasts and bacteria responsible for sour rot in the Niagara Peninsula, designated viticultural area, Ontario, Canada through the sampling of infected clusters across the region, and through pathogenicity assays used to determine which isolates are capable of symptom production. Visual symptoms of sour rot were rated and volatile acidity was measured in g acetic acid/L as the chemical indication of disease. Through field sampling of the microbial population, the relative frequency of sour rot-associated microorganisms was considered in correlation with the factors of grape cultivar and spatial location, as well as over time. The organisms associated with symptom production were considered in sequential inoculations *in vitro* to outline differences in pathogenicity as they grew in succession. Environmental conditions were sampled over four years in a vineyard along with grape ripeness and sour rot disease progress to consider correlations between the environment, ripeness, and disease symptoms. Symptom-producing microbes were incubated at different temperatures and in grapes of different ripeness *in vitro* to further study these factors and disease progress. Field trials were conducted to test the efficacy of KMS (potassium metabisulfite) and Milstop (potassium bicarbonate) fruiting-zone sprays in the reduction of sour rot severity. These treatments were tested *in vitro* in a disk diffusion assay and a grape incubation assay, to determine their efficacy in inhibiting the growth and symptom production of the microorganisms associated with sour rot.

CHAPTER 2: THE ETIOLOGY OF GRAPE SOUR ROT IN THE NIAGARA PENINSULA, DESIGNATED VITICULTURAL AREA

2.1 INTRODUCTION

Sour rot is a late-season bunch rot affecting grapes. First noted in 2005 in the Niagara Peninsula, designated viticultural area, Ontario, Canada in *Vitis vinifera* cv. Riesling, it has been identified as an economic issue for the grape growers and winemakers of Ontario (Ker, 2006). Sour rot has been cited in the Niagara Peninsula for more than 1.5 million dollars in lost crops, when weather conditions promoted infection during a given season, such as rain storms and hail in 2008 (Agrocorp, 2008). Thin-skinned, tightly packed cultivars, such as Riesling, are known to be particularly susceptible to sour rot (Zoecklein et al., 2000).

Sour rot develops as grapes accumulate sugar and approach ripeness. Rot begins at the site where the berry attaches to the pedicel or at an injury site (Gravot et al., 2001). Sour rot organisms are not shown to penetrate grape skin directly (Bisiach et al., 1986). Other pathogens may be implicated in causing injury, for example *Botrytis cinerea* (Marchetti et al., 1984) and *Erisiphe necator* (Gadoury et al., 2007). Grape berry moths and wasps can also puncture skins and allow the introduction of sour rot pathogens (Bisiach et al., 1986). Water stress can lead to a decrease in berry size, and osmotic pressure can cause a sudden increase when there is rain, leading to microfissures that serve as entrance points for the sour rot microflora (Barata et al., 2012a; Gravot et al., 2001). *Drosophila melanogaster* is always associated with the disease in the vineyard and is implicated as a vector (Barata et al., 2012c; Gravot et al., 2001; Guerzoni and Marchetti, 1987; Marchetti et al., 1984).

Sour rot is characterized by grape skin discolouration, from pink to brown in white grapes and purple to brick red in red grapes (Gravot et al., 2001), as well as disaggregation leading to berry detachment from the pedicel and oozing juice (Guerzoni and Marchetti, 1987). The major chemical indicator of disease is volatile acidity, detected as elevated acetic acid content, measured

from 0.3-12.4 g/L in rotten juice (Guerzoni and Marchetti, 1987; Marchetti et al., 1984). In Ontario, grapes may be rejected at wineries if they contain acetic acid at a concentration greater than 0.24 g/L (Grape Growers of Ontario, 2010). Even low levels of acetic acid at harvest indicate the presence of spoilage organisms which may multiply during fermentation (Barata et al., 2008a) and is something wineries want to avoid.

Ecological surveys have shown sour rot to be the result of a community interaction among acetic acid bacterial species and yeasts such as *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata)*, *Candida zemplinina*, *Metschnikowia pulcherrima*, *Pichia*, *Rhodotorula*, *Saccharomycopsis*, and *Zygosaccharomyces* (Barata et al., 2008a; Zoecklein et al., 2000). Filamentous fungi such as *Penicillium*, *Aspergillus*, *Alternaria*, *Cladosporium*, and *Rhizopus* may be isolated from berries with sour rot symptoms but are not capable of initiating a sour rot infection (Bisiach et al., 1986). More recent literature in France has associated *Gluconobacter* and *Acetobacter* with sour rot, and only acetic acid bacteria and the yeasts *Hanseniaspora uvarum* and *Candida zemplinina* (syn. *Starmerella bacillaris*) were capable of initiating infection during *in vitro* inoculations with wounding (Duarte et al., 2012; Gravot et al., 2001).

There is great heterogeneity in the numbers of yeasts and bacteria recovered from sour rotted grapes from one region to another (Zoecklein et al., 2000). In this study, population sampling was used to determine which microbial species were associated with sour rot in the Niagara Peninsula, designated viticultural area, Ontario, Canada. Genetic sequencing was used to identify members of the microbial population, hypothesized to be composed of ascomycetous yeasts and acetic acid bacteria. Pathogenicity assays were used to determine what contribution each made toward the symptoms of sour rot. The null hypotheses of the pathogenicity assays were that there was no difference in the symptom production of each microorganism compared to the uninoculated control, no difference in the symptom production in wound-inoculated and pedicel-inoculated grapes, nor was there a difference in acetic acid production by the identified microorganisms.

2.2 MATERIALS AND METHODS

2.2.1 Microbial survey from grapes infected with sour rot. Grape clusters with sour rot symptoms were collected from commercial vineyards across the Niagara Peninsula, designated viticultural area, Ontario, Canada. In 2010, 15 sites were sampled and 52 sites were visited in 2011. At least five clusters displaying sour rot symptoms were collected from each commercial vineyard. Clusters were frozen up to 60 days at -20°C. Tissue from 50 infected berries was macerated in a 150 mL flask for 5-10 minutes in equal parts peptone water to grape juice (1 g/L peptone plus 0.1% Tween-20). The resulting suspension was diluted in a series with peptone water. Aliquots of 100 µL were plated in duplicate from dilutions with a factor of $1x10^3$, $1x10^4$, and $1x10^5$. Yeast peptone dextrose agar (YPD+) amended with 100 mg/mL streptomycin and 100 mg/mL chloramphenicol was used to select for yeasts and fungi. Glucose yeast calcium carbonate agar (GYC+) amended with 100 mg/mL cycloheximide was used to select for bacteria. Media preparation details are provided in Appendix I. After five days incubation at 22°C, colonies were grouped according to morphology at 400x magnification. Isolates from each morphological group were arbitrarily selected from each sample and streaked onto unamended YPD for molecular identification and pathogenicity testing.

2.2.2 Identification. Whole-colony PCR was performed for yeast isolates. A 200 µL pipette tip was used to collect a colony, which was mixed directly into the PCR reaction as a template. Yeast isolates were identified through the amplification and sequencing of ITS1 and ITS2 and the 5.8S rRNA gene. Yeast rDNA was amplified using the universal fungal primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') (White et al., 1990).

Boiled-cell PCR was performed for bacterial isolates. The bacterial colonies were suspended in 100 µL ddH_2O in 200 µL microtubes frozen for 30 minutes at -30ºC, then boiled for five minutes, then frozen and thawed again before being centrifuged for at least two minutes to pellet the cell fragments. An aliquot of 10 µL of the supernatant was added to the PCR reaction as

a template and ddH_2O was reduced in the reaction mix accordingly. Bacterial isolates were identified through the amplification and sequencing of a fragment of the 16S rRNA gene. Bacterial rDNA was amplified using the primers 968f (5'AACGCGAAGAACCTTAC3') and 1401r (5'CGGTGTGTACAAGACCC3') (Watanabe et al., 2001).

The PCR reaction mix for yeasts and bacteria was as follows: 33.75 µL ddH_2O (or 23.75 µL with 10 µL boiled cell template), 10 µL 5X buffer (Promega GoTaq Flexi, USA), 3 µL 25 mM $MgCl_2$, 1 µL 10 mM dNTP mix (Qiagen, Netherlands), 1 µL 100 µM forward primer, 1 µL 100 µM reverse primer, 0.25 µL 5 u/µL Promega GoTaq Polymerase, aliquoted into 200 µL microtubes.

Thermocycling followed the protocol: 94ºC for seven minutes to lyse whole cells and activate the polymerase, then 36x (94ºC for one minute to melt the template, 55ºC for yeasts or 50ºC for bacteria for 50 seconds to anneal the primers, then 72ºC for two minutes for extension), with a final extension at 72ºC for ten minutes, finishing with a 4ºC hold.

DNA from all samples was visualized electrophoretically then purified using the Qiaquick PCR Purification kit (Qiagen, Netherlands). Purified DNA was run alongside the Norgen Highranger DNA ladder (Norgen, Canada) in a 1% agarose gel at 80 volts for 50 minutes. The concentration of DNA was estimated visually using ethidium bromide staining by comparison to the standards in the DNA ladder. DNA was diluted to an approximate concentration of 5-10 ng/µL and this was combined with a 2 µM solution of the forward primer. The DNA was sent to Robarts Research Institute DNA Sequencing Facility (University of Western Ontario, Canada) for sequencing using a dye termination protocol. The resultant chromatograms were annotated by hand and were compared to a sequence database for identification using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/blast), with nearest matches having a sequence identity of at least 95%. Detailed methods can be found in Appendix II. Sequences were submitted to the NCBI Nucleotide database, and are provided in Appendix III.

2.2.3 Pathogenicity assay. Seasonal store-bought green seedless table grapes were used as a model host for potential sour rot pathogens. Green seedless varieties include Thompson Seedless (also known as Sultana), Sugraone, Princess, and Autumn King (California Table Grape Commission, 2010). Pathogenicity assay ratings in the model host were comparable to those found in *Vitis vinifera* cv. Riesling (Appendix IV). Healthy model host berries, with an intact pedicel, were pre-washed at room temperature in a solution of Dawn dish detergent (Procter & Gamble, USA) to remove pesticide residues and rinsed three times with tap water. Berries were sterilized by agitating in 10% commercial bleach (0.5% sodium hypochlorite) for ten minutes, then were rinsed twice with sterile dH_2O. The berries were arranged on sterilized galvanized wire mesh with 1 cm holes, in bleach-sterilized plastic bins that functioned as an incubation chamber. Paper towel moistened with dH_2O in the bottom of the containers maintained a high humidity when the chambers were sealed. Four replicate chambers were used corresponding to a complete random block design with five grapes used in each replicate for each isolate.

In 2010, to test the pathogenicity of each isolate, colonies were taken directly from YPD plates from field collections described in Section 2.2.1, after three to five days growth, and were rubbed onto the skin of the berries using an inoculating loop. On average, 10^6 cells were applied to each grape, determined by counting a suspension of the loopful using a haemocytometer. Each grape was then punctured through the inoculation site three times with a sterile dissecting needle. Unwounded and uninoculated grapes served as negative controls. The berries were incubated at 22°C for 14 days and sour rot symptoms were assessed using a visual rating scale of zero-four: zero= 0% infected tissue, one= <10% infected tissue, two= 10-25% infected tissue, three= 25-75% infected tissue, four= >75% infected tissue (Figure 2.1). A zero rating indicated the isolate did not colonize the wounded grape flesh, while a rating of four indicated the isolate penetrated the wound and colonized the entire grape, exhibiting softening and browning of the flesh throughout.

After incubation, microbes were re-isolated onto YPD by cutting open the infected grapes and streaking a loopful of the slurry. The berries were crushed and the acetic acid concentrations of four replicates were determined using the K-ACET Acetic Acid Assay kit (Megazyme, Ireland).

The methods were refined in 2011 to determine differences between pedicel inoculation and wound inoculation. Suspensions of each representative isolate, collected from that year of disease, were prepared in sterile dH_2O and diluted to a concentration of 10^8 cells/mL. A droplet of 10 µL of the suspension (containing 10^6 CFU) was applied directly over wounds created by a dissecting needle, or over the pedicel region of non-injured grapes.

2.2.4 Data analyses. Replicates were averaged using Microsoft Excel. ANOVA was performed using Microsoft Excel 2000/XLSTAT-Pro (Version 7.2, 2003, Addinsoft, Inc., USA), and the Student Newman–Keuls (SNK) multiple range test was employed for mean separations with a confidence interval of 95%. Based on the confidence interval, an ANOVA-calculated *p*-value below 0.05 was considered to indicate data which could reliably lead to the rejection of the null hypothesis.

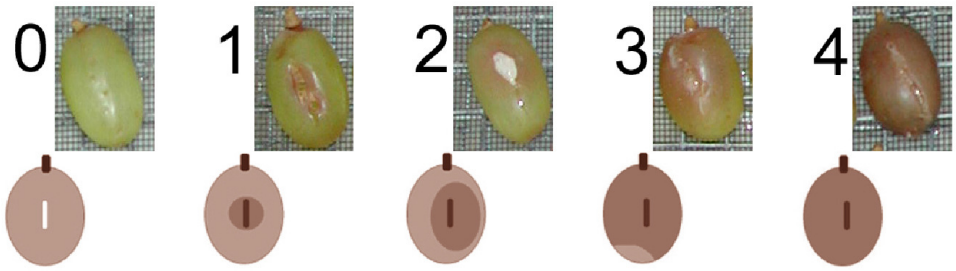


Figure 2.1 Rating scale to quantify symptoms observed in green seedless table grapes, the model host incubated in a moist chamber to function as a pathogenicity assay for sour rot. Pictures were taken 14 days after incubation at 22°C.

2.3 RESULTS

2.3.1 Microbial survey from grapes infected with sour rot. Bacteria and yeasts were isolated from sour rotted grapes across the Niagara Peninsula, designated viticultural area, Ontario, Canada in 2010 and 2011. The most frequently recovered isolates were identified as acetic acid bacteria at

73% of sites in 2010 and 98% of sites in 2011 (Table 2.1). Acetic acid bacterial species were grouped in the plate counts as they were not reliably distinguished from each other by morphology alone. From a sample of ten isolates selected for genetic sequencing, *Gluconobacter oxydans* dominated while *Gluconobacter cerinus* and *Acetobacter malorum* were less abundant (Table 2.2).

Along with the acetic acid bacteria, the yeasts *H. uvarum* and *C. zemplinina* were frequently recovered from sour rotted grapes at 100% and 80% of sites in 2010 and 97% and 86% in 2011, respectively (Table 2.1). One isolate in 2011, grouping morphologically with *Candida zemplinina* was identified as *Zygoascus hellenicus* (syn. *Candida steatolytica*) (Barata et al., 2012b). Other yeasts, *Pichia membranifaciens* (20% of sites in 2010 and 62% in 2011), *Aureobasidium pullulans* (47% of sites in 2010 and 19% in 2011), *Rhodotorula glutinis* (6% of sites in 2010 and 4% in 2011), and bacteria, *Bacillus subtilis* (73% of sites in 2010 and 21% of sites in 2011), and *Pseudomonas fluorescens* (20% of sites in 2010 and 6% of sites in 2011) were recovered from fewer sites. Other species were detected very rarely and were not listed (Table 2.1).

Table 2.1 Isolates recovered from sour rotted grapes from commercial vineyards in the Niagara Peninsula, designated viticultural area, in 2010 and 2011 (N = 15 in 2010 and 52 in 2011). Yeast isolates were identified through the sequencing of ITS1 and ITS2 and the 5.8S rRNA gene. Bacterial isolates were identified through the sequencing of a fragment of the 16S rRNA gene. Genbank accession numbers are provided in Table 2.2.

Identification	% of sites detected	
	2010	2011
Acetic Acid Bacteria: *Gluconobacter oxydans* *Gluconobacter cerinus* *Acetobacter malorum*	73.3	98.1
Bacillus subtilis	73.3	21.2
Pseudomonas fluorescens	20.0	5.8
Hanseniaspora uvarum	100.0	96.2
Candida zemplinina *(Zygoascus hellenicus)*	80.0	86.5
Pichia membranifaciens	20.0	61.5
Aureobasidium pullulans	46.7	19.2
Rhodotorula glutinis	6.7	3.8
	N = 15	N = 52

Table 2.2 Genbank accession numbers of sequences amplified from species isolated from sour rotted grapes and deposited into the NCBI Nucleotide database. Isolate numbers are provided using Roman numerals for identification. Full sequences and source modifiers are provided in Appendix III. Yeast isolates were identified through the sequencing of ITS1 and ITS2 and the 5.8S rRNA gene. Bacterial isolates were identified through the sequencing of a fragment of the 16S rRNA gene. Pathogenicity of isolates was studied in Figure 2.2.

Identification and Isolate Number	Accession Number
Gluconobacter oxydans I	KP010384
Gluconobacter oxydans II	KP010385
Gluconobacter oxydans III	KP010386
Gluconobacter oxydans IV	KP010387
Gluconobacter oxydans V	KP010388
Gluconobacter oxydans VI	KP010389
Gluconobacter oxydans VII	KP010390
Gluconobacter cerinus I	KP010382
Gluconobacter cerinus II	KP010383
Acetobacter malorum	KP010380
Bacillus subtilis	KP010381
Pseudomonas fluorescens	KP010391
Hanseniaspora uvarum I	KP010400
Hanseniaspora uvarum II	KP010401
Hanseniaspora uvarum III	KP010402
Hanseniaspora uvarum IV	KP010403
Hanseniaspora uvarum V	KP010404
Hanseniaspora uvarum VI	KP010405
Hanseniaspora uvarum VII	KP010406
Hanseniaspora uvarum VIII	KP010407
Hanseniaspora uvarum IX	KP010408
Candida zemplinina I	KP010393
Candida zemplinina II	KP010394
Candida zemplinina III	KP010395
Candida zemplinina IV	KP010396
Candida zemplinina V	KP010397
Candida zemplinina VI	KP010398
Candida zemplinina VII	KP010399
Zygoascus hellenicus	KP010411
Pichia membranifaciens	KP010409
Aureobasidium pullulans	KP010392
Rhodotorula glutinis	KP010410

2.3.2 Pathogenicity assay. Wild-isolated strains of potential sour rot pathogens, identified in Section 2.3.1 were inoculated on injured table grapes. Only the acetic acid bacteria and *H. uvarum* were considered strong pathogens with an average pathogenicity rating as high as four (Figure 2.2). The grapes inoculated with the acetic acid bacteria smelled of acetic acid, while *H. uvarum*-infected grapes were scented with the odour of ethyl acetate. None of the other organisms produced odours typical of sour rot.

With wound inoculation, there were no significant differences in the pathogenicity among isolates of *H. uvarum*, with isolates having a mean pathogenicity rating as high as 4.0, whereas isolates of *G. oxydans* varied in pathogenicity with some being comparable to that of *H. uvarum* and others being significantly less pathogenic, as low as 2.7. One tested isolate of *A. malorum* had a mean pathogenicity rating of 3.9, comparable to *H. uvarum* and the most pathogenic isolates of *G. oxydans* (Figure 2.2). Two isolates of *G. cerinus* were less pathogenic, but still comparable to *H. uvarum* and the most pathogenic isolates of *G. oxydans* with ratings of 3.8 and 3.4 (Figure 2.2). *C. zemplinina* isolates were significantly less pathogenic than *H. uvarum* and most isolates of *G. oxydans*, and showed the most variability in their pathogenicity, ranging from mean pathogenicity ratings of 0.9 to 2.5 (Figure 2.2). *B. subtilis, P. fluorescens, P. membranifaciens*, and *R. glutinis,* produced significantly less severe symptoms with mean pathogenicity ratings of 1.2, 0.7, 1.2, and 0.8, respectively (Figure 2.2). *A. pullulans* colonized the grape skin leading to deep fissures, yet did not penetrate deeply into the grape pulp and did not produce tissue softening, leading to an average rating of 1.9, also significantly lower than *H. uvarum* or *G. oxydans* (Figure 2.2).

Suspensions were used as opposed to whole-colony inoculations to test the difference between wound-inoculation and pedicel-inoculation. Only the acetic acid bacteria penetrated unwounded berries at the pedicel attachment site to initiate the symptoms of sour rot (Figure 2.3). *G. oxydans* had a mean pathogenicity rating of 2.8 in pedicel inoculations, while the mean rating was higher at 3.4 when it was applied directly to a wound. *H. uvarum* had a mean pathogenicity rating of 2.3 in wound-inoculated berries and *C. zemplinina* had a rating of 1.1. In pedicel-

inoculated grapes the mean pathogenicity rating of both *H. uvarum* and *C. zemplinina* was 0.0, which was the same as the rating in uninoculated control grapes (Figure 2.3).

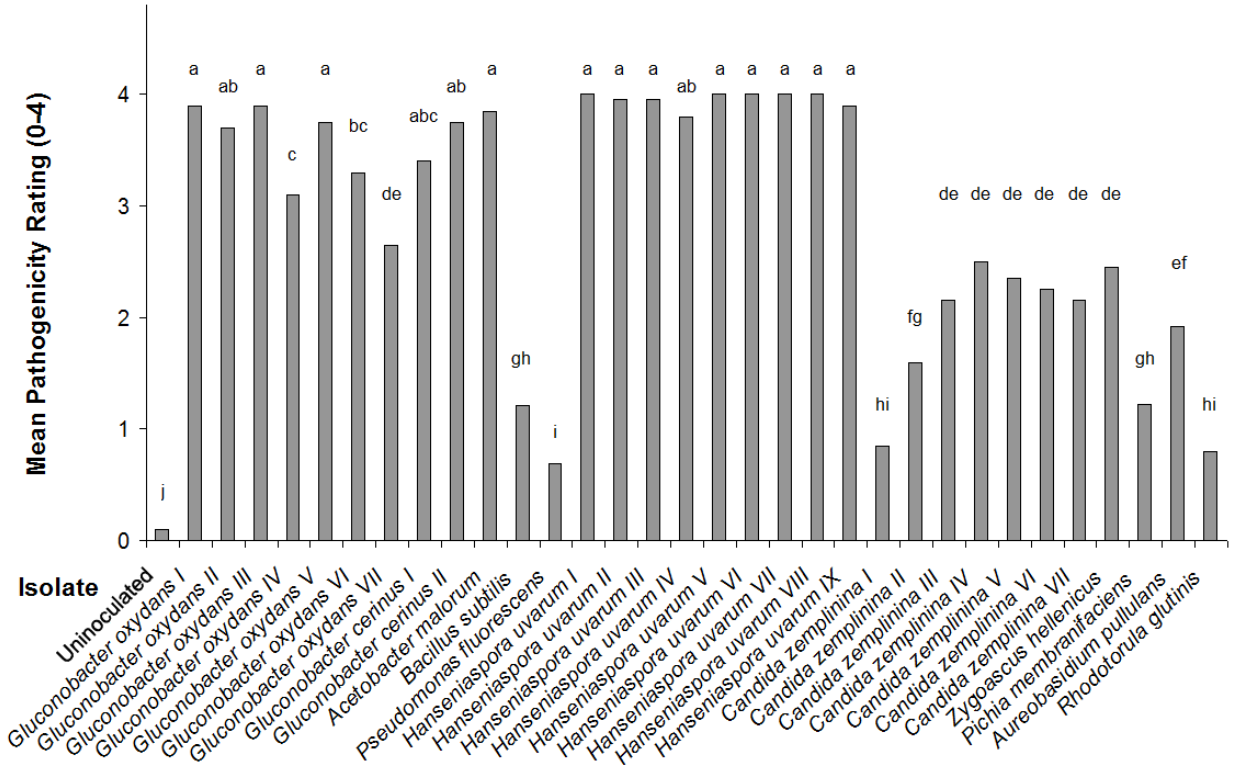


Figure 2.2 Mean pathogenicity ratings of isolates taken from sour rotted grapes in the Niagara Peninsula, designated viticultural area, Ontario, Canada in 2010, incubated for 14 days at 22°C using injured table grapes. Four replicated chambers were employed with four berries rated for each isolate in each chamber (N = 16 berries). ANOVA $F(3,37) = 125.85$, $p = <0.0001$. Bars with the same letters are not significantly different according to the Student Newman-Keuls multiple range test.

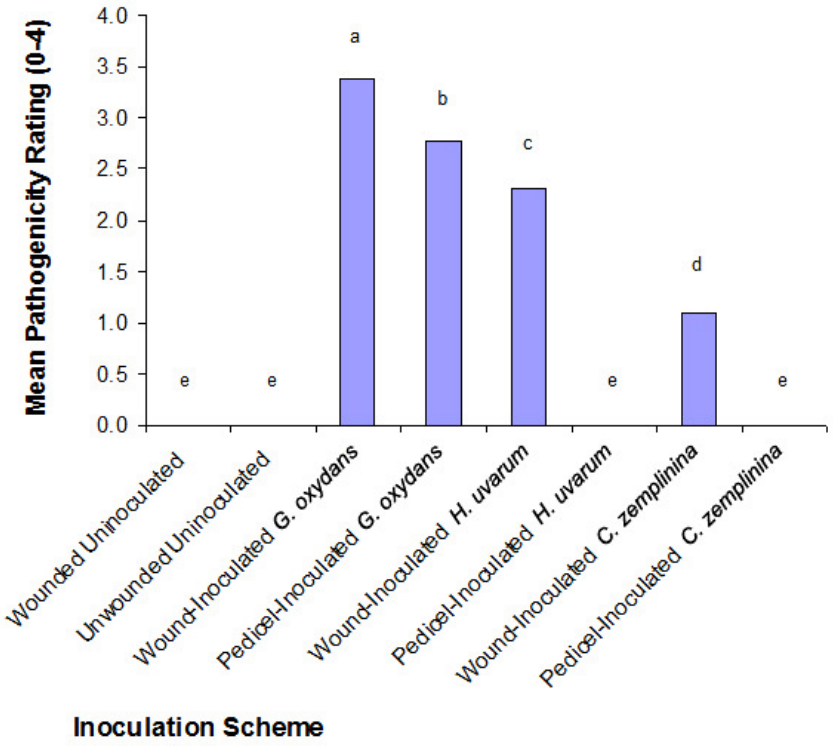


Figure 2.3 Pathogenicity assay results with the strong and variable pathogens, *Gluconobacter oxydans*, *Hanseniaspora uvarum,* and *Candida zemplinina,* after 14 days of incubation at 22°C. Isolates were recovered from sour rotted grapes in the Niagara Peninsula, designated viticultural area, Ontario, Canada in 2011. For each inoculation scheme, four replicates of five berries were inoculated with a suspension through a wound or using unwounded berries at the pedicel attachment site (N = 20 berries for each inoculation scheme). ANOVA $F(3,7) = 310.85$, $p = <0.0001$. Bars with the same letters are not significantly different according to the Student Newman-Keuls multiple range test.

Acetic acid was measured from the grapes incubated in the pathogenicity assay. *G. oxydans* was able to produce the most volatile acidity in the host, 2.85 g acetic acid/L, significantly greater than *A. malorum* producing 1.80 g acetic acid/L (Figure 2.4). The yeasts associated with sour rot symptoms in the pathogenicity assay, *H. uvarum* and *C. zemplinina*, did not produce significantly more acetic acid than was measured from the uninoculated control grapes. *H. uvarum* produced 0.87 g acetic acid/L, and *C. zemplinina* 0.19 g acetic acid/L, which was less than what was detected in the uninoculated control (0.37 g acetic acid/L) (Figure 2.4).

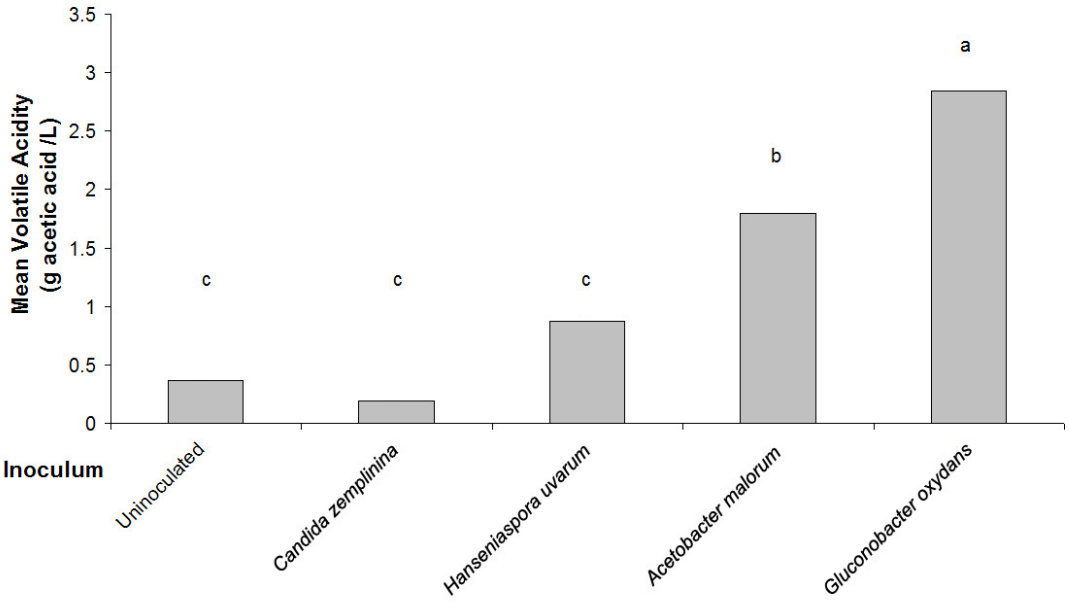


Figure 2.4 Mean volatile acidity measured in g acetic acid/L from grapes incubated 14 days in a pathogenicity assay at 22°C, inoculated with isolates taken from the Niagara Peninsula, designated viticultural area, Ontario, Canada, in 2010. N = 16 crushed berries for each isolate and duplicated readings. ANOVA $F(3,4) = 978.03$, $p = 0.024$. Values with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

2.4 DISCUSSION

Acetic acid bacteria were among the most commonly isolated organisms recovered from sour rotted grapes in 2010 and 2011 (Table 2.1), establishing the role of these bacteria in sour rot in Ontario, Canada. This agrees with observations of the disease in other parts of the world, in which acetic acid bacteria are the primary organisms associated with sour rot symptoms (Barata et al., 2012a; Gravot et al., 2001; Oliva et al., 1999). In early publications these bacteria were overlooked (Guerzoni and Marchetti, 1987) when researchers did not use culturing media such as GYC, which is specifically suited to acetic acid bacterial growth (Du Toit and Pretorius, 2002). Acetic acid bacteria are ubiquitous in winemaking as they readily oxidize ethanol into acetic acid. Concentrations of 10^2-10^3 cells/mL can be found on sound grapes and up to 10^6 cells/mL in damaged grapes (Bartowsky and Henschke, 2008).

Gluconobacter was detected more frequently than *Acetobacter* (Table 2.1). The sugar-rich environment of the grape is conducive to the proliferation of *Gluconobacter* since it oxidizes hexose sugars, while *Acetobacter* prefers alcohols as a metabolic substrate (Bartowsky and Henschke, 2008). In previous research, the acetic acid bacterium, *Acetobacter pasteurianus*, survived in a viable but not culturable (VBNC) state in stored red wine (Du Toit et al., 2005). The bacterium could be detected through epifluorescence, but not through culturing (Du Toit et al., 2005). Similarly, there may be a population of VBNC bacteria associated with sour rotted grapes. If this is the case, direct molecular methods could result in a more reliable quantification of the organisms present in the sour rot community. For example, a DNA microarray could be developed that includes probes from the sequences of the 16S region of all recognizable species of *Gluconobacter* and *Acetobacter* (Zhou, 2003).

The dominance of the yeasts *H. uvarum* and *C. zemplinina* in sour rotted grapes (Table 2.1) agrees with the work of Gravot et al. (2001) in Bordeaux. *H. uvarum* was found at the most sites in 2010, surpassing even the acetic acid bacteria (Table 2.1). *H. uvarum* is cited as the most prevalent organism recovered from both healthy and infected grapes in all parts of the world (Barata et al., 2008b; Villa and Longo, 1996). *H. uvarum* was identified using genetic sequencing (Table 2.2), although spores were not visualized to confirm the morphotype was distinct from the imperfect form, *K. apiculata*, which was the form reported by Gravot et al. (2001). The perfect form, *H. uvarum* was previously described in musts from Ontario vineyards, enumerated using cultural methods (Holloway et al., 1990).

The less frequent recovery of yeasts such as *A. pullulans* and *Rhodotorula* (Table 2.1) agrees with research in Portugal (Barata et al., 2012b), and France (Gravot et al., 2001), respectively. In Portugal, bacteria such as *Bacillus* and *Pseudomonas* were associated with grapes epiphytically (Barata et al., 2012a), and were also recovered from sour rotted grapes in the Niagara Peninsula (Table 2.1). As with acetic acid bacteria, the quantification of all species associated with sour rotted grapes may benefit from the use of direct molecular methods,

especially when considering that certain yeasts and bacteria require differential and selective media (Barata et al., 2008b).

Even though the sour rot complex was isolated and identified from different grape varieties around the world, the same organisms were found to contribute to the disease: acetic acid bacteria and ascomycetous yeasts, verified by the current study and matching the initial hypothesis. All of the yeasts associated with sour rot can be detected as a part of the natural community of healthy grapes, and have rarely been considered as possible phytopathogens (Marchetti et al., 1984). This poses problems when considering strategies for disease control, as there is no single microbial target. Future studies should consider agricultural products that could potentially act upon these organisms in the field.

Few studies have shown data relating the symptoms of sour rot to specific microbial species using pathogenicity assays. In the present study, the development of an assay protocol and rating scale was essential to understanding which organisms were responsible for sour rot symptoms, to discriminate between pathogens and microbial epiphytes (Figure 2.1). The null hypothesis was rejected with an ANOVA-calculated *p*-value of <0.0001, indicating significant differences in the pathogenicity ratings of the isolates and the uninoculated control. Through the pathogenicity assay, the pathogenic potential of the acetic acid bacteria and *H. uvarum* was established in injured grapes, while *C. zemplinina* isolates were found to have variable pathogenicity ratings with some isolates bearing significantly lower mean values. The isolates of *B. subtilis, P. fluorescens, P. membranifaciens*, *R. glutinis,* and *A. pullulans* could be considered very weak pathogens with significantly lower mean pathogenicity ratings (Figure 2.2). These results built upon the experiments performed by Gravot et al. (2001), which found acetic acid bacteria and *H. uvarum* to be the largest contributors to sour rot symptoms, also noting the contribution of *C. zemplinina*. This research group also made the observation that rot often begins its development around the stem attachment site, and not only due to injuries in the grape cuticle. The current study confirms this observation. With an ANOVA-calculated *p*-value of

<0.0001, the null hypothesis that there was no difference in the symptom production in wound-inoculated and pedicel-inoculated grapes, could be rejected. Acetic acid bacteria were able to penetrate the berry around the pedicel with the production of sour rot symptoms. Yeasts were not able to initiate an infection through pedicel inoculation, with the same pathogenicity rating in pedicel-inoculated grapes as in the uninoculated control grapes. Yeasts required injury to produce sour rot symptoms (Figure 2.3). The measurement of the acetic acid concentration of grapes infected with sour rot led to the rejection of the null hypothesis that there was no difference in acetic acid production by the identified microbes (Figure 2.4). The ANOVA-calculated *p*-value was 0.024. The yeasts *C. zemplinina* and *H. uvarum* did not produce significantly more volatile acidity than the uninoculated control, measured in g acetic acid/L. The increased production of acetic acid could be solely attributed to the acetic acid bacteria (Figure 2.4).

C. zemplinina has variable biochemical properties by strain, especially with respect to the production of alcohols and esters (Romano et al., 1997). This metabolic diversity may be directly linked to its variability in sour rot symptom production (Figure 2.2). It is possible that *C. zemplinina* shows some opportunistic qualities in the sour rot complex. This yeast was recovered with regularity from the sour rot microbial community (Table 2.1), yet did not always produce sour rot symptoms (Figure 2.2). *C. zemplinina* produces lower volatile acidity than the wine yeast, *Saccharomyces cerevisiae*, especially in high-solute fermentations (Magyar and Toth, 2011; Tofalo et al., 2012). This agrees with the low volatile acidity detected in berries infected with *C. zemplinina*, 0.19 g acetic acid/L, not significantly different than the uninoculated control (Figure 2.4). *C. zemplinina* is a fructophilic yeast (Magyar and Toth, 2011), and so it is not likely to compete with the other organisms involved in sour rot for its preferred substrate, possibly explaining its common recovery from sour rotted grapes (Figure 2.1). To study further the role of this yeast in the sour rot complex, sequential inoculations could be utilized in the pathogenicity

assays to determine to what extent it reduces the symptoms produced by other associated microbes, as its proliferation could reduce acetic acid.

It is difficult to consider *H. uvarum* a true pathogen, as it has not been shown to produce pectinases (Masoud and Jespersen, 2006), an important virulence factor for the penetration of fruit. This supports the present observations. The yeast cells required an injury site in order to penetrate the grape berries (Figure 2.3). All enzyme activities are strain-dependent (Strauss et al., 2001). However, there was very little pathogenic variability in the *H. uvarum* isolates collected in this survey (Figure 2.2). *H. uvarum* produces high levels of acid proteases and can reduce the protein content of grape juice by one third within seven days under fermentation conditions (Dizy and Bisson, 2000). This may be a unique contribution to the metabolic environment of the sour rot complex, liberating nutrients for other species to utilize. This property could be further studied in a sequential inoculation experiment.

Rot caused by *H. uvarum* is often darker in colour than that produced by acetic acid bacteria (Gravot et al., 2001), which was observed in the pathogenicity assays (Figure 2.2 and 2.3). This may be due to the great enzyme production by the yeast, while acetic acid bacteria have reduced metabolic capabilities (Du Toit and Pretorius, 2002). *H. uvarum* isolates could be grown in a minimal medium and compared to growth within the grape, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) could identify some of the proteins present during sour rot infection. Non-denaturing polyacrylamide gel electrophoresis could identify proteins in their native state and active quaternary structure. Genome sequencing would be required to initiate understanding of all the enzymatic capabilities of *H. uvarum*, while the full genome of *G. oxydans* is known (Prust et. al., 2005).

H. uvarum can produce high levels of ethyl acetate (Romano et al., 1997), up to 250 mg/L in wine fermentations (Moreira et al., 2008), and subject to strain-specific properties it may produce very high levels of volatile acidity in fermentations, up to 37.5 g acetic acid/L (Andorra et al., 2010). In the present experiment, *H. uvarum* only produced a mean of 0.87 g acetic acid/L

(Figure 2.4). Acetic acid is a wine spoilage compound with 1.3 g/L being its legal limit in Ontario wines (Cliff and Pickering, 2006). The smell of ethyl acetate was noted in the berries inoculated with *H. uvarum* in the pathogenicity assay. Further experiments could quantify the levels of ethyl acetate using gas chromatography-olfactometry-mass spectrometry (GC-O-MS). Ethyl acetate is a wine spoilage compound with a sensory threshold of 60 mg/L in white musts and 115 mg/L in red musts (Corison et al., 1978).

Acetic acid bacteria lack a functional Embden-Meyerhof Parnas glycolysis pathway. These bacteria possess alcohol dehydrogenase and acetaldehyde dehydrogenase, cytoplasmic membrane-bound enzymes catalyzing acetic acid production (Bartowsky and Henschke, 2008). Since acetic acid production is required for the metabolism and proliferation of acetic acid bacteria, it is no surprise these organisms were primarily responsible for volatile acidity observed in the grape pathogenicity assay (Figure 2.4). *G. oxydans* produced 2.85 g acetic acid/L, significantly greater than *A. malorum* producing 1.80 g acetic acid/L (Figure 2.4). *Gluconobacter* prefers glucose as a substrate, while not all strains of *Acetobacter* can utilize glucose, using ethanol as a substrate (Drysdale and Fleet, 1988; Du Toit and Pretorius, 2002), which may be limited in the sugar-rich environment of the grape. The yeasts involved in the sour rot complex are capable of producing ethanol (Andorra et al., 2010) for use by such *Acetobacter* strains. The species and strain variability noted in acetic acid bacteria (Drysdale and Fleet, 1988), along with their infection of grapes not inoculated with yeasts that may be liberating necessary metabolites, could explain the variable results in the pathogenicity assay (Figure 2.2).

Acetic acid bacteria were able to initiate sour rot symptoms when inoculated on the junction of the berry and the pedicel. This is likely because they were able to penetrate grapes through the region of pedicel attachment (Figure 2.3), which may be attributed to their motility and small size compared to yeast cells (Du Toit and Pretorius, 2002). Along with the production of acetic acid, the acetic acid bacteria oxidize sugars into gluconic acid and ketogluconic acids (Drysdale and Fleet, 1989), which bind to sulfur dioxide, altering its efficacy (Bartowsky and

Henschke, 2008). Sulfur dioxide is an important additive used to limit contaminants in grape juice being prepared for fermentation (Loureiro and Malfeito-Ferreira, 2003), and therefore the presence of acetic acid bacteria can have wide-ranging detrimental effects during winemaking.

Acetic acid bacteria may also contaminate fermentations and slow the growth of *S. cerevisiae* (Drysdale and Fleet, 1989). Overall, in order to ensure wine quality, acetic acid bacteria are among the most important organisms to control during winemaking. Their primary role in sour rot of wine grapes is the reason this bunch rot is subject to such strict rejection limits. Future research into the agricultural control of the sour rot complex of acetic acid bacteria, *H. uvarum* and *C. zemplinina* is necessary to protect the crops of grape growers in Ontario, Canada and around the world.

CHAPTER 3: THE ECOLOGY OF GRAPE SOUR ROT IN THE NIAGARA PENINSULA, DESIGNATED VITICULTURAL AREA

3.1 INTRODUCTION

In the Niagara Peninsula, designated viticultural area, Ontario, Canada, sour rot in grapes is an economic problem (Ker, 2006). Crop loss occurs particularly in wet years, which promotes sour rot (Loureiro and Malfeito-Ferreira, 2003). This late-season bunch rot develops in grapes as they accumulate sugar and approach ripeness (Gravot et al., 2001). The disease is associated with grape injury, which can occur due to water stress or other physical injury to the berry. When under water stress berries decrease in size, and when exposed to wet weather, berries can dramatically increase in size leading to small splits that serve as entrance points for the microbes that cause disease (Barata et al., 2012a; Gravot et al., 2001). This may be why cultivars with thin skin and tightly packed clusters, such as Riesling and Pinot Noir, are particularly susceptible to sour rot (Zoecklein et al., 2000). Hail, insects and other pathogens can also cause injury directly to the berries, providing entry to the microbes (Barata et al., 2012a).

Sour rot begins as a discolouration at an injury site or where the pedicel attaches to the grape (Bisiach et al., 1986; Gravot et al., 2001). Internal tissues become disaggregated, while the berry detaches from the pedicel and juice becomes liberated (Guerzoni and Marchetti, 1987). The major chemical markers of disease are increased acetic acid content, and an increase in the ethanol-acetic acid ester, ethyl acetate (Guerzoni and Marchetti, 1987; Marchetti et al., 1984). These compounds, considered wine oxidation faults, are associated with wine spoilage (Barata et al., 2012a), and lead to the rejection of grapes with high volatile acidity at the winery.

Sour rot symptoms are associated with an increase in the number of yeasts and bacteria that constitute the wine microbial consortium (Barata et al., 2012a). These microbes are able to survive under anaerobic conditions. The yeasts particularly implicated are the ascomycetes *Hanseniaspora uvarum*, *Candida zemplinina*, and *Pichia* (Barata et al., 2008b; Bisiach et al., 1986; Gadoury et al.,

2007; Gravot et al., 2001; Guerzoni and Marchetti, 1987; Zoecklein et al., 2000). Species of acetic acid bacteria in the genera *Gluconobacter* and *Acetobacter* are also associated, and are the primary agents causing sour rot symptoms (Barata et al., 2012a; Gravot et al., 2001).

The yeasts and bacteria colonize grape surfaces as the berries ripen, up to 10^3-10^5 cells/mL on mature, sound grapes, and can be accompanied by saprotrophic moulds. Microbial populations are not consistent year to year or from winery to winery. The mixed population is affected by rainfall, temperature, grape variety, and agrochemicals, and tends to localize around sites where juice may escape (Loureiro and Malfeito-Ferreira, 2003). When damage occurs to the grapes, populations can increase up to 10^8 cells/mL (Barata et al., 2008b). The microbes require a source of carbohydrates and other nutrients, and adherence is affected by the waxy cuticle of the grape while the skin keeps most of the nutrient-rich juice inside. Grape damage favours the sour rot organisms, ascomycete yeasts with wine spoilage potential, as well as acetic acid bacteria (Barata et al., 2012a).

The Niagara Peninsula has been divided into designated viticultural areas based on the Vintners' Quality Alliance Ontario standards. These areas are sub-appellations, divided based on the concept of terroir, which states that local geography can change the climate under which grapes grow, which has an effect on wine flavour (Schlosser et al., 2005). The ten sub-appellations of the Niagara Peninsula are the Lakeshore, Creek Shores, Niagara Lakeshore, Beamsville Bench, Twenty Mile Bench, Short Hills Bench, Vinemount Ridge, St David's Bench, Four Mile Creek and Niagara River (Figure 3.1) (Shaw, 2014). These geographic areas can be generalized into the Lakeshore, Escarpment, and Lakeshore Plains. The Lakeshore is nestled between Lake Ontario and the higher elevation of the Escarpment, usually with a band of cloud cover and more lake effect, moderating temperatures for cooler daytime temperatures and warmer nights. The Escarpment, or "Bench" region, is a northern-facing slope of the Niagara Escarpment, which has good drainage and experiences less of the lake effect. The Lakeshore Plain, dominated by Four Mile Creek is a

flat area with low elevation, little lake effect, and great sun exposure (Schlosser et al., 2005; Shaw, 2014).

In the present study, samples were taken from sour rotted grapes from two cultivars, *Vitis vinifera* cv. Riesling and cv. Pinot Noir, in eight out of the ten sub-appellations of the Niagara Peninsula. These were analyzed for differences in their respective microbial communities to correlate the population overall with cultivar as well as to qualify population differences according to spatial characteristics and microclimate amongst all sites. The null hypothesis was that there were no differences in the composition of the population between cultivars. Grapes were sampled over time from cv. Riesling and cv. Pinot Noir at two sites to study the microbial population changes according to grape maturity and the onset of veraison, as well as the onset of sour rot symptoms. The null hypothesis was that there were no changes in the population structure over time. Pathogenicity assays were performed *in vitro*, with sequentially inoculated microorganisms associated with sour rot, to consider changes to symptoms in grapes during an ecological succession of microorganisms. An ecological succession is an evolution in a biological community structure over time (Fleming et al., 2014), in this case the community of microorganisms associated with grapes as they ripen. The null hypothesis was that there were no changes in sour rot symptoms when the microorganisms were applied in different sequences representing different possible successions. Volatile acidity in the form of the concentration of acetic acid was measured from the grapes incubated in the pathogenicity assay as a chemical indicator of disease. The null hypothesis was that there were no differences in the concentration of acetic acid when the microorganisms were applied in different sequences representing different possible successions.

3.2 MATERIALS AND METHODS

3.2.1 Population survey. Sour rotted grape clusters were collected from six cv. Pinot Noir and eight cv. Riesling vineyards across the Niagara Peninsula, designated viticultural area, Ontario, Canada, from September through October, 2011 (Figure 3.1). The vineyards were located in eight of ten sub-appellations in the Niagara Peninsula. Pinot Noir was sampled from Beamsville Bench,

Twenty Mile Bench, Short Hills Bench, Niagara Lakeshore, and Four Mile Creek. Riesling was sampled from Niagara Lakeshore, Four Mile Creek, Niagara River, Vinemount Ridge, Creek Shores, and Short Hills Bench. Sour rot severity was not evaluated in these vineyards. Five clusters displaying sour rot symptoms were sampled and combined from each commercial vineyard. Isolations were conducted by freezing the infected clusters, removing only the infected berries with pedicel attached and surface-sterilizing each berry with brief exposure to 95% ethanol and flame. Fifty berries displaying infected tissue were suspended in 1 mL peptone water/berry (1 g/L peptone with 0.1% Tween-20), and macerated for five minutes. Aliquots of 100 µL were plated in duplicate from dilutions with factors of $1x10^3$, $1x10^4$, and $1x10^5$. Yeast peptone dextrose agar (YPD+) amended with 100 mg/mL streptomycin and 100 mg/mL chloramphenicol was used for the growth and enumeration of yeasts, and glucose yeast calcium carbonate agar (GYC+) amended with 100 mg/mL cycloheximide was used for the growth and enumeration of bacteria. After five days growth at 22°C, colonies were grouped according to morphology at 400x magnification, and individuals were counted according to the medium upon which they grew best. Yeasts and bacteria were isolated from each morphological group by streaking each representative colony onto unamended YPD.

Yeast isolates were identified through the amplification and sequencing of ITS1 and ITS2 and the 5.8S rRNA gene, amplified using the universal fungal primers ITS1 (5'TCCGTAGGT GAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') (White et al., 1990). Bacterial isolates were identified through the amplification and sequencing of a fragment of the 16S rRNA gene, using the primers 968f (5'AACGCGAAGAACCTTAC3') and 1401r (5'CGGTGTGTACA AGACCC3') (Watanabe et al., 2001). Complete methods are provided in Chapter 2, Appendix I, and Appendix II. Sequences are presented in Appendix III.

3.2.2 Timecourse sampling. Changes over time were monitored in the wild microbial population after veraison in 2012 from two commercial vineyards, *Vitis vinifera* cv. Riesling in the Twenty Mile Bench sub-appellation and cv. Pinot Noir in the Creek Shores sub-appellation.

Riesling was sampled on August 17, August 24, September 7, and September 16, 2012. Pinot Noir was sampled on August 17, August 24, September 7, September 17, and October 3, 2012. On successive sampling dates, five clusters were obtained from four replicated plots at each vineyard which were used throughout the sampling period. Sampled clusters represented typical symptomology in the vineyard, determined by rating the severity of 25 clusters/plot (% cluster infected). On each sampling date, 50 arbitrarily selected berries, with pedicel attached, were removed from each cluster and were rinsed for 15 minutes in 1 mL peptone water/berry. Dilutions were plated as in Section 3.2.1 onto YPD+ and GYC+ media. Individuals of each morphological group were counted as in Section 3.2.1 after five days growth at 22°C.

3.2.3 Sequential inoculation pathogenicity assay. Green seedless table grapes were used as a model host. Healthy grapes, with an intact pedicel, were pre-washed in Dawn dish detergent (Procter & Gamble, USA), rinsed three times with tap water, agitated in 10% commercial bleach (0.5% sodium hypochlorite) for ten minutes, then rinsed twice with sterile dH_2O. The grapes were arranged on sterilized galvanized wire mesh with 1 cm holes, in bleach-sterilized plastic bins that functioned as an incubation chamber. Moistened paper towel on the bottom of the chambers maintained a high humidity when the chambers were sealed. Four replicate chambers were used corresponding to a complete random block design with five grapes used in each replicate for each isolate. Five grapes were uninoculated and five uninjured as an untreated control.

Wild isolates of *G. oxydans*, *C. zemplinina*, and *H. uvarum*, identified through genetic sequencing as in Chapter 2, were maintained on YPD and used as inocula. *H. uvarum* was genetically identified in the perfect form, although spores were not visualized. Suspensions of each isolate were prepared in sterile dH_2O and diluted to a concentration of 10^8 cells/mL. The berries were injured with a dissecting needle and 10 µL of the first pathogen was applied directly to the wound. After 48 hours, the second pathogen was applied in the same manner. If only one pathogen was used, it was reapplied 48 hours later. Symptoms were assessed in the injured

grapes after ten days of incubation at 22°C using a rating scale of zero-four: zero= 0% infected tissue, one= <10% infected tissue, two= 10-25% infected tissue, three= 25-75% infected tissue, four= >75% infected tissue (Figure 2.1). After ten days incubation, five grapes per treatment-replicate were crushed and the acetic acid concentration of each was determined using the K-ACET Acetic Acid Assay kit (Megazyme, Ireland).

3.2.4 Data analyses. In the survey and timecourse sampling, relative frequencies were calculated for the six most dominant species, to account for up to 100% of the population, with some outlier species unaccounted for at certain sites or on certain sampling dates. The relative frequencies (q_r) of microbial species from the plate counts were calculated by comparing the total individuals sampled in each species (r*) to the total number of individuals in the sampled population (N) (q_r= r*/N) (Good, 1953). Relative frequencies were expressed as percentages by multiplying q_r by 100.

In the survey, population differences between the cultivars were tested using the Welch statistic in lieu of ANOVA testing to account for differences in variation caused by different sample sizes. In the Welch test, the denominator of the F ratio is adjusted for the heterogeneity in variance. The *p*-value is interpreted with the same approach as in ANOVA (Addinsoft, Inc., XLSTAT-Pro, 2003). A confidence interval of 95% was employed. A *p*-value of less than 0.05 indicated the null hypothesis could be reliably rejected.

In the timecourse sampling and pathogenicity assay, the replicates were averaged using Microsoft Excel. ANOVA was performed using Microsoft Excel 2000/XLSTAT-Pro (Version 7.2, 2003, Addinsoft, Inc., USA), and the Student Newman–Keuls (SNK) multiple range test was employed for mean separations with a confidence interval of 95%. An ANOVA-calculated *p*-value below 0.05 was considered to indicate data that could reliably lead to the rejection of the null hypothesis.

3.3 RESULTS

3.3.1 Population survey. Sour rotted clusters were sampled for yeast and bacterial populations from 14 sites across the Niagara Peninsula, designated viticultural area, with two cultivars and eight sub-appellations represented (Figure 3.1). Six dominant morphogroups were recovered and representative isolates were identified by genetic sequencing: *Aureobasidium pullulans*, *Bacillus subtilis*, *Pichia membranifaciens*, *Candida zemplinina*, *Hanseniaspora uvarum*, and acetic acid bacteria (Figure 3.2). The acetic acid bacterial population consisted of mainly *Gluconobacter oxydans*, but also *Gluconobacter cerinus* and *Acetobacter malorum*. All of the species of acetic acid bacteria displayed variation by strain in pigmentation. Strains of both *Acetobacter* and *Gluconobacter* varied from colourless to pink or brown. The fungal pathogen *Botrytis cinerea* was detected at 8 of 14 sites. Its effects on the composition of the yeast and bacterial population were non-significant (Appendix VII).

Relative frequencies were calculated using morphogroup counts to compare the dominance of each species at each site (Figure 3.2). Acetic acid bacteria and *H. uvarum* were recovered from all sites. Relative frequencies of these organisms varied from site to site with acetic acid bacteria displaying a frequency as low as 4.0% in Riesling at site "11" and as high as 96.7% in Pinot Noir at site "3", and *H. uvarum* displaying a frequency as low as 1.1% in Riesling at site "7" and as high as 72.8% also in Riesling at site "9" (Figure 3.2). The mean relative frequencies of each morphogroup were compared using the Welch statistic to determine if the frequencies were different when comparing Pinot Noir and Riesling cultivars. The relative frequency of *H. uvarum* was significantly different between cultivars, with a mean of 40.6% in Riesling, and a mean of 10.1% in Pinot Noir. The mean relative frequencies of the other morphogroups were not significantly different between these cultivars (Table 3.1).

H. uvarum was the dominant species at sites "8", "9", "11", "12", and "13" with frequencies of 45.0, 72.8, 48.0, 42.6, and 66.9%, respectively (Figure 3.2), with higher relative frequencies than any other species sampled from the sites. Acetic acid bacteria showed dominance at sites "1", "3", "4", "7", and "10" with frequencies of 43.7, 96.7, 84.8, 90.7, and

60.1%, respectively (Figure 3.2). When the acetic acid bacteria displayed dominance above 80%, as at sites "3", "4", and "7", the relative frequency of *H. uvarum* reached its lowest frequencies of 1.7, 4.5, and 1.1%, respectively (Figure 3.2).

C. zemplinina was found at 12 of 14 sites and was the dominant species at site "5", with a relative frequency of 54.8%, and site "14" with a relative frequency of 62.3% (Figure 3.2). *P. membranifaciens* was recovered from 8 of 14 sites and was dominant at site "6" with a relative frequency of 23.7%. *P. membranifaciens* was recovered from sites "5", "6", "12", and "13", with frequencies above 2.0% (Figure 3.2). All of these sites were geographically clustered in the Creek Shores and Short Hills Bench sub-appellations (Figure 3.1). *B. subtilis* was recovered from 3 of 14 sites, with dominance at site "2" with a relative frequency of 39.4% (Figure 3.2). The sites containing *B. subtilis* were all located along the Niagara Escarpment in the Beamsville Bench, Twenty Mile Bench, and Short Hills Bench sub-appellations (Figure 3.1). *A. pullulans* was recovered at 3 out of 14 sites with a relative frequency as low as 0.2% at site "1" and as high as 17.0% at site "11" (Figure 3.2).

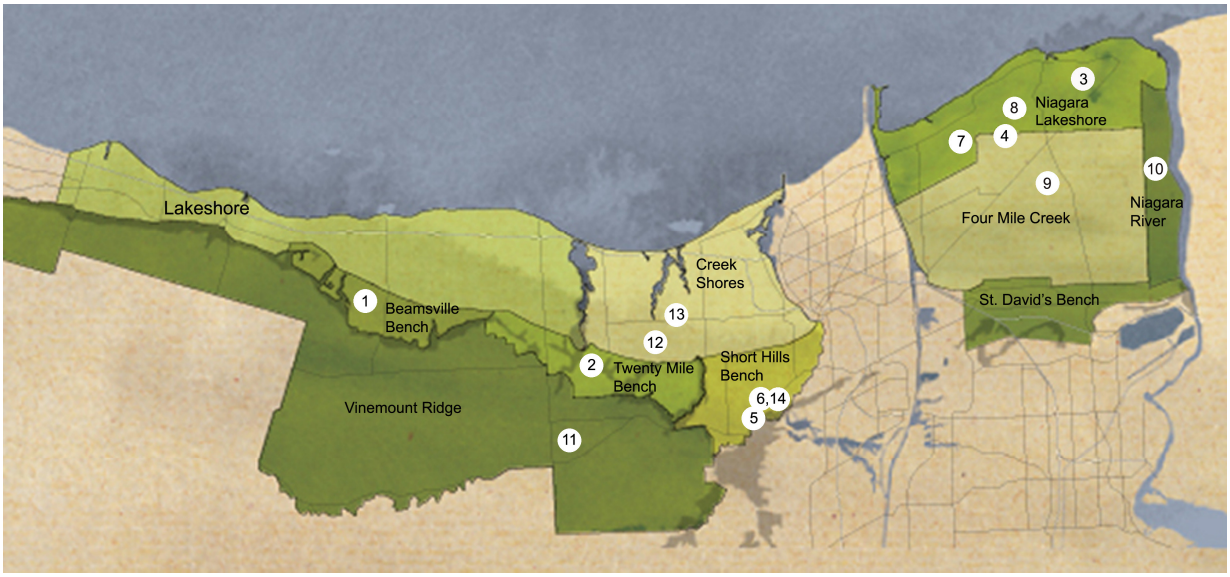


Figure 3.1 Locations in the Niagara Peninsula, designated viticultural area, Ontario, Canada, used to sample the microbial ecology of *Vitis vinifera* cv. Pinot Noir and cv. Riesling infected with sour rot. Results are reported in Figure 3.2. The map is reproduced with permission of the Vintners' Quality Alliance Ontario.

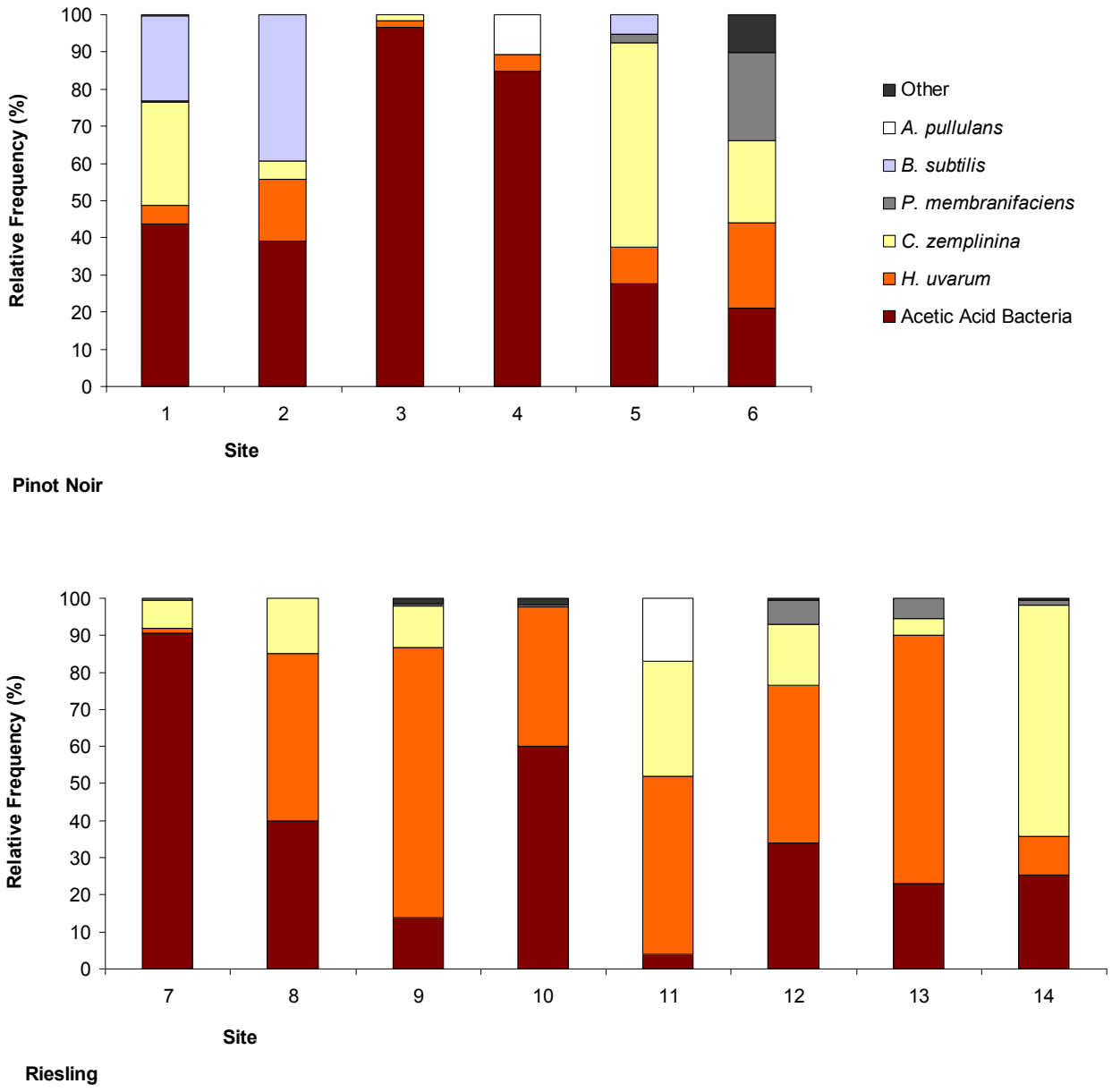


Figure 3.2 Relative frequency (%) of the most common organisms isolated from sour rotted grapes in *Vitis vinifera* cv. Pinot Noir (top) and cv. Riesling (bottom). Site numbers correspond to locations shown in Figure 3.1. Raw data is given in Appendix VII.

Table 3.1 Mean relative frequencies of the six most dominant morphogroups recovered from *Vitis vinifera* cv. Pinot Noir and cv. Riesling. Samples were taken from sour rotted grapes from 14 sites in the Niagara Peninsula, designated viticultural area (Figure 3.1 and 3.2). Raw data is given in Appendix VII. Significant data is marked with an *.

Colony	Cultivar	Mean Relative Frequency	Welch's statistic
Acetic Acid Bacteria	Pinot Noir	52.2	$F(1,10.15) = 0.97$
	Riesling	36.4	$p = 0.347$
H. uvarum	Pinot Noir	10.1	$F(1,8.90) = 10.60$
	Riesling	40.6	$p = 0.010$ *
C. zemplinina	Pinot Noir	18.6	$F(1,10.58) = 0.00$
	Riesling	18.5	$p = 0.997$
P. membranifaciens	Pinot Noir	4.4	$F(1,5.60) = 0.42$
	Riesling	1.8	$p = 0.541$
B. subtilis	Pinot Noir	11.3	$F(1,5.00) = 2.80$
	Riesling	0.1	$p = 0.155$
A. pullulans	Pinot Noir	1.8	$F(1,12.00) = 0.01$
	Riesling	2.1	$p = 0.912$

3.3.2 Timecourse sampling. In the Niagara Peninsula, designated viticultural area, Ontario, Canada, microbial population samples were taken over time from four replicated plots in each of two commercial vineyards, one cv. Riesling and one cv. Pinot Noir in 2012. Sour rot was first detected on September 7 at both sites. As depicted in Figure 3.3, at both the Riesling and Pinot Noir sites, grape clusters started in the pre-harvest period with a high relative frequency of the oxidative yeast, *A. pullulans*, the filamentous fungus, *Alternaria alternata*, and the bacterium, *Pseudomonas fluorescens*. At the Riesling site, *A. pullulans* started the season with a mean relative frequency of 43.5%, *A. alternata* started with a mean relative frequency of 39.2%, and *P. fluorescens* with a mean relative frequency of 13.8%. At the Pinot Noir site, *A. pullulans* started the season with a mean relative frequency of 34.9%, *A. alternata* started with a mean relative frequency of 18.4%, and *P. fluorescens* with a mean relative frequency of 44.6% (Figure 3.3).

The ascomycete yeasts and acetic acid bacteria associated with sour rot were not detected in either vineyard before symptoms of sour rot were observed. On September 7, along with the

first symptomatic grapes, acetic acid bacteria and *H. uvarum* were observed to take dominance. In the Riesling vineyard, *H. uvarum* had a mean relative frequency of 58.7%, and the acetic acid bacteria a mean relative frequency of 17.4%. In the Pinot Noir vineyard, *H. uvarum* had a mean relative frequency of 43.2%, and the acetic acid bacteria a mean relative frequency of 51.4% (Figure 3.3). These frequencies were significantly higher than the mean relative frequencies observed on the earlier sampling dates (Table 3.2). The oxidative yeast, *A. pullulans* still showed presence on September 7 in Riesling, with a mean relative frequency of 17.8% (Figure 3.3), although this was significantly lower than the previous two sampling dates (Table 3.2). The mean relative frequencies of *A. pullulans*, *A. alternata*, and *P. fluorescens* otherwise tailed off (Figure 3.3) with significantly lower values (Table 3.2).

C. zemplinina was not observed in the vineyards until the sampling date after sour rot symptoms appeared, September 16 in Riesling and September 17 in Pinot Noir. On this date in Riesling, *C. zemplinina* displayed a mean relative frequency of 19.8%, and in Pinot Noir, a mean relative frequency of 46.0% (Figure 3.3). In Pinot Noir, this was significantly higher than preceding observations (Table 3.2). Subsequently, the relative frequency of acetic acid bacteria dropped significantly in the Pinot Noir vineyard (Table 3.2) to a mean relative frequency of 24.1% on September 17, and 12.1% on October 3 (Figure 3.3). The mean relative frequencies of acetic acid bacteria in the Riesling vineyard and *H. uvarum* in both the Riesling and Pinot Noir vineyards remained statistically similar after first observation on September 7 (Table 3.2).

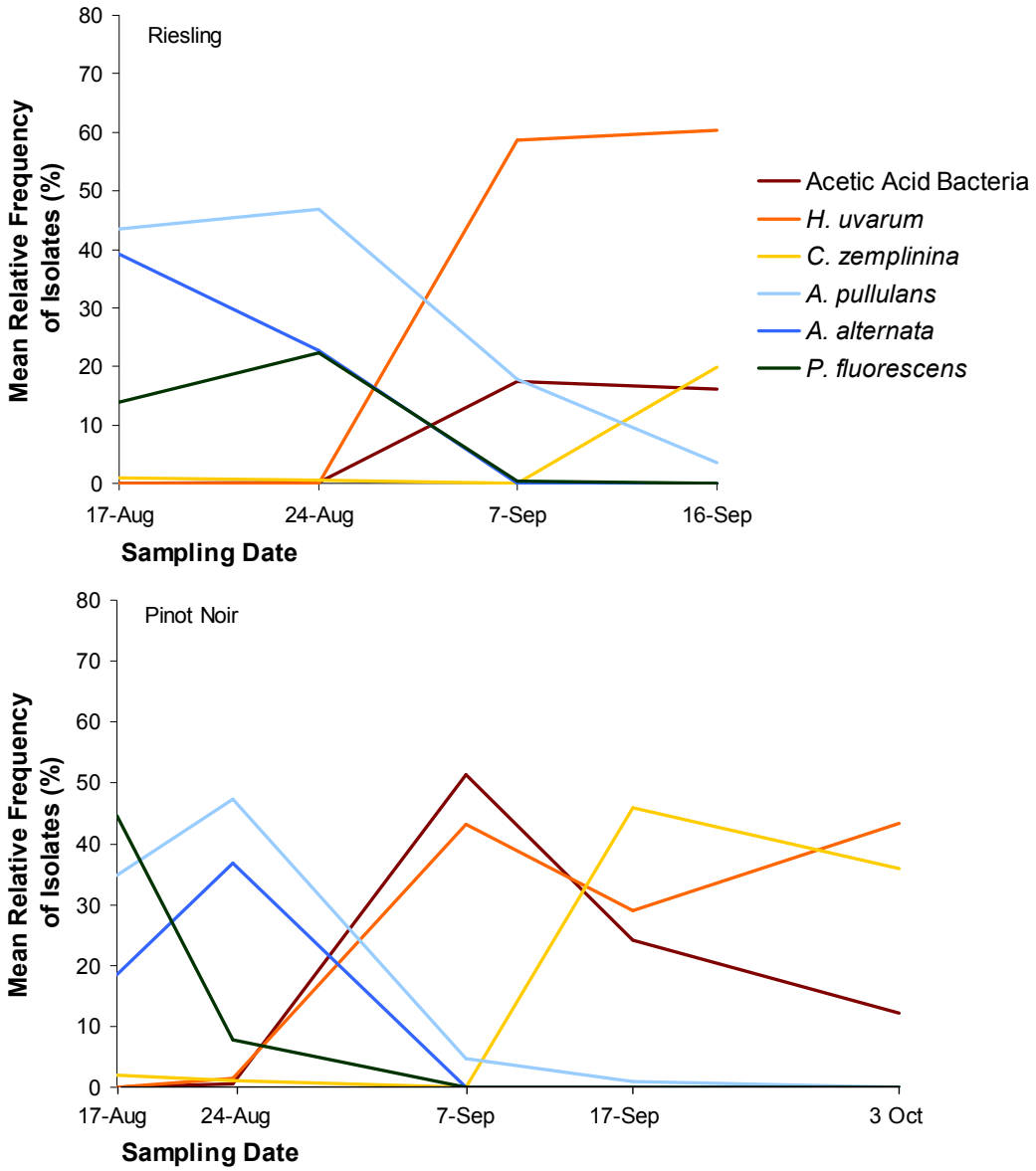


Figure 3.3 Mean relative frequency (%) of the most common organisms isolated from grapes over time. Samples were collected from four replicated plots in commercial vineyards of *Vitis vinifera* cv. Riesling (above) and cv. Pinot Noir (below) in 2011 (N = 20 clusters on each sampling date). Sour rot was first detected on September 7. Student-Newman-Keuls means separations and *p*-values for differences in each species over time are presented in Table 3.2.

Table 3.2 Analysis of Variance statistics considering differences in the mean relative frequencies of organisms sampled over time in two vineyards, cv. Riesling and cv. Pinot Noir. Mean ripeness is also presented in °Brix. Corresponding means are presented in Figure 3.3. Significant data is marked with an *. Horizontal cells with the same letters are not significantly different according to the Student Newman-Keuls multiple range test.

		Cultivar Riesling				
Organism	**ANOVA**	**SNK Group on each Sampling Date**				
		17-Aug	**24-Aug**	**7-Sep**	**16-Sep**	
Acetic Acid Bacteria	$F(3,3) = 7.15$ $p = 0.005$ *	b	b	a	a	
H. uvarum	$F(3,3) = 16.85$ $p = 0.0002$ *	b	b	a	a	
C. zemplinina	$F(3,3) = 2.66$ $p = 0.091$	non-significant				
A. pullulans	$F(3,3) = 5.59$ $p = 0.011$ *	a	a	b	b	
A. alternata	$F(3,3) = 4.32$ $p = 0.025$ *	a	ab	b	b	
P. fluorescens	$F(3,3) = 3.51$ $p = 0.045$ *	ab	a	b	b	
Mean Ripeness (°Brix)		13.33	15.39	17.11	17.79	
		Cultivar Pinot Noir				
Organism	**ANOVA**	**SNK Group on each Sampling Date**				
		17-Aug	**24-Aug**	**7-Sep**	**17-Sep**	**3-Oct**
Acetic Acid Bacteria	$F(3,4) = 15.83$ $p = < 0.0001$ *	c	c	a	b	bc
H. uvarum	$F(3,4) = 6.13$ $p = 0.003$ *	b	b	a	a	a
C. zemplinina	$F(3,4) = 18.19$ $p = < 0.0001$ *	b	b	b	a	a
A. pullulans	$F(3,4) = 6.37$ $p = 0.003$ *	a	a	b	b	b
A. alternata	$F(3,4) = 7.41$ $p = 0.001$ *	a	b	bc	c	c
P. fluorescens	$F(3,4) = 13.75$ $p = < 0.0001$ *	a	b	b	b	b
Mean Ripeness (°Brix)		12.89	16.41	18.53	21.31	After Harvest

3.3.3 Sequential inoculation pathogenicity assay. Wild isolates genetically identified as *C. zemplinina*, *H. uvarum*, and the acetic acid bacterium, *G. oxydans*, were recovered from the Niagara Peninsula in 2011. These organisms were applied to four replicates of five wounded green seedless table grapes. After 48 hours, the organisms were reapplied so that each of the organisms was paired with each of the other organisms in succession after disturbance by bleach sterilization. No symptoms developed in any of the uninoculated berries. Regardless of the order of inoculation, after ten days of incubation at 22°C, symptoms were usually significantly greater when *G. oxydans* was present, as determined by a rating scale of zero to four. *H. uvarum* alone was capable of producing symptoms that rated as highly as those with *G. oxydans* (Figure 3.4b).

G. oxydans alone had a mean pathogenicity rating of 3.38 after ten days. When *H. uvarum* succeeded *G. oxydans*, the mean pathogenicity rating was 3.40, and when *C. zemplinina* succeeded *G. oxydans*, the mean rating was 3.11, which was not significantly different (Figure 3.4a). *H. uvarum* had a mean pathogenicity rating of 2.32 alone, and 2.86 succeeded by *G. oxydans*, which was significantly higher than *H. uvarum* succeeded by *C. zemplinina* with a mean pathogenicity rating of 2.08 (Figure 3.4b). The mean pathogenicity rating of *C. zemplinina* alone was 1.10, and 1.49 succeeded by *H. uvarum*. The rating was significantly higher when *C. zemplinina* was succeeded by *G. oxydans* at 2.88 (Figure 3.4c).

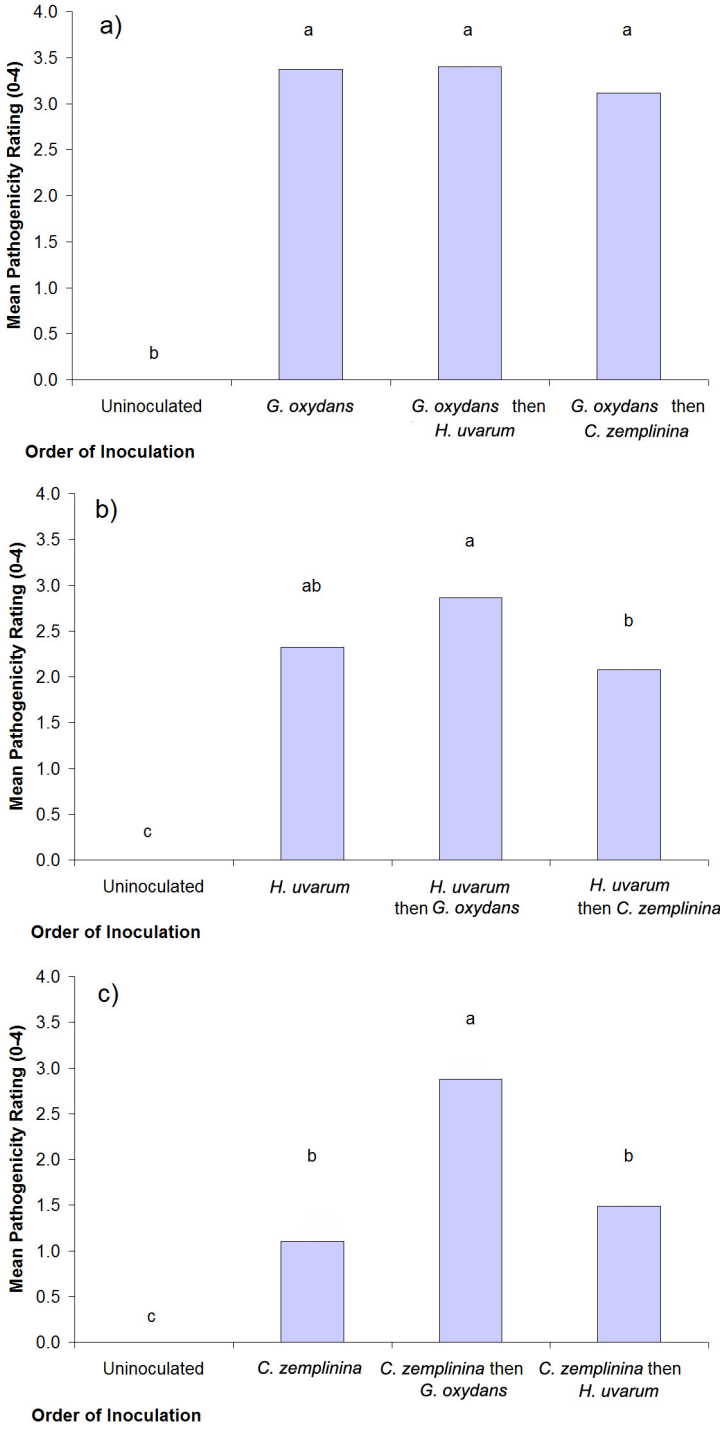


Figure 3.4 Mean pathogenicity ratings of *Gluconobacter oxydans*, *Hanseniaspora uvarum*, and *Candida zemplinina* in sequential inoculations. Green seedless table grapes were wounded and sequentially inoculated with a 48 hour difference, incubated ten days at 22°C then rated on a scale of zero to four (N = 20 berries per order of inoculation). ANOVA a) $F(3,3) = 295.16$, $p = <0.0001$, b) $F(3,3) = 39.58$, $p = <0.0001$, c) $F(3,3) = 17.22$, $p = <0.0001$. Bars with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

Acetic acid was measured from the grapes sequentially inoculated and incubated ten days in the pathogenicity assay (Figure 3.4). Background acetic acid was measured in the injured, uninoculated grapes in the amount of 0.21 g acetic acid/L. Trends were very similar in volatile acidity compared to the pathogenicity ratings, with significantly higher volatile acidity when the acetic acid bacterium, *G. oxydans*, was present. *G. oxydans* alone had a mean volatile acidity of 3.37 g acetic acid/L. When succeeded by the yeasts, the volatile acidity was not significantly different. *G. oxydans* succeeded by *H. uvarum* had a mean volatile acidity of 3.85 g acetic acid/L. *G. oxydans* succeeded by *C. zemplinina* had a mean volatile acidity of 3.04 g acetic acid/L (Figure 3.5a).

Alone, *H. uvarum* had a mean volatile acidity of 0.46 g acetic acid/L alone, and 0.40 g acetic acid/L succeeded by *C. zemplinina.* When *H. uvarum* was succeeded by *G. oxydans*, the volatile acidity was significantly higher at 2.97 g acetic acid/L (Figure 3.5b). The mean volatile acidity of *C. zemplinina* was 0.16 g acetic acid/L alone and 0.26 g acetic acid/L when *H. uvarum* succeeded *C. zemplinina*. The mean volatile acidity was significantly higher when *G. oxydans* succeeded *C. zemplinina* at 2.16 g acetic acid/L (Figure 3.5c).

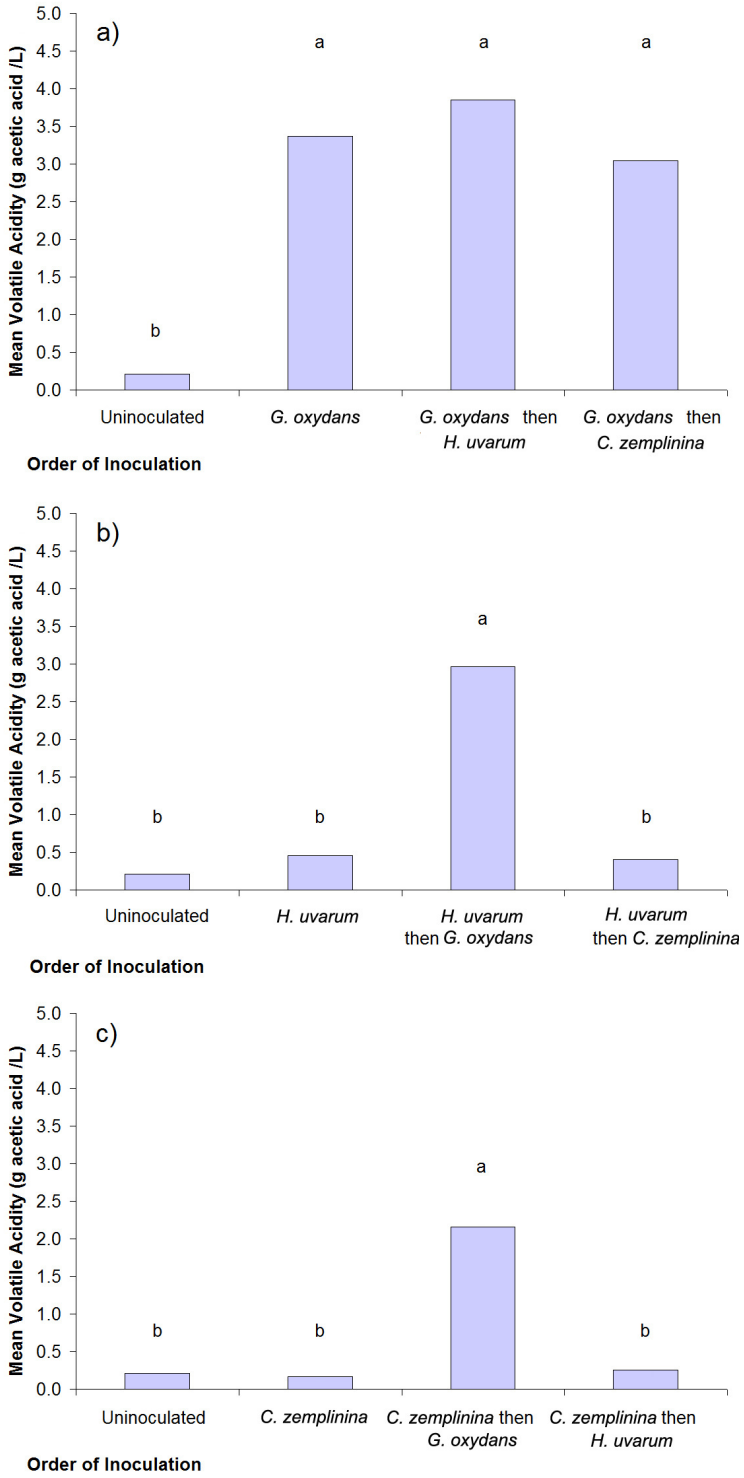


Figure 3.5 Mean volatile acidity (g acetic acid/L) from grapes sequentially inoculated with sour rot-isolated strains of *Gluconobacter oxydans, Hanseniaspora uvarum*, and *Candida zemplinina*. Green seedless table grapes were wounded and sequentially inoculated. After ten days incubation at 22°C, acetic acid was measured (N = 20 berries per order of inoculation and duplicated readings). ANOVA a) $F(3,3) = 24.85$, $p = <0.0001$, b) $F(3,3) = 118.56$, $p = <0.0001$, c) $F(3,3) = 14.57$, $p = <0.0001$. Bars with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

3.4 DISCUSSION

Microbial populations in grapes are affected by rainfall, temperature, grape variety, and agrochemicals (Loureiro and Malfeito-Ferreira, 2003), and increase in numbers with grape maturity (Combina et al., 2005). Approaching harvest, the most common bacterial and yeast isolates found across the Niagara Peninsula, designated viticultural area, Ontario, Canada, in sour rotted grapes in 2011 were acetic acid bacteria, and the yeasts *H. uvarum* and *C. zemplinina* (Figure 3.2), agreeing with literature (Barata et al., 2012a; Gravot et al., 2001). The relative frequencies (q_r) of each sour rot-associated species were calculated by comparing the total individuals sampled in each species (r*) to the total number of individuals in the sampled population (N) (q_r= r*/N) (Good, 1953). Relative frequencies of each species were sampled from multiple sites and two cultivars across the Niagara Peninsula to determine any correlations with grape cultivar and microclimate. There was a statistically higher relative frequency of *H. uvarum* recovered from cv. Riesling compared to cv. Pinot Noir, with a Welch statistic *p*-value of 0.010, taking into account the effect sample size had on variation in the data (Table 3.1). Therefore, the null hypothesis that there were no differences in the composition of the population between cultivars could be rejected. Similarly, the literature has presented data for yeast population differences by cultivar. In Slovenia, *A. pullulans* was isolated more frequently from two red cultivars compared to a white cultivar (Raspor et al., 2006). This is in contrast to research in China, where three grape cultivars were sampled for microbial populations in four regions. *H. uvarum* was found to be most frequent, but varied more by region than by grape cultivar, with a lower frequency in regions with cool and dry conditions (Li et al., 2010). The present study also took the factors of spatiality and microclimate into consideration.

The present study observed that geographical features may have been correlated with the relative frequency of organisms, according to microclimate (Figure 3.1 and 3.2). Acetic acid bacteria were observed to have the greatest relative frequency at sites “3”, “4”, and “7”, clustered in the Niagara Lakeshore sub-appellation (Figure 3.1 and 3.2), recognized to have wetter

conditions (Schlosser et al., 2005). At these sites, *H. uvarum* showed its lowest relative frequencies, contradicting the research of Li et al., in which *H. uvarum* had a higher frequency in wet regions (2010). A major difference between these papers is that Li et al. (2010) studied healthy and undamaged grapes, while the samples in the present study were infected with sour rot.

B. subtilis was also recovered with higher relative frequency from sites that clustered geographically, mainly from sites "1", "2", and "5" in the Bench sub-appellations of the Niagara Escarpment (Figure 3.1 and 3.2). The Niagara Escarpment has good drainage due to elevation and slope and also experiences less of the lake effect (Schlosser et al., 2005). However, the recovery of *B. subtilis* may have had more to do with a third factor, the pest management program of the vineyard. If these were organic vineyards they may spray the biological control agent labeled Serenade MAX, which contains a strain of *B. subtilis* (AgraQuest Inc., 2010), introducing this inoculum to the vineyard. Interestingly, this product is labeled for the management of sour rot (AgraQuest Inc., 2010), which may indicate its competitiveness against organisms such as acetic acid bacteria and *H. uvarum*. To more fully integrate the population data with all possible factors, replicated samples would need to be taken from the vineyards over a timecourse noting grape ripeness and development of sour rot, and the pest management program of the vineyard should be taken into consideration. Population data could also be more sensitive by directly sampling DNA. For example, using an approach such as pyrosequencing, in which adapter-ligated PCR products from a mixed template are bound to microbeads that can each be sequenced in separate picotiter wells (Will et al., 2010). The present approach of culturing could not reliably detect strains with fewer than 20,000 colony forming units (CFU)/mL in the original sample, the highest sensitivity being $1x10^3$ dilution plates. Less than one cell would grow on each plate. Live culturing also could not detect viable but not culturable (VBNC) samples.

Grape populations were sampled by culturing arbitrarily selected grapes in a replicated timecourse from a *Vitis vinifera* cv. Riesling and a cv. Pinot Noir vineyard in the period after veraison in 2012. Three species were found to be dominant in the sampling dates preceding the first detection of sour rot: an oxidative yeast, *A. pullulans*, a filamentous fungus, *A. alternata*, and a bacterium, *P. fluorescens* (Figure 3.3). *A. pullulans* and *P. fluorescens* were shown to have minimal effect on the symptoms of sour rot in pathogenicity assays (Figure 2.2). *A. alternata* infects grapes mainly around the pedicel producing lesions, visible mycelia and conidia (Swart and Holz, 1994), which are symptoms unrelated to sour rot. The relative frequencies of these species were significantly reduced by September 7, when symptoms of sour rot were first observed. In Riesling, the mean relative frequency of *A. pullulans* had an ANOVA-calculated *p*-value of 0.011, *P. fluorescens* a *p*-value of 0.045, and *A. alternata* a *p*-value of 0.025, over time (Table 3.2). In Pinot Noir, the mean relative frequency of *A. pullulans* had an ANOVA-calculated *p*-value of 0.003, *P. fluorescens* a *p*-value of <0.0001, and *A. alternata* a *p*-value of 0.001, over time (Table 3.2). Therefore, the null hypothesis there were no changes to the population structure over time could be rejected. These results were similar to a study in Bordeaux, France, where variation in the yeast population was mainly attributable to berry development stage (Renouf et al., 2005). *A. pullulans* was more dominant at berry set and was not found at harvest (Renouf et al., 2005).

Acetic acid bacteria and *H. uvarum* were found at their highest relative frequency on the date sour rot symptoms were first detected, September 7 (Figure 3.3). Acetic acid bacteria and *H. uvarum* were both shown to cause the symptoms of sour rot (Figure 2.2). In Riesling, the mean relative frequency of acetic acid bacteria had an ANOVA-calculated *p*-value of 0.005 and the mean relative frequency of *H. uvarum* a *p*-value of 0.0002, over time. In Pinot Noir, the mean relative frequency of acetic acid bacteria had an ANOVA-calculated *p*-value of <0.0001 and the mean relative frequency of *H. uvarum* a *p*-value of 0.003, over time (Table 3.2). Therefore, the null hypothesis that there were no changes to the population structure over time could be

rejected. This is similar to previous research which observed that fermentative yeasts such as *H. uvarum* and *C. zemplinina* tended to dominate as grapes approached harvest, when the acetic acid bacterium, *G. oxydans,* could also be detected (Renouf et al., 2005). An increase in the population of these species is associated with grape damage and these species are found associated with sour rotted grapes (Barata et al., 2012a).

On the sampling date after sour rot detection, September 17, the relative frequency of acetic acid bacteria dropped significantly in Pinot Noir, while the relative frequency of *H. uvarum* remained constant and *C. zemplinina* gained dominance (Figure 3.3), with a statistically significant increase in relative frequency indicated by an ANOVA-calculated *p*-value of <0.0001 over time (Table 3.2). The increase in the relative frequency of *C. zemplinina* was not significant in Riesling with an ANOVA-calculated *p*-value of 0.091 over time (Table 3.2), and the relative frequency of acetic acid bacteria and *H. uvarum* remained steady in this cultivar between September 7 and 16 (Figure 3.3 and Table 3.2). Therefore, the null hypothesis that there were no changes to the population structure over time could be rejected. Particularly in Pinot Noir, there was an indication of an ecological succession of grapes first inhabited by epiphytes such as *A. pullulans*, *A. alternata*, and *P. fluorescens* at the beginning of the ripening season, replaced by acetic acid bacteria and *H. uvarum* once sour rot symptoms were detected, followed yet by an increase in *C. zemplinina* after symptom detection (Figure 3.3). In Riesling, trends were similar, yet the succession of acetic acid bacteria and *H. uvarum* by *C. zemplinina* was not found to be significant in 2012 (Figure 3.3). Sampling could be initiated earlier in the season, at berry set, to determine the first organisms to inhabit berries, outlining the primary succession.

C. zemplinina is a fructophilic yeast (Andorra et al., 2010; Ciani and Ferraro, 1998), a property which may affect its dominance once glucose becomes scarcer in sour rot-affected berries. *C. zemplinina* was shown to minimally produce symptoms associated with sour rot (Figure 2.2), and was shown to be a low acetic acid producer (Figure 2.4), confirming previous

research in fermentations (Magyar and Toth, 2011). A succession of acetic acid bacteria followed by *C. zemplinina* may signify a change in the vigour of sour rot symptom production as the disease progresses, and *C. zemplinina* may be an opportunist of the sour rot environment. Further research could elucidate the effect of microbial succession on disease symptoms through sequential inoculation in the laboratory, as this study sought to do. To explore the correlation of factors other than ripening with the microbial population, weather could be monitored from the sampling vineyards and rain events and temperature could be taken into consideration. Also, sampling could be performed at more vineyard sites to provide replication of cultivars under different environmental conditions, to demonstrate whether the differences in the succession of microbes could be associated with the factor of grape cultivar.

To study the effect of microbial succession on sour rot symptoms, grapes were sequentially inoculated in *in vitro* pathogenicity assays incubated 10 days at 22°C. It was determined that regardless of the order of succession of organisms, sour rot symptoms were primarily associated with *G. oxydans* (Figure 3.4), although *H. uvarum* alone was also able to produce symptoms that were not significantly different compared to the yeast succeeded by *G. oxydans* (Figure 3.4b). Successions including *C. zemplinina* and *H. uvarum* without *G. oxydans* had significantly lower pathogenicity ratings than those with *G. oxydans* (Figure 3.4b and 3.4c). ANOVA-calculated *p*-values were <0.0001, which indicated that the null hypothesis, that there were no changes in sour rot symptoms when the microorganisms were applied in sequences representing different possible successions, could be rejected. Similarly, the successions including *G. oxydans* displayed significantly higher levels of acetic acid compared to the successions that did not include *G. oxydans* (Figure 3.5). With ANOVA-calculated *p*-values of <0.0001, this indicated that the null hypothesis, that there were no differences in the concentration of acetic acid when the microorganisms were applied in different sequences representing different possible successions, could be rejected.

The findings that sour rot symptoms were mainly produced by acetic acid bacteria conform to the more recent literature (Barata et al., 2012a) and the capacity for *H. uvarum* to cause sour rot has also been observed (Gravot et al., 2001). *Gluconobacter* has been seen to dominate earlier stages of rot and even colonize sound berries, while *Acetobacter* grew in later stages of sour rot (Barata et al., 2012a; Du Toit and Pretorius, 2002), using ethanol as opposed to sugar as a substrate (Bartowsky and Henschke, 2008). *H. uvarum* and *C. zemplinina* were shown to produce ethanol (Andorra et al., 2010), indicating they may promote acetic acid bacterial growth if inoculated earlier in succession. Some strains of *C. zemplinina* have been shown to produce lower acetic acid values than even *Saccharomyces cerevisiae*, and the species is currently studied in co-inoculations or step-wise inoculations in wine fermentations to reduce acetic acid (Ciani and Ferraro, 1998). *C. zemplinina* may similarly reduce acetic acid if inoculated in succession with the organisms mainly responsible for sour rot symptoms. The ability of yeasts to promote acetic acid bacterial growth or mitigate acetic acid production were not observed in the present study, but perhaps a longer period of incubation than two days between successive inoculations *in vitro* is necessary. In the field, it took ten days for a succession to occur between acetic acid bacteria and *C. zemplinina*, with *C. zemplinina* eventually outnumbering the bacteria (Figure 3.3). As well, studying more species and strains could improve understanding of the sour rot microbial community, since the yeasts and bacteria associated with sour rot have been noted for their strain-dependent qualities (Drysdale and Fleet, 1988; Romano et al., 1997; Strauss et al., 2001). Sequential inoculation experiments could also be scaled up into a field study by spraying inoculum over replicated plots in the vineyard. Sour rot disease symptoms could be compared on a timeline amongst different inoculation sequences, with the microbes growing under fluctuating weather conditions, which is more true to the challenges facing grape growers. If the timing of the microbial succession could be understood alongside the progress of sour rot symptoms in the field, treatments targeting the sour rot organisms could be planned for the management of disease.

CHAPTER 4: ENVIRONMENTAL CONTRIBUTIONS TO GRAPE SOUR ROT IN THE NIAGARA PENINSULA, DESIGNATED VITICULTURAL AREA

4.1 INTRODUCTION

Sour rot can be an economic problem in wine grapes in the cool climate region of the Niagara Peninsula, designated viticultural area, Ontario, Canada, with crop losses varying from year to year. Wet weather during pre-harvest periods tends to promote the disease (Loureiro and Malfeito-Ferreira, 2003), but there is no research indicating the exact weather parameters in which the disease thrives. Sour rot can affect whole bunches and is considered late-season because it develops when sugars accumulate after veraison, as grapes approach ripeness (Gravot et al., 2001). The disease is associated with grape injury. This can be caused by insect pests and pathogens which weaken the grape skin, or meteorologically by rain and hail (Barata et al., 2012a). Cultivars with thin skins and tightly packed clusters, such as Pinot Noir and Riesling, can be particularly susceptible to injury and therefore sour rot (Zoecklein et al., 2000).

Beginning as a discolouration at an injury site or where the grape attaches to the pedicel (Bisiach et al., 1986; Gravot et al., 2001), sour rot leads to tissue disaggregation, while the berry detaches from the pedicel and juice starts to leak (Guerzoni and Marchetti, 1987). Elevated volatile acidity, particularly an increase in acetic acid and the acetic acid-ethanol ester, ethyl acetate, are the major chemical markers of disease (Guerzoni and Marchetti, 1987; Marchetti et al., 1984). Acetic acid smells of vinegar, while ethyl acetate has a solvent smell similar to nail polish remover, both of which can be readily detected in highly affected vineyards. Acetic acid and ethyl acetate are associated with wine spoilage (Barata et al., 2012a). Infected clusters with volatile acidity above 0.24 g/L acetic acid are commonly rejected by wineries (Grape Growers of Ontario, 2010).

The symptoms of sour rot are associated with increased growth of yeasts and bacteria able to survive under anaerobic conditions during vinification (Barata et al., 2012a). This includes

Hanseniaspora uvarum (syn. *Kloeckera apiculata*), *Candida zemplinina*, and *Pichia membranifaciens* (Barata et al., 2008a; Bisiach et al., 1986; Gravot et al., 2001), and species of acetic acid bacteria, *Gluconobacter* and *Acetobacter*, which are the primary agents causing sour rot symptoms (Barata et al., 2012a; Gravot et al., 2001). Previous research in the Niagara Peninsula, designated viticultural area, Ontario, Canada associated *H. uvarum*, *Gluconobacter oxydans*, and *Acetobacter malorum* with symptoms in sour rotted grapes (Huber et al., 2012).

Loureiro and Malfeito-Ferreira (2003) reported that as grapes ripen, the yeasts and bacteria colonize surfaces up to 10^3-10^5 cells/mL on mature, sound berries. The mixed population is affected by rainfall, temperature, grape variety, and agrochemicals, and tends to localize around sites where there is juice leakage or gummy secretions (Loureiro and Malfeito-Ferreira, 2003). Damage to the grape can increase the microbial load up to 10^8 cells/mL (Barata et al., 2008a).

The current study observed weather and ripening conditions in the field over four years and associated these conditions with sour rot disease progress, to depict which factors were correlated to the disease. The factors of temperature and berry maturity were translated into *in vitro* experiments. The organisms associated with sour rot were incubated in a model host at different temperatures. The null hypothesis was that the temperature would not have an effect on sour rot disease progress. These organisms were also incubated in wine grapes of different maturities. The null hypothesis was that ripeness would not have an effect on sour rot symptoms, measured by a pathogenicity rating scale and the concentration of acetic acid.

4.2 MATERIALS AND METHODS

4.2.1 Correlation of daily mean temperature, total daily rainfall and berry maturity to sour rot development in the vineyard. Daily mean temperature, total daily rainfall, and berry maturity (°Brix) were monitored in a commercial vineyard of *Vitis vinifera*, cv. Riesling in 2009, 2010, 2011, and 2012. Vines were trained to low cane with vertical shoot positioning in the Twenty Mile Bench sub-appellation of the Niagara Peninsula, designated viticultural area, Ontario, Canada (Figure 3.1). Insecticide and fungicide applications and viticultural practices

were completed by the grower cooperator according to standard practices. Four replicated panels of five vines were randomly selected for sampling fruit maturity and sour rot disease progress was monitored in four different replicate panels. At intervals starting at veraison, 100 grape berries were arbitrarily selected and collected from each fruit maturity sampling plot and soluble solids were measured in °Brix, using a laboratory refractometer (Reichert Abbe Mark II, USA). Disease progress of sour rot was assessed in the vineyard on 25 arbitrarily selected grape clusters per plot using a modified Barratt-Horsfall rating scale of severity (0, 1, 3, 5, 10, 25, 50, 75, 90, 95, 97, 99, or 100% of the cluster infected) (Horsfall and Barratt, 1945). Incidence of sour rot was reported from the same plots as a whole number representing the number of infected clusters from the 25 observed. When temperature and rainfall were monitored at the cv. Riesling vineyard, a WatchDog 2700 weather station (Spectrum Technologies, Canada) was used on site from within the research plot, with sensors above the fruiting zone. At 15 minute intervals, temperature in degrees Celsius was measured to the nearest tenth of a degree, and precipitation to the nearest tenth of a millimetre (litre per square metre). Daily mean temperature and total daily rainfall were then calculated.

4.2.2 Temperature range pathogenicity assay. Four incubators were held at each temperature range: 5-10, 10-15, 15-20 and 20-25°C, and pathogenicity experiments were replicated *in vitro* with incubators each holding a second temperature. Intact green seedless table grapes, with pedicel attached, were pre-washed in Dawn dish detergent (Procter & Gamble, USA), rinsed three times with tap water, agitated in 10% commercial bleach (0.5% sodium hypochlorite) for twenty minutes, then rinsed twice with sterile, distilled water and suspended on sterilized galvanized wire mesh with 1 cm holes placed in plastic bins lined with moist paper towels to maintain humidity. An experimental unit was comprised of five berries wounded by poking three times with a sterile dissecting needle, which was replicated in three bins, for a total of six replications in each temperature range. Field-isolated *G. oxydans* and *H. uvarum*, sampled from the Niagara Peninsula, designated viticultural area, in 2011 and genetically identified as in

Chapter 2, were maintained on yeast peptone dextrose agar (YPD). These isolates were applied to the wounds in aliquots of 10 µL from suspensions of 10^8 cells/mL prepared in dH_2O. Five uninjured and five injured but uninoculated berries served as negative controls for each replicate. iButton temperature loggers were used to monitor the temperature in each chamber (Hoskin Scientific, Canada). Visual symptoms of sour rot were assessed in each berry each day using a rating scale of zero to four as in Figure 2.1 (zero=0% infected area, one=<10% infected area, two= 10-25% infected area, three=25-75% infected area, four=>75% infected area).

4.2.3 Ripeness pathogenicity assay. Immature berries from secondary clusters of *Vitis vinifera* cv. Pinot Noir were collected from a commercial vineyard in 2011. Berries were removed from clusters with pedicel attached, and were sorted into maturity classes according to colour (very green, slightly rosy, half red, deep red). Riesling berries of greater maturity were also collected as a positive control, shown previously to display the symptoms of sour rot (Appendix IV). Ten berries from each maturity class were macerated and soluble solids (°Brix) were measured using a portable refractometer. Ten berries per replicate of each maturity class were surface-sterilized, injured, and arranged as in Section 4.2.2 in four replicated plastic bins under high humidity. Berries were incubated for ten days at 22°C. A mixed suspension of 10^8 cells/mL was used as the inoculum, with 2.5 x 10^7 cells/mL each of *H. uvarum*, *C. zemplinina*, *A. malorum*, and *G. oxydans*. Isolates were collected from Niagara Peninsula vineyards, genetically identified as in Chapter 2, and maintained on yeast peptone dextrose agar, YPD. *H. uvarum* was genetically identified in the perfect form, although spores were not visualized. Disease was assessed using a rating scale of zero to four, described in Section 4.2.2. After final disease assessment, all grapes within a treatment-replicate were crushed and the K-ACET Acetic Acid Assay kit (Megazyme, Ireland) was used to measure the acetic acid concentration.

4.2.4 Data analyses. The curves representing sour rot disease progress were analyzed using Area Under Disease Progress Curve analyses (AUDPC), where the area under each curve was measured using the midpoint rule, and the areas under the curves were analyzed for significant

differences by ANOVA (Simko and Piepho, 2012). ANOVA was performed using Microsoft Excel 2000/XLSTAT-Pro (Version 7.2, 2003, Addinsoft, Inc., USA), and the Student Newman–Keuls (SNK) multiple range test was employed for mean separations with a confidence interval of 95%. An ANOVA-calculated *p*-value below 0.05 was considered to indicate data that could reliably lead to the rejection of the null hypothesis.

4.3 RESULTS

4.3.1. Correlation of daily mean temperature, total daily rainfall and berry maturity to sour rot development in the vineyard. The season of 2009 was a very cold, with below average temperatures and a killing frost on October 16 before harvest on October 26. Fruit maturity was delayed, veraison occurring on September 3. Rain was infrequent in September, with only four rain events. Three were clustered toward the end of the month, for an average precipitation of 1.1 mm per day. In September, mean daily temperatures were below 20°C, and in October below 14°C. With dry, cool conditions, sour rot was not detected until October 18 and reached only a mean severity of 0.02% and an incidence of 2.3/25 (Figure 4.1 and Table 4.1).

Veraison occurred on August 20 in 2010. In September, there was persistent rainfall, with two to three precipitation events per week for an average of 3.1 mm per day. There was a day of very heavy precipitation (34.8 mm) on September 29, just before harvest. In September, mean daily temperatures were between 10°C and 26°C, with harvest occurring on October 1, before temperatures dipped below 10°C. Sour rot was first detected on September 14 and incidence and severity continued to increase throughout the sampling period. At harvest, the mean incidence and severity of sour rot were 16.3/25 and 12.2%, respectively (Figure 4.2 and Table 4.1)

In 2011, veraison occurred on August 27. Rainfall was frequent, with seven continuous days of precipitation in late September with an average of 4.2 mm per day in that month. There was a very heavy rainfall (30.7 mm) on September 23. It also rained before harvest occurred on October 15, with 13.7 mm of rain on October 12. Temperatures usually remained between 14°C and 29°C during ripening, dipping down to 10°C only during periods of rainfall after October 1.

There was a very warm day of 28.8°C on September 3 and rain of 19.5 mm on September 4, just before sour rot was detected on September 5. Sour rot continued to increase, reaching a mean incidence of 23.3/25, and severity of 55.1% by harvest on October 15 (Figure 4.3 and Table 4.1).

In 2012, veraison occurred on August 17. Mean daily temperatures stayed between 15°C and 26°C until September 19, dipping down to between 10°C and 19°C until October 5. Rainfall occurred more infrequently than in 2010 or 2011, averaging every five days in September, but with a large volume, for an average rainfall of 4.9 mm per day. There were very heavy rainfalls on September 4 of 36.9 mm and September 8 of 42.7 mm, around the time sour rot was first detected on September 7. Harvest occurred on October 3 with a mean sour rot incidence of 17/25 and severity of 12.3% (Figure 4.4 and Table 4.1).

Mean soluble solids at first sour rot detection were 19.1°Brix in 2009, 18.0°Brix in 2010, 14.8°Brix in 2011, and 17.8°Brix in 2012 (Table 4.1).

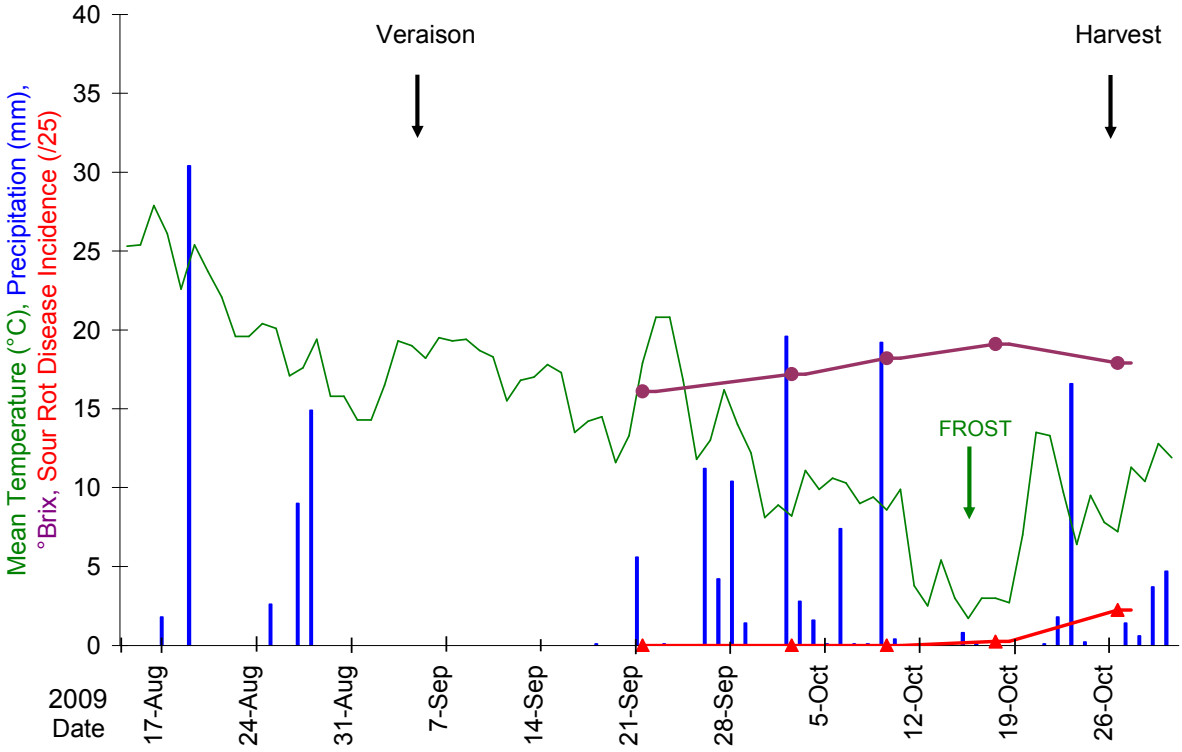


Figure 4.1 Mean incidence of sour rot (red, infected clusters/25), daily mean temperature (green, °C), total daily rainfall (blue, mm), and mean fruit maturity (purple, °Brix) in a commercial Riesling block in 2009. Veraison is noted on September 3 and harvest on October 26.

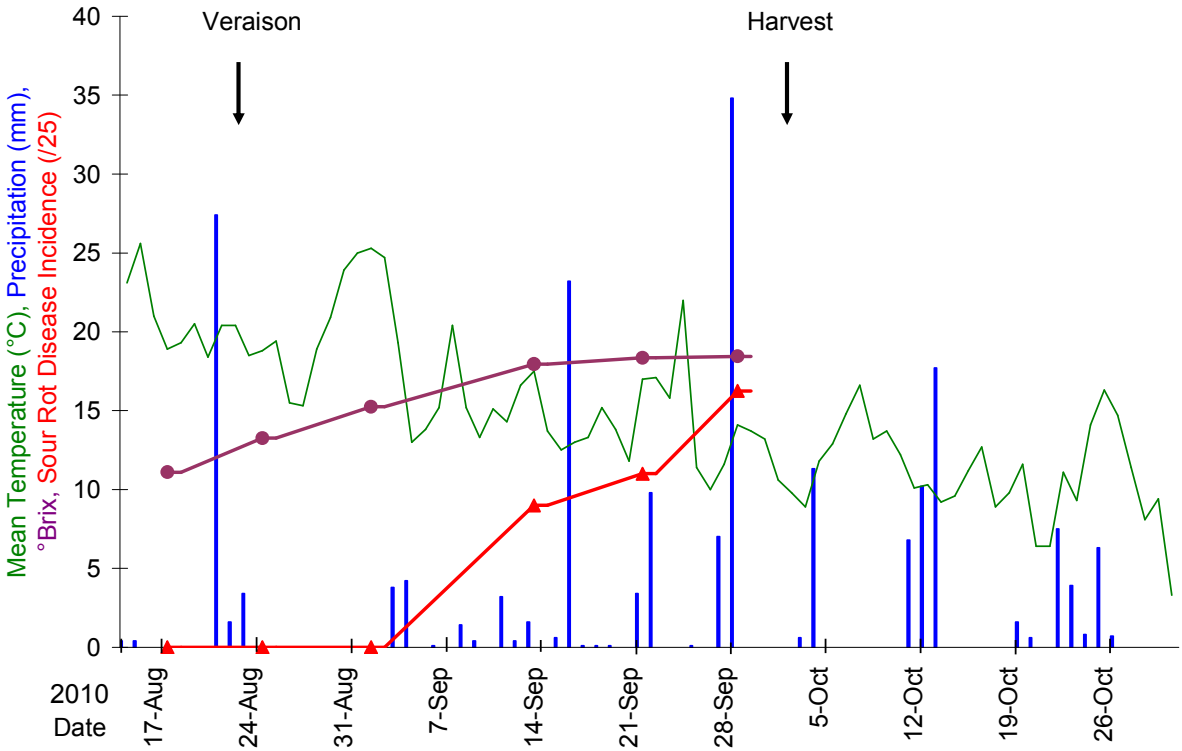


Figure 4.2 Mean incidence of sour rot (red, infected clusters/25), daily mean temperature (green, °C), total daily rainfall (blue, mm), and mean fruit maturity (purple, °Brix) in a commercial Riesling block in 2010. Veraison is noted on August 20 and harvest on October 1.

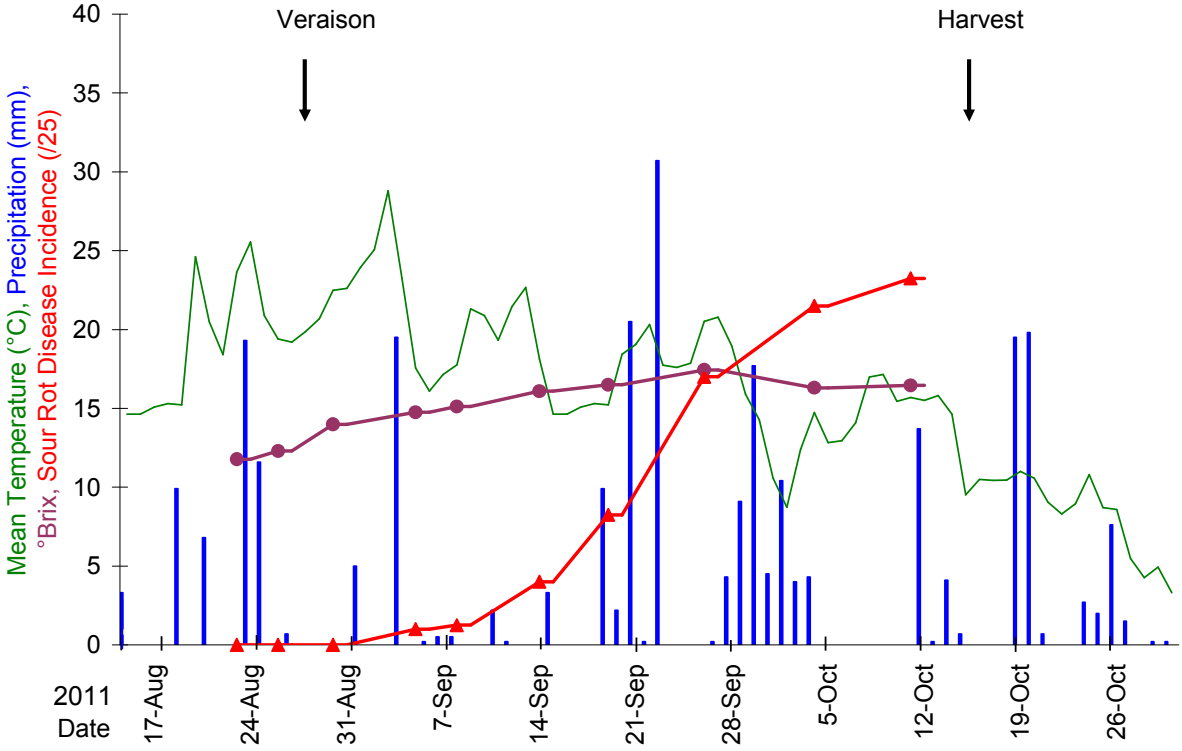


Figure 4.3 Mean incidence of sour rot (red, infected clusters/25), daily mean temperature (green, °C), total daily rainfall (blue, mm), and mean fruit maturity (purple, °Brix) in a commercial Riesling block in 2011. Veraison is noted on August 27 and harvest on October 15.

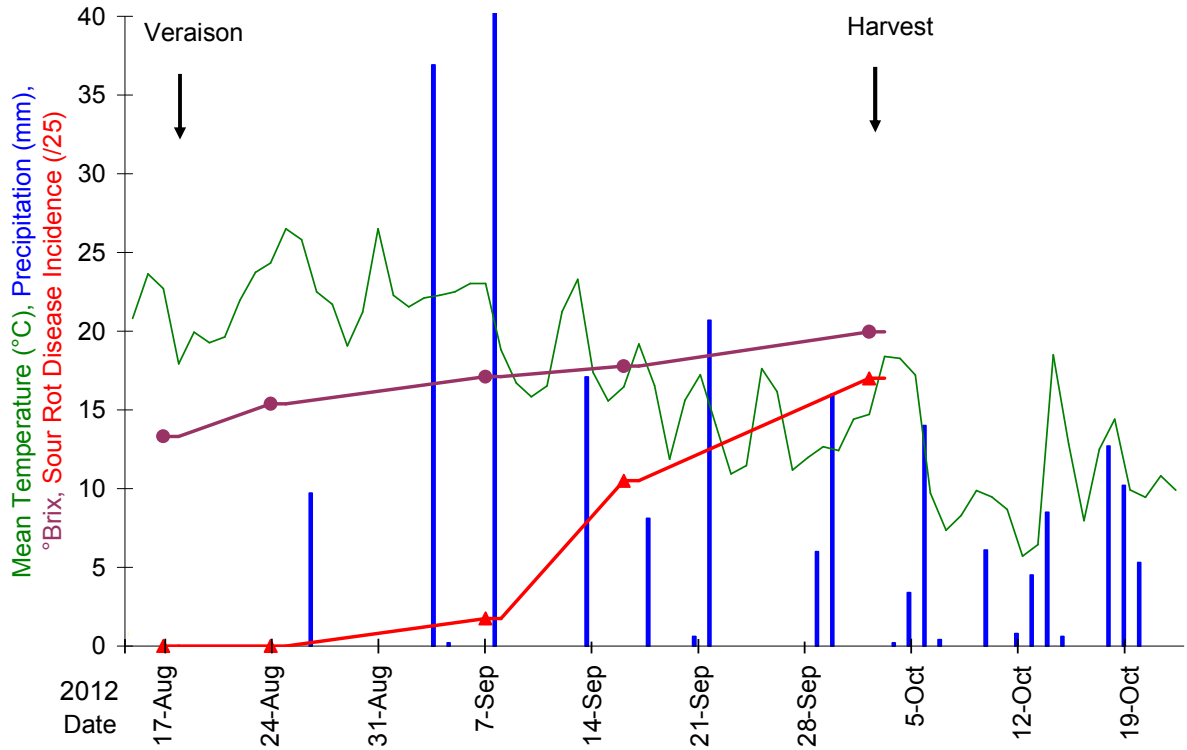
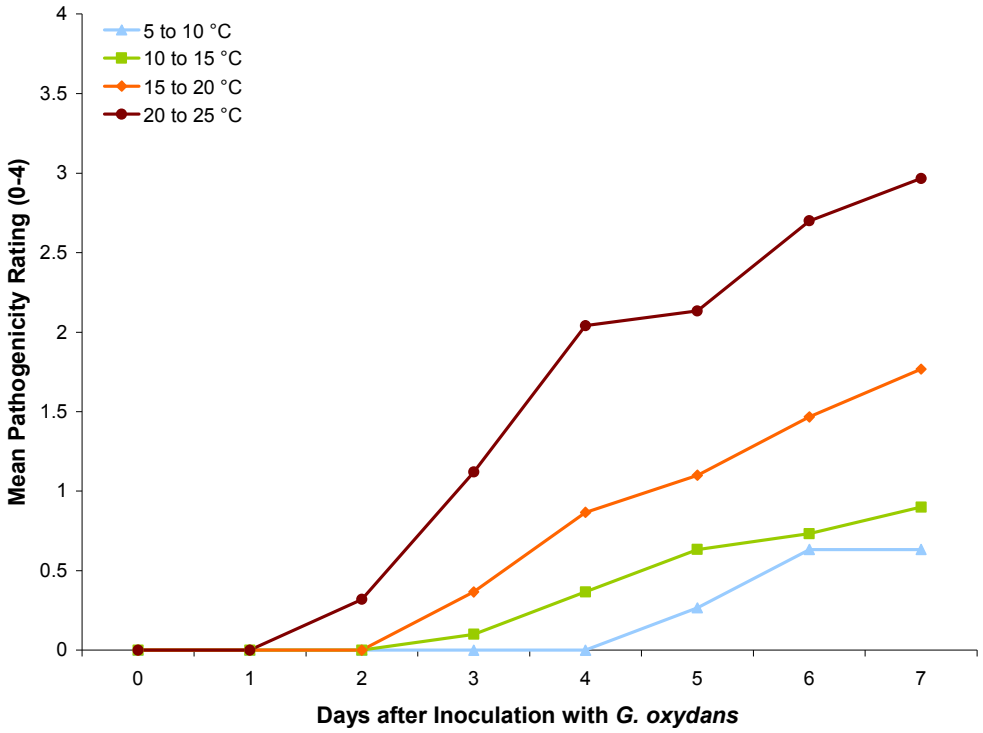


Figure 4.4 Mean incidence of sour rot (red, infected clusters/25), daily mean temperature (green, °C), total daily rainfall (blue, mm), and mean fruit maturity (purple, °Brix) in a commercial Riesling block in 2012. Veraison is noted on August 17 and harvest on October 3.

Table 4.1 Mean soluble solids (°Brix) (N = 400 berries per sampling date), sour rot severity and sour rot incidence (N = 100 clusters per sampling date) sampled from a commercial Riesling block in 2009, 2010, 2011, and 2012. Data is visualized in Figure 4.1, 4.2, 4.3, and 4.4, respectively.

Sampling Date	Mean Brix (°)	Mean Sour Rot Severity (%)	Mean Sour Rot Incidence (/25)
22-Sep-09	16.1	0.0	0.0
3-Oct-09	17.2	0.0	0.0
10-Oct-09	18.2	0.0	0.0
18-Oct-09	19.1	0.0	0.3
27-Oct-09	17.9	0.0	2.3
18-Aug-10	11.1	0.0	0.0
25-Aug-10	13.3	0.0	0.0
2-Sep-10	15.3	0.0	0.0
14-Sep-10	18.0	2.0	9.0
22-Sep-10	18.4	4.1	11.0
29-Sep-10	18.5	12.2	16.3
23-Aug-11	11.8	0.0	0.0
26-Aug-11	12.3	0.0	0.0
30-Aug-11	14.0	0.0	0.0
5-Sep-11	14.8	1.3	1.0
8-Sep-11	15.1	1.0	1.3
14-Sep-11	16.1	5.7	4.0
19-Sep-11	16.5	15.6	8.3
26-Sep-11	17.4	32.3	17.0
4-Oct-11	16.3	47.8	21.5
11-Oct-11	16.5	55.1	23.3
17-Aug-12	13.3	0.0	0.0
24-Aug-12	15.4	0.0	0.0
7-Sep-12	17.1	0.5	1.8
16-Sep-12	17.8	5.0	10.5
2-Oct-12	20.0	12.3	17.0

4.3.2 Temperature range pathogenicity assay. After seven days of incubation in wounded green seedless table grapes at 20-25°C, *G. oxydans* reached a mean pathogenicity rating of 2.97 on a scale of zero to four, and *H. uvarum* a rating of 2.27. Pathogenicity ratings were less after seven days of incubation at lower temperature ranges. *G. oxydans* reached a mean pathogenicity rating of 1.77 at 15-20°C, 0.90 at 10-15°C, and 0.63 at 5-10°C after seven days (Figure 4.5). The Area Under Disease Progress Curves for each temperature range were significantly different according to ANOVA testing in *G. oxydans* (Table 4.2). *H. uvarum* had a mean pathogenicity rating of 1.47 at 15-20°C, 0.57 at 10-15°C, and 0.43 at 5-10°C after seven days (Figure 4.5). The Area Under Disease Progress Curves demonstrated a significant difference for the temperature ranges of 20-25°C and 15-20°C, while the ranges of 10-15°C and 5-10°C were grouped by the Student-Newman-Keuls multiple range test in *H. uvarum* (Table 4.2). Disease onset began on day two in both organisms at 20-25°C (Figure 4.5). Disease onset began later at lower temperatures, on day three in both organisms at 15-20°C and day four in *H. uvarum* at 10-15°C and 5-10°C, while disease onset began on day three in *G. oxydans* at 10-15°C, and day 5 at 5-10°C (Figure 4.5).



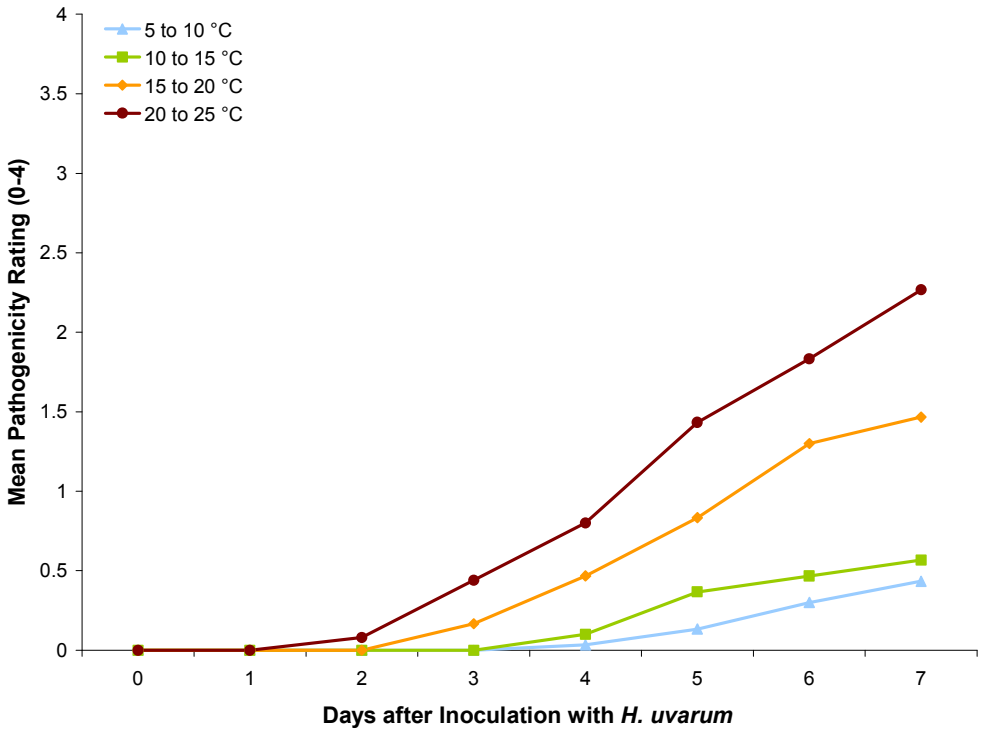


Figure 4.5 Temperature pathogenicity assay using wounded green seedless table grapes inoculated with isolates of *Gluconobacter oxydans* (above) and *Hanseniaspora uvarum* (below) isolated from sour rotted grapes in the Niagara Peninsula, designated viticultural area. Grapes were incubated at different temperatures and assessed daily on a scale of zero to four (zero=0% infected area, one=<10% infected area, two= 10-25% infected area, three=25-75% infected area, four=>75% infected area). Mean pathogenicity of isolates is shown (N = 30 berries for each temperature). Statistics are presented in Table 4.2.

Table 4.2 Area Under Disease Progress Curve (AUDPC) values calculated for the temperature pathogenicity assay (Figure 4.5) incubated for seven days using wild *Gluconobacter oxydans* and *Hanseniaspora uvarum* as inocula in green seedless table grapes. ANOVA *G. oxydans* $F(5,3) = 133.18$, $p = <0.0001$, *H. uvarum* $F(5,3) = 19.30$, $p = <0.0001$. AUDPC means are presented. Significant data is marked with an *. Curve values with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

Gluconobacter oxydans *		*Hanseniaspora uvarum* *	
Temperature (°C)	Mean AUDPC	Temperature (°C)	Mean AUDPC
5-10	1.22 d	5-10	0.48 c
10-15	2.28 c	10-15	1.03 c
15-20	4.68 b	15-20	3.58 b
20-25	9.68 a	20-25	5.72 a

4.3.3 Ripeness pathogenicity assay. Soluble solids concentration of berries, measured in °Brix, and signifying berry ripeness, had a statistically significant effect on the symptoms of sour rot in a mixed inoculation pathogenicity assay using *H. uvarum*, *C. zemplinina*, *A. malorum* and *G. oxydans*. Green berries had a mean of 9.7°Brix, slightly rosy berries 11.2°Brix, half red berries 13.4°Brix, and deep red berries 15.5°Brix. Riesling berries with a mean of 16.7°Brix served as a positive control. These maturity classes were utilised in Figure 4.6 and 4.7.

Sour rot developed very little in the least mature berries with a mean pathogenicity rating of 0.88 in the half red berries, 0.52 in the slightly rosy berries, and 0.11 in the green berries. As berry maturity increased, the severity of sour rot increased significantly in a stepwise manner, as high as a pathogenicity rating of 2.89 in the deep red berries, and 3.54 in the Riesling berries. The pathogenicity rating was more than three times more in berries with a mean of 15.5°Brix compared to the less mature berry stage with a mean of 13.4°Brix, after ten days incubation with the mixed inoculum (Figure 4.6).

Mean volatile acidity measured 0.19 g acetic acid/L in the half red berries, 0.14 g acetic acid/L in the slightly rosy berries, and 0.16 g acetic acid/L in the green berries. These numbers were more than seven times less than in the deep red berries which had a mean volatile acidity of 1.47 g acetic acid/L, while the Riesling berries had a mean volatile acidity of 1.63 g acetic acid/L after ten days incubation (Figure 4.7).

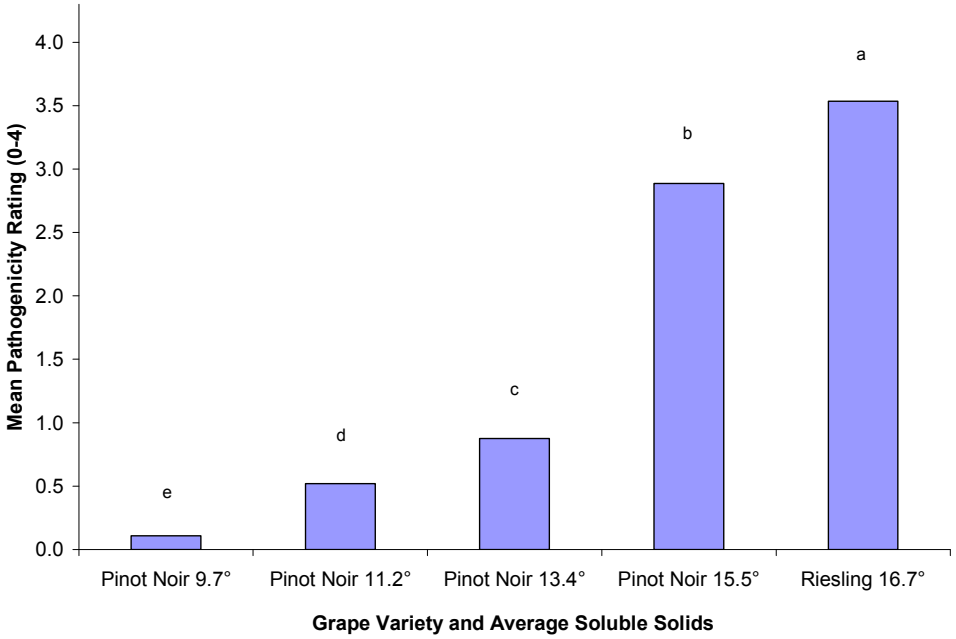


Figure 4.6 Mean pathogenicity, rated on a scale of zero to four, observed in wine grapes of different soluble solids concentration (°Brix) incubated ten days at 22°C. Grapes were injured and inoculated in four replicated chambers with a wild-isolated mixture of *H. uvarum*, *C. zemplinina*, *A. malorum* and *G. oxydans*. N = 40 berries of each average ripeness. ANOVA $F(3,4) = 257.87$, $p = <0.0001$. Bars with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

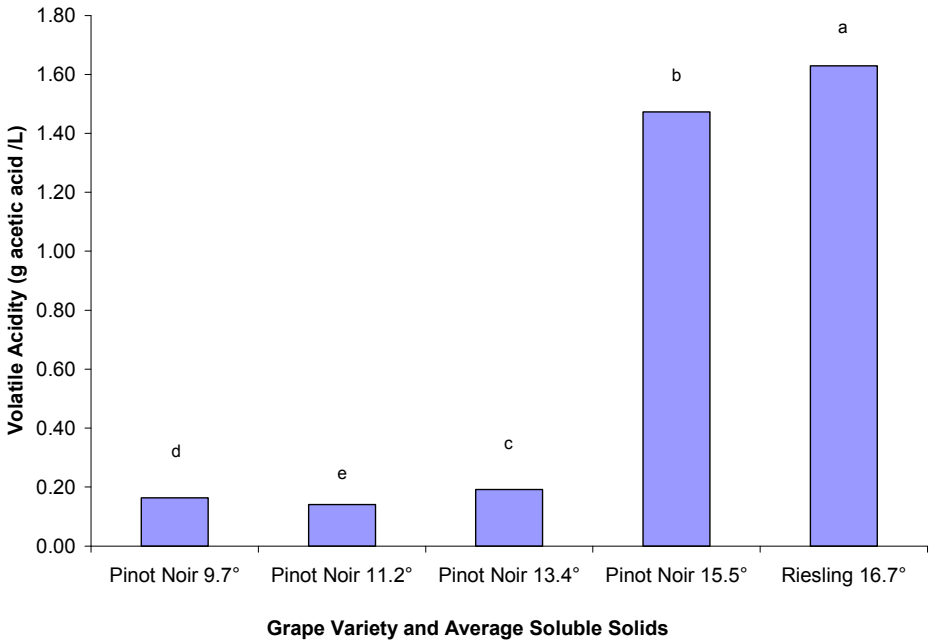


Figure 4.7 Mean volatile acidity measured in the form of acetic acid from wine grapes of different soluble solids concentration (°Brix) incubated ten days at 22°C. Grapes were injured and inoculated in four replicated chambers with a wild-isolated mixture of *H. uvarum*, *C. zemplinina*, *A. malorum* and *G. oxydans*. N = 40 berries of each average ripeness and duplicated acetic acid measurements. ANOVA $F(3,4) = 42\ 222.80$, $p = <0.0001$. The Student Newman-Keuls multiple range test demonstrates that bars with the same letter are not significantly different.

4.4 DISCUSSION

Daily mean temperature, total daily rainfall, berry maturity (°Brix) and sour rot development (incidence /25 clusters) were monitored over four years in *Vitis vinifera* cv. Riesling. Sour rot severity was also assessed. Generally, there was a higher incidence and greater severity of sour rot in years with warmer daily mean temperatures and with more total daily rainfall during the period of ripening between veraison and harvest. 2010 was a little cooler during the period of ripening, compared with 2011 and 2012, having more days with a mean temperature between 10-15°C during the month of September. There was more total daily rainfall during this month in 2011 (Figure 4.2, 4.3, and 4.4). 2011 had the highest mean sour rot severity by harvest on October 15, of 55.1% (Table 4.1). 2010 and 2012 were harvested on October 1 and October 3, with similar mean sour rot severities of 12.2% and 12.3%, respectively (Table 4.1). It is possible to hypothesize the higher severity of sour rot in 2011 was correlated with a later harvest date. However, the lowest severity of sour rot at harvest was observed on the latest harvest date of October 26 in 2009 (Figure 4.1), with a mean severity of 0.02% (Table 4.1). The disease did not reach a high enough severity in 2009 for clusters to be rejected at wineries due to volatile acidity. Rain arrived late in the season in 2009 and this year was cold amongst the four years of study, below 15°C after veraison and reaching frost in October before grapes were harvested (Figure 4.1). A correlation between warmer daily mean temperatures (approaching 20°C) and more total daily rainfall with a higher mean sour rot severity was indicated (Figures 4.1, 4.2, 4.3, 4.4, and Table 4.1).

The organisms primarily responsible for sour rot are *H. uvarum*, *C. zemplinina*, *Gluconobacter*, and *Acetobacter* (Barata et al., 2012a; Bisiach et al., 1986; Gravot et al., 2001). The cardinal temperatures of these organisms have been reported based on their growth. According to literature, a strain of *H. uvarum* had a maximum temperature of 36.87°C and a minimum temperature of 4.71°C, with an optimum temperature of 24.51°C. *Candida stellata* (syn. *C. zemplinina*) had a maximum temperature of 37.00°C and a minimum temperature of

2.81°C, with an optimum temperature of 24.77°C (Salvado et al., 2011). Acetic acid bacteria also have a wide range of temperature tolerance, with weak growth as low as 8-10°C, and thermotolerant strains up to 37-40°C (Du Toit and Pretorius, 2002), with an optimum temperature of 25-30°C (Carrascosa et al., 2011). Therefore, although the sour rot yeasts and bacteria can grow at temperatures below 10°C, a temperature between 20°C and 30°C is optimal for their growth, similar to the present findings that sour rot was observed at a higher severity in warmer weather (Figures 4.1, 4.2, 4.3, and 4.4). Further research conducted in the present study incubated the sour rot organisms in susceptible grapes at different temperatures to determine the effect of temperature on symptom production.

In 2010, 2011, and 2012, there were events of heavy rainfall, measured to be more than 30 mm in one day (Figure 4.2, 4.3, and 4.4). In 2010 there was 34.8 mm total daily rainfall on September 29, in 2011 there was 30.7 mm on September 23, and in 2012 there was 36.9 mm on September 4 and 42.7 mm on September 8. In 2012, these precipitation events occurred around the time sour rot was first detected on September 7. Osmotic pressure can cause a sudden increase in berry size when there is a high volume of rain, leading to microfissures that can serve as entrance points for the sour rot microbes (Barata et al., 2012a; Gravot et al., 2001) especially in tightly packed clusters with thin skin (Zoecklein et al., 2000), such as Riesling used in the present study. Heavy rain and precipitation can also cause direct injury to the grape berries or promote other pests and pathogens which weaken the grape skin (Barata et al., 2012a). Damage to the grape berry is the main factor influencing the number and kinds of microbes recovered through grape sampling, favouring ascomycete yeasts and acetic acid bacteria associated with sour rot and wine spoilage (Barata et al., 2012a). Further studies could use scanning electron microscopy (SEM) to analyze the damage to grape skins after a rain event, and observe the types of microorganisms populating the damaged areas.

Mean soluble solids at first sour rot development were 19.1°Brix on October 18 in 2009, 18.0°Brix on September 14 in 2010, 14.8°Brix on September 5 in 2011, and 17.8°Brix on

September 7 in 2012 (Table 4.1). Sour rot development began at a lower mean ripeness in 2011, the year with warmer daily mean temperatures and higher total daily rainfall before harvest (Figure 4.3), and sour rot developed at a higher ripeness in 2009, the year with coldest daily mean temperatures (Figure 4.1). Environmental conditions and grape ripeness are interrelated factors when considered in correlation to the symptoms of sour rot. Further research, as performed in the present study, would incubate the sour rot organisms under ideal conditions in grapes of decreasing ripeness, to ascertain a minimum soluble solids concentration necessary to support their growth.

To formulate a null hypothesis, such as that measures of temperature, precipitation, and ripeness do not correlate with sour rot incidence in the vineyard, one would have to develop a statistical model. For example, the technique of multiple regression could be used (Smith et al., 2007), which takes into consideration all of the independent environmental variables to predict the dependent variable of disease incidence using the equation of a line or curve. The Smith et al. (2007) model found precipitation to have a low correlation with the dependent variable, using relative humidity instead. Some disease models do include rainfall as an independent variable (McFadden-Smith et al., 2000), although this may be dependent on the disease, Smith et al. studying Sclerotinia peanut blight and McFadden-Smith et al. studying sour cherry black knot. The variables of interest for modelling sour rot in wine grapes would be mean, maximum, and minimum air temperature, total, mean, and maximum precipitation, and relative humidity and leaf wetness (McFadden-Smith et al., 2000; Smith et al., 2007). A factor also of interest would be grape cultivar, which would necessitate repeating data collection in multiple vineyards, so that the data could be valid for more than just one cultivar. Mean daily temperature, total daily rainfall, and disease incidence sampling was initiated in a cv. Pinot Noir crop in the Niagara Peninsula, designated viticultural area (Appendix VI), with similar methodology as in cv. Riesling (section 4.2.1). In 2012, harvest was earlier in Pinot Noir on September 19 with a Brix of 21.3° (Appendix VI), compared with Riesling on October 3 with a Brix of 20.0° (Figure 4.4).

If the incidence of sour rot in all susceptible cultivars could be predicted according to correlation with local climate variables and the variable of grape ripeness, the periods in which to treat the disease, with management practices and agrochemical applications, could be targeted.

To test the correlation observed in the field between sour rot severity and daily mean temperatures approaching and above 20°C (Figure 4.1, 4.2, 4.3, and 4.4, and Table 4.1), field-isolated strains of the yeast, *H. uvarum*, and the acetic acid bacterium, *G. oxydans*, were employed in an *in vitro* pathogenicity assay. These strains were inoculated in injured green seedless table grapes and incubated at different temperature ranges: 5-10°C, 10-15°C, 15-20°C, and 20-25°C. Both organisms had an earlier disease onset and higher pathogenicity rating after seven days incubation at progressively higher temperature ranges, up to the upper limit tested at 25°C (Figure 4.5). Therefore, the incubations at higher temperature ranges had significantly greater Area Under Disease Progress Curve values (Table 4.2) with ANOVA-calculated *p*-values of <0.0001 for both organisms, with the exception of *H. uvarum* at the lowest temperatures, showing no statistical difference between incubations at 5-10°C and 10-15°C. The null hypothesis that temperature would not have an effect on sour rot disease progress could be rejected based on this data. The organisms still produced minor symptoms under 10°C (Figure 4.5), indicating weak growth in accordance with the minimum cardinal temperatures of the organisms outlined in the literature (Du Toit and Pretorius, 2002; Salvado et al., 2011). This was in accordance with present field observations (Figure 4.1, 4.2, 4.3, and 4.4). Future research would utilize more microbial strains in replicated pathogenicity assays, since the organisms associated with sour rot have been shown to display strain-dependent qualities (Drysdale and Fleet, 1988; Romano et al., 1997; Strauss et al., 2001).

In the vineyard, soluble solids were as low as a mean of 14.8°Brix, and as high as 19.1°Brix when sour rot symptoms were first observed (Table 4.1). Pathogenicity assays using grapes of different ripeness classes were incubated under high humidity at 22°C for 10 days. A significantly higher mean pathogenicity rating was observed in grapes with a higher degree of

ripeness (Figure 4.6), and along with visual symptoms, significantly more acetic acid was detected (Figure 4.7). With ANOVA-calculated *p*-values of <0.0001 for both *in vitro* experiments, the null hypothesis that ripeness would not have an effect on sour rot symptoms, measured by a pathogenicity rating scale and the concentration of acetic acid, could be rejected. The mean pathogenicity rating was more than three times less in berries with a mean of 13.4°Brix compared to the next more mature berry stage with a mean of 15.5°Brix (Figure 4.6), and the volatile acidity was more than seven times less (Figure 4.7). This indicates the critical period of grape maturity to be in the range of 13.4°Brix to 15.5°Brix for susceptibility to sour rot at 22°C in a high humidity incubation chamber. The experiment could be replicated to narrow this range. Higher temperatures could be tested in future experiments, as well as monitoring and modifying humidity during incubation. Sour rot is known to sweep vineyards as grapes reach maturity, when sugar becomes available for the metabolism of the microbial population (Gravot et al., 2001; Marchetti et al., 1984). The increased sugar content may also increase osmotic potential in the grapes, influencing injury due to water stress and providing entrance to fermentative yeasts and acetic acid bacteria (Barata et al., 2012a; Gravot et al., 2001). Further studies using SEM as well as testing berry firmness would be necessary to confirm this hypothesis.

CHAPTER 5: MANAGEMENT OF GRAPE SOUR ROT AND IMPACT OF TREATMENTS ON SOUR ROT ORGANISMS

5.1 INTRODUCTION

Sour rot develops in grapes as they accumulate sugar after veraison (Gravot et al., 2001). The disease can be a significant economic problem in cool climates such as the Niagara Peninsula, designated viticultural area, Ontario, Canada, accountable for more than 1.5 million dollars in crop loss in years when weather conditions, such as rain and hail in 2008, promote infection (Agrocorp, 2008). Sour rot begins as a discolouration at an injury site or the pedicel attachment site (Bisiach et al., 1986; Gravot et al., 2001). Internal tissues disintegrate and skin weakens, leading to berry detachment from the pedicel and oozing juice (Guerzoni and Marchetti, 1987).

The major chemical markers of the disease are an elevated acetic acid content, the main contributor to volatile acidity, and an increase in the ethanol-acetic acid ester, ethyl acetate (Guerzoni and Marchetti, 1987; Marchetti et al., 1984). In Ontario, grapes may be rejected at the winery or in the vineyard if volatile acidity (VA), measured in the concentration of acetic acid, exceeds 0.24 g/L (Grape Growers of Ontario, 2010). Even low levels of acetic acid at harvest indicate the presence of spoilage organisms which may multiply during early fermentation (Barata et al., 2008a).

The symptoms of sour rot are associated with increased numbers of certain yeasts and bacteria, especially those that constitute the wine microbial consortium, surviving under anaerobic winemaking conditions (Barata et al., 2012a). Ecological surveys have shown sour rot to be a community interaction between acetic acid bacterial species and yeasts such as *Kloeckera apiculata* (teleomorph *Hanseniaspora uvarum*), *Candida zemplinina*, *Metschnikowia pulcherrima*, and *Pichia* (Barata et al., 2008a; Barata et al., 2012b; Bisiach et al., 1986; Gravot et al., 2001). In the Niagara Peninsula, designated viticultural area, *H. uvarum*, *Gluconobacter oxydans*, and *Acetobacter malorum* have been shown to cause the disease (Huber et al., 2012).

While they are often associated with sour rotted fruit, filamentous fungi such as *Penicillium*, *Aspergillus*, *Alternaria*, *Cladosporium*, and *Rhizopus* are not capable of initiating a sour rot infection (Bisiach et al, 1986).

The growth of sour rot organisms in the field is associated with grape injury, which can occur due to biotic and abiotic factors. Other pathogens may be implicated in causing injury, for example *Botrytis cinerea* (Marchetti et al., 1984) and *Erisiphe necator* (Gadoury et al., 2007). Water stress is an abiotic factor causing injury, and the disease is associated with precipitation (Loureiro and Malfeito-Ferreira, 2003). Wetted berries can suddenly increase in size after a decrease during dehydration, leading to small splits in the cuticle that serve as entrance points for the sour rot microbes (Barata et al., 2012a; Gravot et al., 2001). This may contribute to the susceptibility of cultivars with thin skins and tightly packed clusters (Zoecklein et al., 2000), such as Riesling, studied presently.

Sour rot symptoms begin after veraison in the weeks before harvest, with pre-harvest intervals limiting permissible treatments (Oliva et al., 1999). Nigro et al. (2006) tested carbonate and bicarbonate, salts with little toxicity to the environment, for activity against sour rot. Carbonate, CO_3^{2-}, forms a solution of pH of 11.3 while bicarbonate, HCO_3^-, forms a solution of pH of 8.4. With two salt applications 90 and 30 days pre-harvest, sour rot was reduced to 40.6, 51.4, and 50.8% of the untreated control by using calcium carbonate, sodium bicarbonate, and sodium carbonate, respectively (Nigro et al., 2006). Milstop (Bioworks, Canada) and Sirocco (AEF Global) are potassium bicarbonate products registered in Canada for the suppression of powdery mildew in grapes. Both have a zero day pre-harvest interval (Ontario Ministry of Agriculture and Food, 2014), and in the present study, Milstop was researched for efficacy in managing sour rot.

Added to the surface of fruit in bins or to grape juice before fermentation, the use of sulfur dioxide is a part of wine manufacturing practices. It eliminates oxidative species of yeast, while fermenting species are more likely to survive (Villa and Longo, 1996), targeting basidiomycetes before the ascomycetes associated with sour rot. However, at high enough

concentrations, the spoilage species are also eradicated, requiring up to 60-70 mg/L sulfur dioxide in the must of white grapes to kill *H. uvarum* (Longo et. al. 1991) before the addition of *Saccharomyces cerevisiae* starter cultures. The molecular form of sulfur dioxide is also known as sulfurous acid, H_2SO_3, which increases in concentration as acidity increases and pH decreases. At the pH of wine, which is three to four, only approximately 5% of sulfur dioxide is present in the molecular form, which is the active antimicrobial form. The other forms, bisulfite and sulfite serve as antioxidants (Du Toit and Pretorius, 2002). The bisulfite form, HSO_3^{1-}, dominates in juice and wine in the pH range of three to four, representing approximately 90-99% of sulfur dioxide. The sulfite form, SO_3^{2-}, is maintained under basic pH (Du Toit et al., 2005). Only the antimicrobial form of sulfur dioxide, H_2SO_3, can enter bacterial or yeast cells, deprotonating in the less acidic internal environment and reacting with intracellular components to halt their metabolism (Margalit, 1997).

Sulfur dioxide can enter a bound and free form. The bound form is completely inactive as the sulfite molecule reacts with carbonyl groups and cannot further react. Carbonyl compounds can originate from grape pulp, for example 5-oxofructose, and δ -gluconolactone, or from yeasts producing compounds such as acetaldehyde, pyruvic acid and 2-oxoglutaric acid. Acetic acid bacteria also produce sulfite-binding molecules including gluconic acid, 5-oxofructose, and dihydroxyacetone (Barbe et al., 2001; Carrascosa et al., 2011; Du Toit and Pretorius, 2002).

Potassium metabisulfite (KMS), $K_2O_5S_2$, is the most commonly used source of sulfur dioxide in winemaking. It was tested in field sprays against *Botrytis* bunch rot in South Australia at a concentration of 4 g/L using pre-inoculated grapes. KMS did not markedly reduce *Botrytis* spore germination in these trials and there was no mention of phytotoxicity (Wicks, 2002). In the present study, KMS applications were tested in Canadian vineyards and in laboratory trials to determine the impact on the yeasts and acetic acid bacteria causing sour rot. KMS was tested in different concentrations and formulations and at different timings. Milstop was also tested,

allowing the comparison of a product with a basic pH. The null hypothesis in the field trials was that plots sprayed with KMS or Milstop formulations would not be different in disease severity nor VA compared to the untreated control plots, nor would different timings result in a different disease severity or VA. The null hypothesis when the products were tested in a disk assay was that the products would not clear the growth of the sour rot organisms on YPD agar, compared to an untreated control. When the treatments were applied to inoculated grapes *in vitro,* the null hypothesis was that the treatments would not reduce the symptoms of sour rot compared to an untreated control.

5.2 MATERIALS AND METHODS

5.2.1 Field applications. Treatments for sour rot were applied to a commercial block of *Vitis vinifera*, cultivar Riesling, in the Niagara Peninsula, designated viticultural area, Ontario, Canada, in the period between veraison and harvest during the 2010 and 2011 seasons. Veraison and harvest occurred on August 20 and October 2 in 2010, and August 27 and October 16 in 2011, respectively. Insecticide and fungicide applications and viticultural practices were completed by the grower cooperator according to standard practices. Treatments were applied according to a randomized complete block design with four repetitions. Each plot consisted of a panel of five vines with 1 m spacing, with one vine between each plot as a barrier zone, and rows were spaced by 2.8 m. Treatments were sprayed using a calibrated CO_2 pressurized backpack sprayer at a rate of 600 L/ha, directly into the fruiting zone on both sides of the vine (Bellspray Inc, USA). The untreated control plots were not sprayed. Milstop (85% potassium bicarbonate, BioWorks, USA) was applied throughout the season according to the label concentration of 5.6 kg/1000L (Table 5.1 and 5.2). KMS (100% potassium metabisulphite) was tested at different concentrations of 2.5, 5, and 10 kg/1000L, and at decreasing intervals before harvest (Table 5.1 and 5.2). Treatments were applied with either a one day pre-harvest interval, or three day pre-harvest interval (PHI). In 2010, all treatments were applied with a one day PHI, except a treatment of KMS 5 kg/1000L consisting of only one spray, applied with a three day PHI (Table

5.1). In 2011, all treatments were applied with a three day PHI except a treatment of KMS 5 kg/1000L consisting of two sprays with a one day PHI, and a similarly formulated treatment consisting of one spray with a one day PHI (Table 5.2). KMS 5 kg/1000L was tested throughout the season in a mixed formulation with Milstop 5.6 kg/1000L (Table 5.2). KMS 5 kg/1000L was also tested in an acidified formulation by mixing with a tartaric acid solution of 5 kg/1000L (Table 5.2). This was with the goal of decreasing the pH of the overall solution to increase the concentration of sulphur dioxide in the active antimicrobial molecular form.

At harvest, one day after the final spray, 25 grape clusters were arbitrarily selected and collected from each plot, excluding the barrier vines. Each cluster was assessed for disease severity using a variation of the Horsfall-Barratt rating scale (0, 1, 3, 5, 10, 25, 50, 75, 90, 95, 97, 99, or 100% cluster infected) (Horsfall and Barratt, 1945).

The 25 clusters per plot were combined and crushed by hand in plastic bags and 1.5 mL of must was centrifuged, frozen at -30°C for one to two months, then thawed at room temperature for an hour. VA was measured from the must in the form of the concentration of acetic acid, using the K-ACET enzyme assay kit (Megazyme, Ireland). The acetate from the must sample was converted into acetyl-CoA, which was then reacted with oxaloacetate to form citrate. To replenish the oxaloacetate, L-malate was oxidized, with NAD^+ reduced to NADH. The NADH was measured spectrophometrically at 340 nm.

Table 5.1 2010 spray schedule of treatments, rates and timings applied to a commercial block of *Vitis vinifera* cv. Riesling and harvested on October 2.

		Application Date (Harvest October 2, 2010)					
Treatment	**Concentration (kg/1000L)**	**Aug 20**	**Sept 5**	**Sept 14**	**Sept 21**	**Sept 29**	**Oct 1**
Milstop	5.6	**x**	**x**	**x**	**x**	**x**	**x**
KMS 10	10	**x**	**x**	**x**	**x**	**x**	**x**
KMS 2.5	2.5	**x**	**x**	**x**	**x**	**x**	**x**
KMS 5	5	**x**	**x**	**x**	**x**	**x**	**x**
KMS 6 Sprays	5	**x**	**x**	**x**	**x**	**x**	**x**
KMS 5 Sprays	5		**x**	**x**	**x**	**x**	**x**
KMS 4 Sprays	5			**x**	**x**	**x**	**x**
KMS 3 Sprays	5				**x**	**x**	**x**
KMS 1 Spray 3 Day PHI	5					**x**	
KMS 1 Spray 1 Day PHI	5						**x**
Untreated Control	No spray						

Table 5.2 2011 spray schedule of treatments, rates and timings applied to a commercial block of *Vitis vinifera* cv. Riesling, harvested October 16.

		Application Date (Harvest October 16, 2011)					
Treatment	**Concentration (kg/1000L)**	**Aug 27**	**Sept 13**	**Sept 20**	**Oct 10**	**Oct 13**	**Oct 15**
Milstop	5.6	**x**	**x**	**x**	**x**	**x**	
KMS + Milstop	5 + 5.6	**x**	**x**	**x**	**x**	**x**	
KMS 10	10	**x**	**x**	**x**	**x**	**x**	
KMS 2.5	2.5	**x**	**x**	**x**	**x**	**x**	
KMS 5	5	**x**	**x**	**x**	**x**	**x**	
Acidified KMS	5 + 5 Tartaric Acid	**x**	**x**	**x**	**x**	**x**	
Tartaric Acid Control	5		**x**	**x**	**x**	**x**	
KMS 5 sprays	5	**x**	**x**	**x**	**x**	**x**	
KMS 4 sprays	5		**x**	**x**	**x**	**x**	
KMS 3 sprays	5			**x**	**x**	**x**	
KMS 2 sprays 3 day PHI	5				**x**	**x**	
KMS 1 spray 3 day PHI	5					**x**	
KMS 2 sprays 1 day PHI	5					**x**	**x**
KMS 1 spray 1 day PHI	5						**x**
Untreated Control	No Spray						

5.2.2 Disk diffusion assay. To assess the sensitivity of microbial isolates to potential sour rot treatments, four replicates of yeast peptone dextrose agar, YPD, were spread with a suspension of wild-isolated *H. uvarum* or *G. oxydans*, so as to cover the entire surface of the plate with confluent growth. Isolates were collected from Ontario vineyards of the Niagara Peninsula in 2011, genetically identified as in Chapter 2, and maintained on YPD. *H. uvarum* was genetically identified in the perfect form, although spores were not visualized. Filter paper was cut into 6 mm diameter circles using a hole puncher, and these were autoclaved. Disks were dipped into solutions of one of KMS 10 g/L (KMS 10), KMS 5 g/L (KMS 5), Milstop 5.6 g/L (Milstop), KMS 5 g/L + Milstop 5.6 g/L (KMS + Milstop), KMS 5 g/L acidified with 5 g/L tartaric acid (Acidified KMS), a tartaric acid control 5 g/L (Acid Control), or distilled water as an untreated control. Four disks were placed on each of the four replicates of the freshly inoculated agar for each treatment and incubated for three days at 22°C. The radius of clearance around each disk was measured in mm (Hadacek and Greger, 2000).

5.2.3 Treatment activity assay *in vitro*. Intact green seedless table grapes with pedicel attached were pre-washed in Dawn dish detergent (Procter & Gamble, USA), sterilized in 10% commercial bleach for twenty minutes, then washed with distilled water. The experimental design was a split plot design with species of inoculum as the main plot and antimicrobial treatment as the sub-plot and four replicates. An experimental unit consisted of three berries which were placed together into a 5 cm diameter plastic deli cup. The deli cups were arranged in plastic bins to maintain humidity. Each berry was wounded by poking three times with a sterile dissecting needle. Immediately after wounding, half of the grapes were pre-treated with the potential sour rot treatments: KMS 10 g/L (KMS 10), KMS 5 g/L (KMS 5), Milstop 5.6 g/L (Milstop), KMS 5 g/L + Milstop 5.6 g/L (KMS + Milstop), acidified KMS 5 g/L with 5 g/L tartaric acid (Acidified KMS), or a tartaric acid control 5 g/L (Acid Control). The grapes were dipped into solutions with sterilized forceps and dripped dry. Treated and untreated grapes were then inoculated directly onto the wounds by applying 10 µL of a solution of wild-isolated *H.*

uvarum or wild-isolated *G. oxydans* (identified as in Section 5.2.2), each prepared to a concentration of 10^8 cells/mL in dH_2O. With untreated and uninoculated controls set aside, the second half of the inoculated grapes were dipped into the potential sour rot treatments freshly prepared 24 hours after inoculation. After 14 days of incubation, symptoms of sour rot were assessed in each berry using a rating scale of zero to four as in Figure 2.1 (zero=0% infected, one=<10% infected, two= 10-25% infected, three=25-75% infected, four=>75% infected).

5.2.4 Data analyses. Replicates were averaged using Microsoft Excel. Field data was transformed by arcsin squareroot to improve normality (Ahrens et al., 1990). ANOVA was performed using Microsoft Excel 2000/XLSTAT-Pro (Version 7.2, 2003, Addinsoft, Inc., USA), and the Student Newman–Keuls (SNK) multiple range test was employed for mean separations with a confidence interval of 95%. An ANOVA-calculated *p*-value below 0.05 was considered to indicate data that could reliably lead to the rejection of the null hypothesis. Mean values were backtransformed for presentation.

5.3 RESULTS

5.3.1 Field applications. 2010 was a year with typical weather conditions during grape ripening, from the period after veraison to grape harvest, compared within a four year study between 2009-2012 (Figures 4.1, 4.2, 4.3, and 4.4). Daily mean temperatures were between 10 and 26°C with a mean total daily rainfall of 3.1 mm per day in September, before grapes were harvested October 2. September in 2011 was warmer, with daily mean temperatures between 14 and 29°C and with more frequent precipitation leading to a mean total daily rainfall of 4.2 mm per day; daily mean temperatures dipped down to between 10 and 20°C in the first half of October, before grapes were harvested on October 16. Disease severity was lower at harvest in 2010 at 12.2% (Table 4.1), than in 2011 at 55.1% (Table 4.1). In both years, results were significant, with sour rot reduced when treatments were applied in the field (Figure 5.1, 5.2, 5.3, and 5.4).

In 2010, under lower disease pressure, Milstop and KMS treatments at all concentrations significantly reduced sour rot severity from 45.1% to 67.6% of the untreated control and there

were no significant differences among treatments (Figure 5.1). All treatments significantly decreased VA compared to the untreated control, which had mean VA of 0.41 g acetic acid/L (Figure 5.1). The treatments reduced the VA to between 0.19 to 0.25 g acetic acid/L. KMS at 5 kg/1000L had the most impact in reducing VA, but all KMS concentrations reduced VA below 0.24 g acetic acid/L (Figure 5.1).

In 2011, only Milstop, KMS 5 kg/1000L, and KMS 10 kg/1000L significantly reduced sour rot severity. Milstop reduced severity to 74.1% of the untreated control, KMS 5 kg/1000L to 56.7%, and KMS 10 kg/1000L to 42.3%. There was no statistical difference in the severity of sour rot between KMS at 5 and 10 kg/1000L and both had significantly less severe sour rot than other treatments (Figure 5.2). All treatments, except tartaric acid, significantly reduced VA in the pressed must. KMS at the highest rate of 10 kg/1000L reduced acetic acid by the greatest margin from 0.78 g acetic acid/L in the untreated control to 0.29 g acetic acid/L in the treated plots (Figure 5.2). Milstop had significantly lower VA (0.34 g acetic acid/L) than KMS 5 kg/1000L (0.58 g acetic acid/L), although sour rot severity was significantly less in the KMS plots, 76.6% that of the Milstop plots. The addition of tartaric acid to KMS resulted in higher VA than KMS alone, from 0.71 g acetic acid/L with tartaric acid to 0.58 g acetic acid/L without. With higher disease pressure, none of the treatments in 2011 reduced VA below 0.24 g acetic acid/L.

In 2010, there were no significant differences in sour rot severity among different timing schedules of KMS 5 kg/1000L (Figure 5.3). The treatment with six sprays, initiated six weeks pre-harvest, had significantly lower VA than treatments that started later (0.19 g acetic acid/L at harvest) (Figure 5.3). In 2011, delaying the initiation of KMS applications from seven weeks to three days pre-harvest (five to two sprays) did not affect the severity of sour rot or VA, although the treatment with four sprays resulted in anomalously high sour rot severity accompanied by the lowest VA of all treatments (Figure 5.4). Applying KMS three days and one day pre-harvest resulted in significantly more severe sour rot and more VA than a single one day pre-harvest spray (Figure 5.4). This repeats the trends in the acetic acid ratings of 2010 (Figure 5.3).

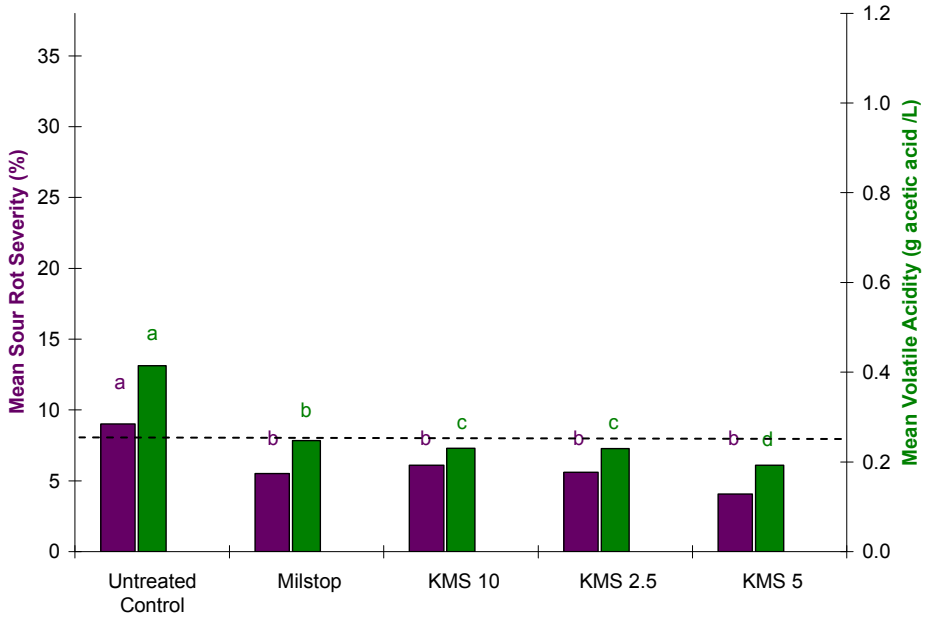


Figure 5.1 Mean sour rot severity and mean acetic acid concentration according to treatments and concentrations applied to plots of *Vitis vinifera* cv. Riesling, harvested October 2, 2010. Four replicated plots were sprayed with details listed in Table 5.1. N = 100 clusters assessed per treatment and duplicated acetic acid measurements. ANOVA severity $F(3,4) = 4.89$, $p = 0.001$, acetic acid $F(3,4) = 431.51$, $p = <0.0001$. Bars with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

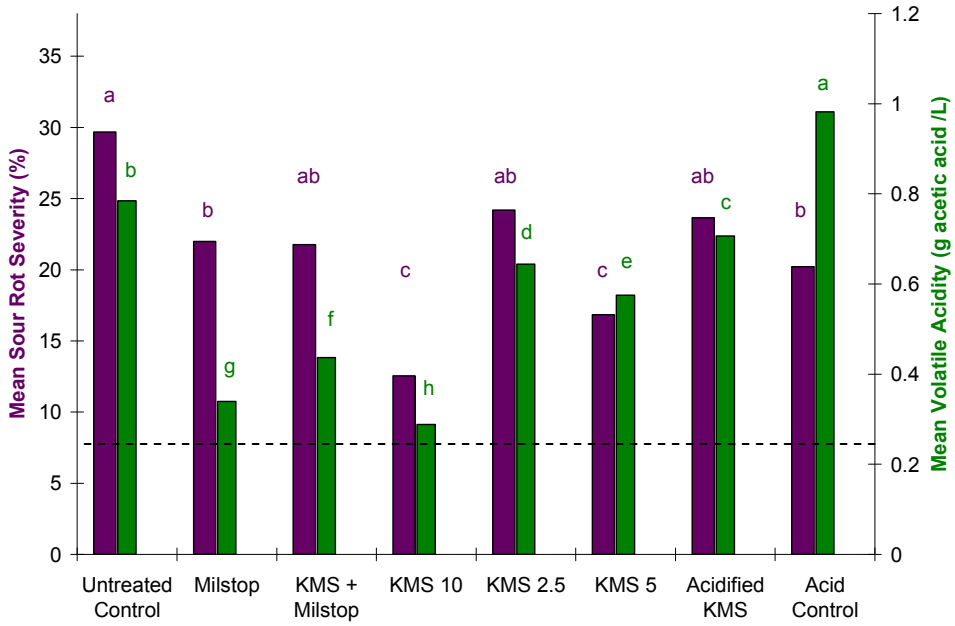


Figure 5.2 Mean sour rot severity and mean acetic acid concentration according to treatments and concentrations applied to four replicated plots of *Vitis vinifera* cv. Riesling, during the 2011 season, harvested October 16. Treatments and concentrations are listed in Table 5.2. ANOVA severity $F(3,7) = 7.79$, $p = <0.0001$, acetic acid $F(3,7) = 1300.42$, $p = <0.0001$. N = 100 clusters assessed per treatment and duplicated acetic acid measurements. Bars with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

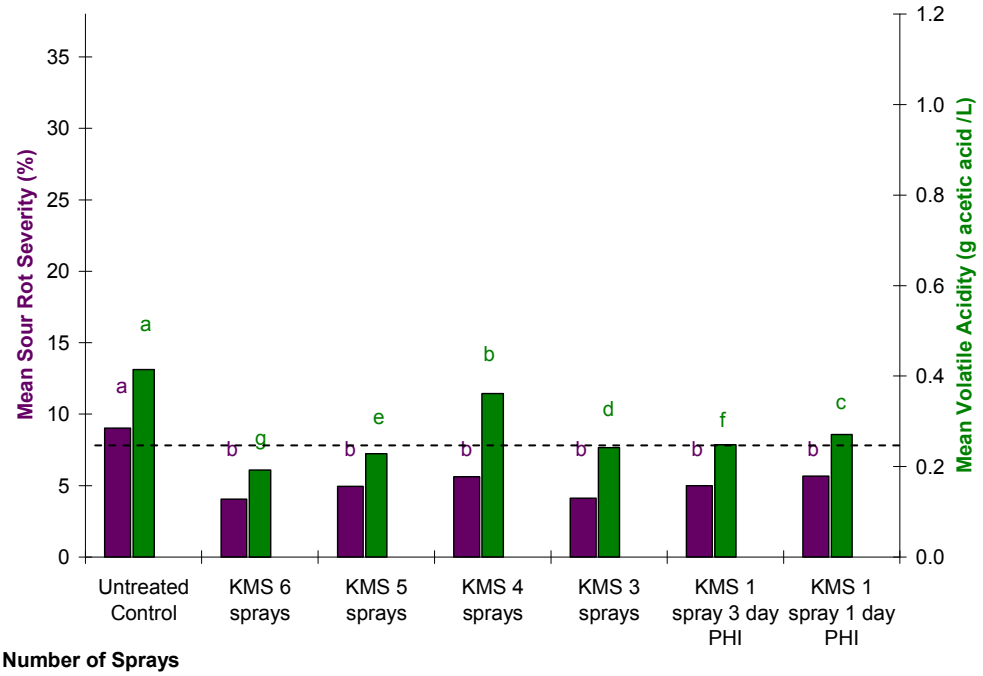


Figure 5.3 Mean sour rot severity and mean acetic acid concentration according to KMS 5 kg/1000L applied at different timing intervals to plots of *Vitis vinifera* cv. Riesling, harvested October 2, 2010. Four replicated plots were sprayed with the timings listed in Table 5.1. ANOVA severity $F(3,6) = 4.23$, $p = <0.0001$, acetic acid $F(3,6) = 660.51$, $p = <0.0001$. N = 100 clusters assessed per treatment and duplicated acetic acid measurements. Bars with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

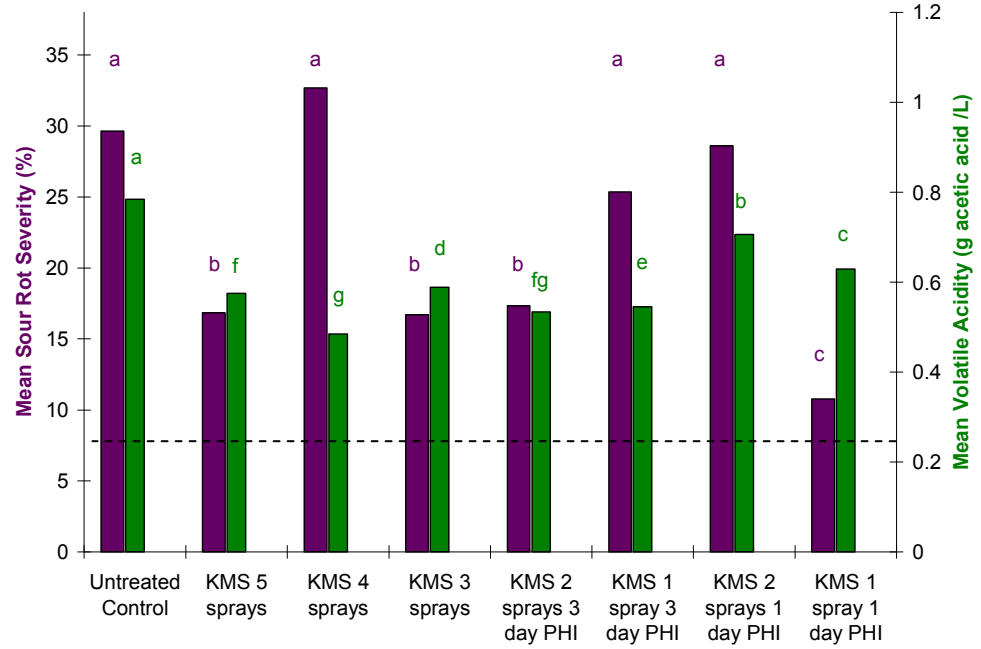


Figure 5.4 Mean sour rot severity and mean acetic acid concentration resulting from decreasing interval timings of KMS 5 kg/1000L applied to four replicated plots of *Vitis vinifera* cv. Riesling in 2011, harvested on October 16. Timings are listed in Table 5.2. ANOVA severity $F(3,4) = 4.89$, $p = <0.0001$, acetic acid $F(3,4) = 431.51$, $p = <0.0001$. N = 100 clusters assessed per treatment and duplicated acetic acid measurements. Bars with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

5.3.2 Disk diffusion assay. In the disk diffusion assay, disks saturated in potential sour rot treatments were applied to YPD agar plates inoculated with wild-isolated *H. uvarum* or *G. oxydans*. After three days growth at 22°C, the organisms grew confluently across the plates, with a clearance area around some disks. Only KMS at a concentration of 10 g/L worked to clear a statistically significant radius of 1.0 mm beyond the disk on the *H. uvarum*-seeded plates. In the *G. oxydans*-inoculated plates, all treatments containing KMS cleared a radius that was significantly greater than the untreated control. KMS at a concentration of 10 g/L provided the greatest clearance of 9.7 mm, significantly greater than other treatments (Figure 5.5). Milstop did not inhibit growth of either organism in the disk assay.

5.3.3 Treatment activity assay *in vitro*. Treatments were applied to wounded berries immediately before inoculation and 24 hours after inoculation with wild-isolated *G. oxydans* and *H. uvarum*. After 14 days incubation at 22°C, grapes were rated for symptoms of sour rot according to a rating scale of zero to four (Figure 2.1). KMS 5 g/L and 10 g/L were the only treatments to significantly reduce symptoms caused by *G. oxydans* when applied before inoculation, from a pathogenicity rating of 3.5 untreated, to ratings of 1.8 and 1.6, respectively. Only KMS 5 g/L reduced symptoms caused by *H. uvarum* when applied before inoculation to a wounded berry, from a pathogenicity rating of 3.0 untreated, to a rating of 1.3 (Figure 5.6).

When applied 24 hours after inoculation, KMS 10 g/L and acidified KMS significantly reduced the symptoms produced by *G. oxydans* from a rating of 3.9 untreated, to ratings of 2.2 and 2.6, respectively. None of the post-infection treatments significantly reduced symptoms produced by *H. uvarum* (Figure 5.7).

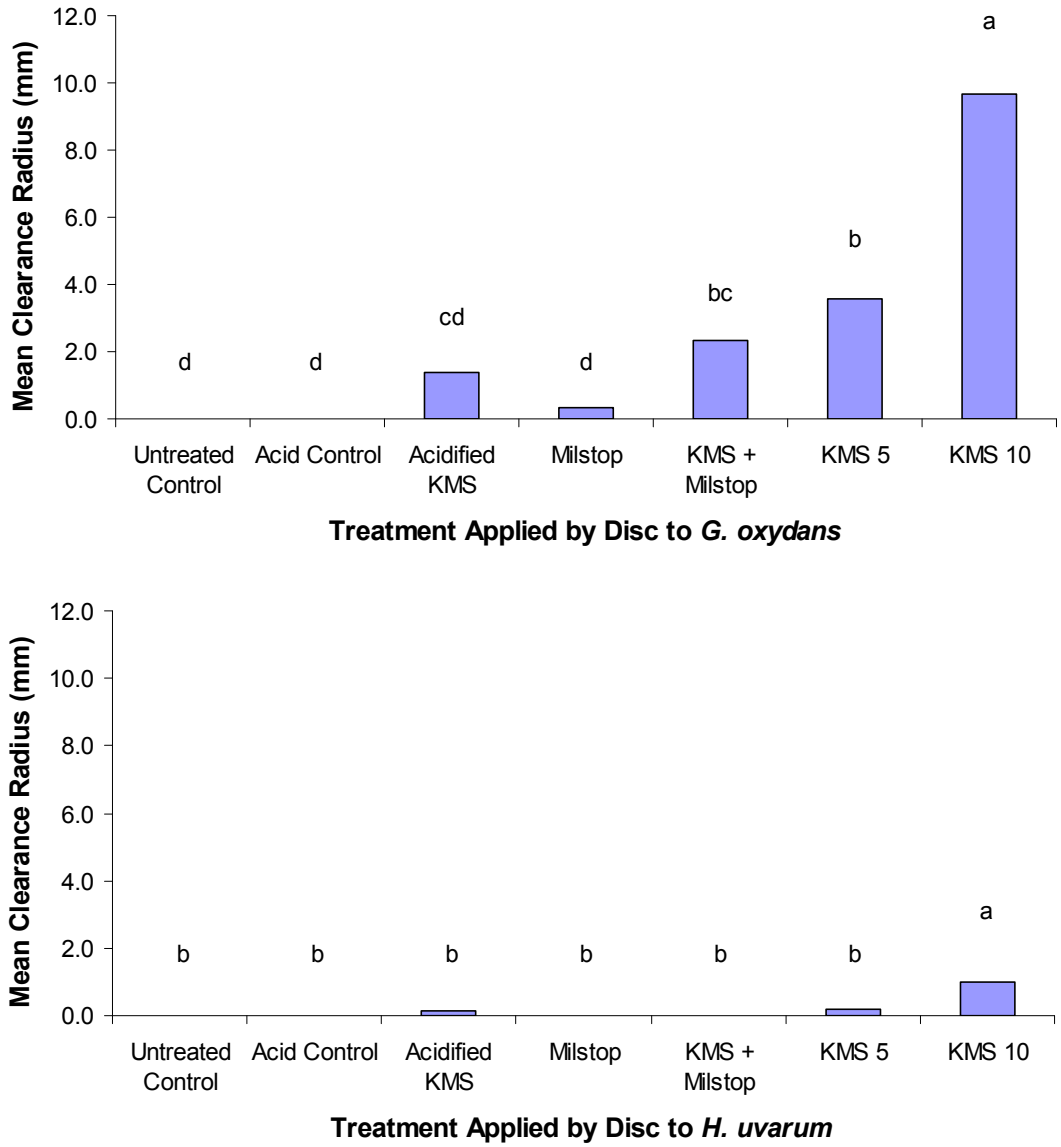


Figure 5.5 Treatments applied by saturated disk to plates of wild-isolated *Gluconobacter oxydans* (above) or *Hanseniaspora uvarum* (below). Plates were incubated three days at 22°C. The mean clearance radius was measured from four disks on each of four replicated plates for each treatment (N = 16). *G. oxydans* ANOVA $F(3,6) = 30.66$, $p = <0.0001$, *H. uvarum* ANOVA $F(3,6) = 14.28$, $p = <0.0001$. Bars with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

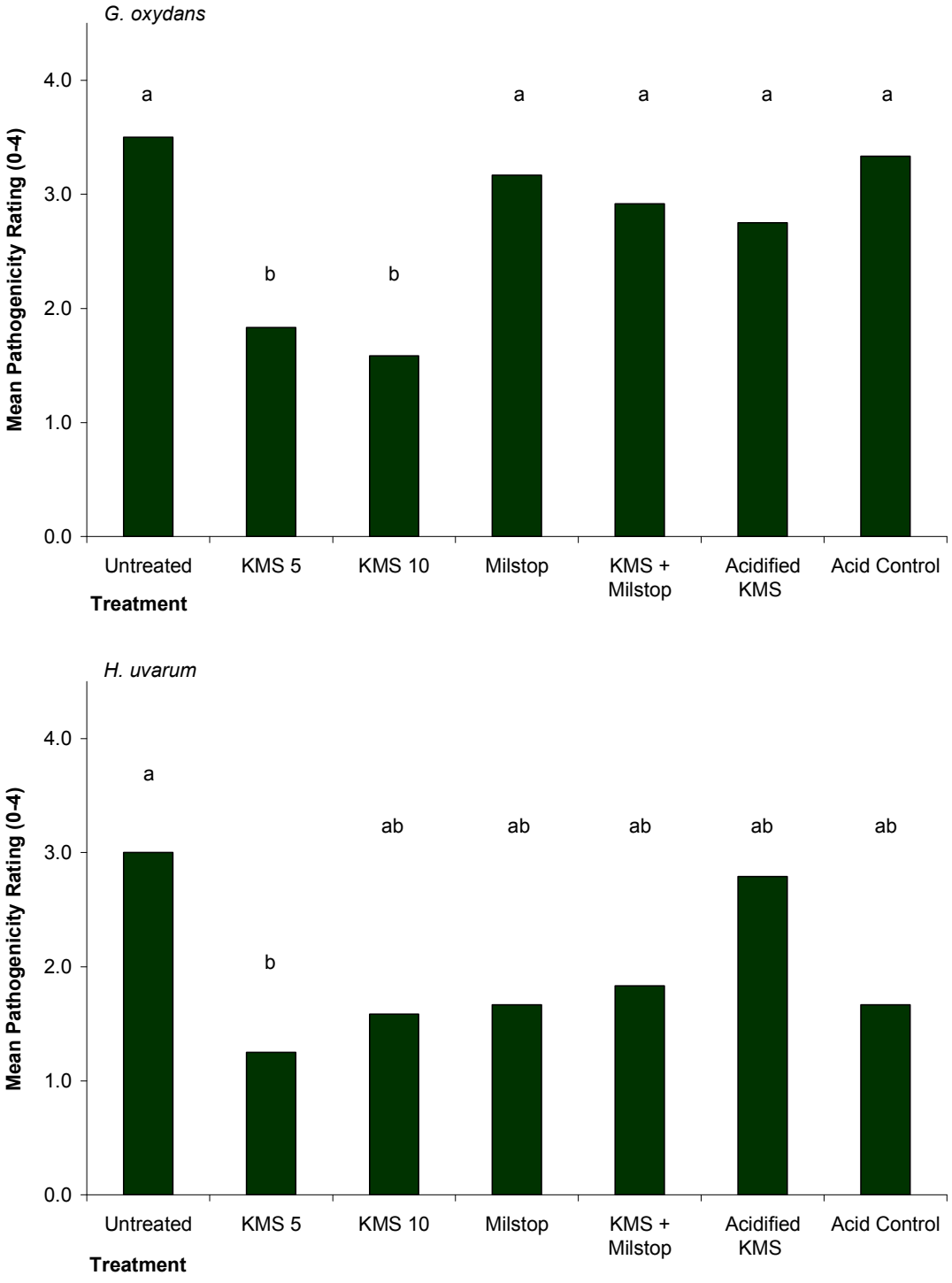


Figure 5.6 Treatments applied immediately before inoculation of wounded grapes with wild isolates of *Gluconobacter oxydans* (above) or *Hanseniaspora uvarum* (below). Grapes were incubated in four replicated chambers for 14 days at 22°C, and the symptoms produced were rated on a scale of zero to four. N = 12 berries per treatment. ANOVA $F(3,6) = 6.15$, $p = <0.0001$ (*G. oxydans*), and $F(3,6) = 2.86$, $p = 0.0144$ (*H. uvarum*). Bars with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

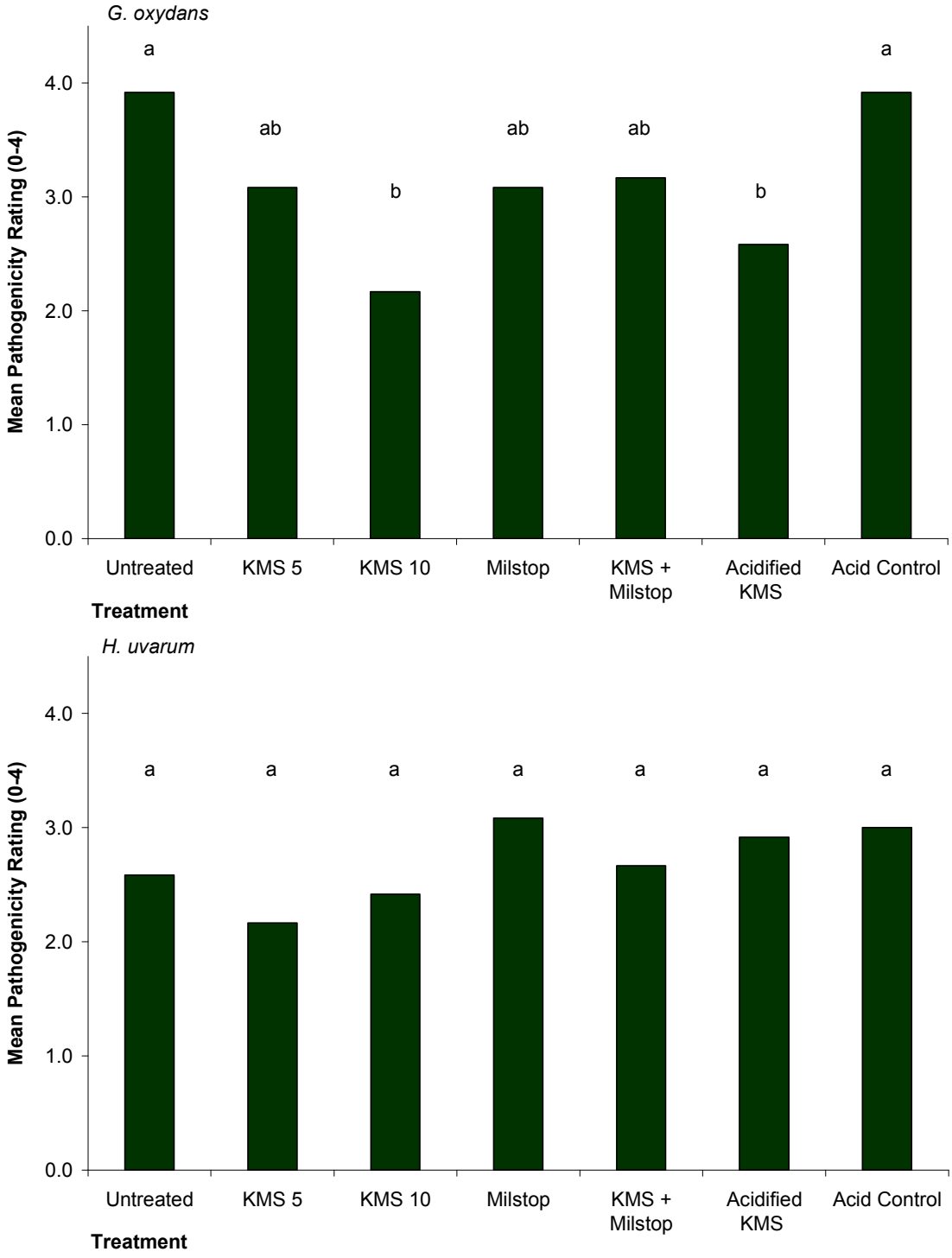


Figure 5.7 Treatments applied 24 hours after inoculation of wounded grapes with wild-isolated *Gluconobacter oxydans* (above) or *Hanseniaspora uvarum* (below). Grapes were incubated in four replicated chambers for 14 days at 22°C, and the symptoms produced were rated on a scale of zero to four. N = 12 berries per treatment. ANOVA $F(3,6) = 5.91$, $p = <0.0001$ (*G. oxydans*), and $F(3,6) = 1.30$, $p =$ n.s. (*H. uvarum*). Bars with the same letter are not significantly different according to the Student Newman-Keuls multiple range test.

5.4 DISCUSSION

In 2011, harvest was on October 16, approximately two weeks later than in 2010, on October 2 (Table 5.1 and 5.2). September had warmer daily mean temperatures in 2011 and there was more total daily rainfall in September and mid-October before the 2011 harvest compared with 2010 (Figure 4.2 and 4.3). Weather conditions were correlated with greater sour rot disease severity of 55.1% in 2011 compared with 12.2% in 2010 (Table 4.1), and with grapes hanging longer before harvest, they may have had more opportunity to develop disease in 2011. With increased disease severity in control plots in 2011 compared with 2010, the differences between sour rot treatments were more noticeable (Figure 5.1, 5.2, 5.3, and 5.4).

Milstop and KMS formulations significantly reduced visual and chemical sour rot symptoms, likely by acting on the microorganisms that cause sour rot. In 2010, the ANOVA-calculated *p*-value amongst different treatment formulations was 0.001 for disease severity and <0.0001 for VA (Figure 5.1). The ANOVA-calculated *p*-value for treatment formulations in 2011 was <0.0001 for disease severity and VA (Figure 5.2). Therefore, the null hypotheses that plots sprayed with KMS or Milstop formulations would not be different compared to the untreated control plots in disease severity or VA, could be rejected. In 2010, all of the formulations worked to reduce sour rot severity from 45.1% to 67.6% of the untreated control and there were no differences among treatments (Figure 5.1). Milstop had a higher VA than any of the KMS treatments. KMS at all concentrations reduced VA to below the rejection threshold of Ontario wineries, which is 0.24 g acetic acid/L (Figure 5.1; Grape Growers of Ontario, 2010). In 2010, KMS 5 kg/1000L resulted in the lowest VA of 0.19 g acetic acid/L. KMS was able to reduce VA below that of Milstop, even though both treatments similarly reduced sour rot severity (Figure 5.1). One may hypothesize this is because KMS, as well as having antimicrobial properties in the molecular form, contains antioxidant properties at higher pHs in the bisulfite and sulfite forms (Du Toit and Pretorius, 2002; Margalit, 1997).

In 2011, KMS 5 kg/1000L and KMS 10 kg/1000L reduced sour rot severity by the greatest margin to 56.7%, and 42.3% of the untreated control, respectively. Milstop also reduced the severity of sour rot to 74.1% of the untreated control, but was not as effective as the KMS treatments (Figure 5.2). Yet, all formulations of KMS and Milstop reduced VA, with KMS 10 kg/1000L reducing it the most to 0.29 g acetic acid/L, compared to the untreated control with a VA of 0.78 g acetic acid/L (Figure 5.2). Disease pressure was much greater in 2011 compared to 2010 in the untreated plots, and this level of VA was above the winery rejection threshold of 0.24 g acetic acid /L (Grape Growers of Ontario, 2010). The Milstop formulation resulted in a VA that was lower than the KMS 5 kg/1000L formulation, negating the hypothesis that KMS is more efficacious due to antioxidant properties. On the other hand, Milstop forms a solution at a pH of 8.4 (Nigro et al., 2006), while KMS in its active molecular form is favoured below a pH of two (Fugelsang and Edwards, 2007). It is possible the basic solution of Milstop had some neutralization action in the acidic sour rot environment. The pH of treatments applied in the field is given in Appendix V.

Since an appreciable amount of KMS is in an active antimicrobial form only below a pH of three, and the pH of the formulation prepared for the field studies was 3.64 (Appendix V), an attempt was made to acidify the KMS solution using tartaric acid. The addition of tartaric acid formed a KMS solution with a pH of 2.54 (Appendix V). Yet, in 2011 the addition of tartaric acid resulted in higher VA than KMS 5 kg/1000L alone, 0.71 g acetic acid/L with acidified KMS, compared to 0.58 g acetic acid/L with KMS 5 kg/1000L (Figure 5.2). It appeared that the acidification of the sour rot environment with a formulation of low pH resulted in a higher VA. This trend was most clearly shown in the tartaric acid control, a formulation which had a pH of 2.25 (Appendix V). Tartaric acid resulted in a VA of 0.98 g acetic acid/L, above that of the untreated control with a VA of 0.78 g acetic acid/L (Figure 5.2).

In 2010, all timings of KMS 5 kg/1000L resulted in a reduction of disease severity and VA compared to the untreated control plot with an ANOVA-calculated p-value of <0.0001 for

each parameter (Figure 5.3). Similarly, all timings in 2011 reduced sour rot severity and VA, each with an ANOVA-calculated *p*-value of <0.0001 (Figure 5.4). Therefore, the null hypotheses that different timings would not result in a different disease severity or VA compared to the untreated control, could be rejected. In 2010, timings were no different from each other in the severity of sour rot, meaning a spray schedule initiated six weeks before harvest had the same effect on severity as one spray the day before harvest (Figure 5.3). Yet, the season-long schedule did result in a lower VA than any of the timings initiated later, and only the treatments with six sprays and five sprays reduced VA below the winery rejection threshold of 0.24 g acetic acid/L (Figure 5.3; Grape Growers of Ontario, 2010). In 2011, spray timing with a three day PHI could be reduced from five sprays to two sprays without an effect on the severity of sour rot or VA (Figure 5.4). In spray timings with a one day PHI, two sprays resulted in more severe sour rot and more VA than one spray (Figure 5.4). This indicates that more sprays may not necessarily be more efficacious. Climatic events such as rain may be shown to have an effect on sour rot development (Figure 4.1, 4.2, 4.3, and 4.4) and it may improve disease management to time treatment sprays according to rain events rather than by a predetermined schedule.

Microorganisms are able to produce compounds with aldehyde and ketone groups, which bind bisulfite, the main form of sulfur dioxide in aqueous solutions. Binding of bisulfite limits the amount of active molecular sulfur dioxide. Acetic acid bacteria, such as *G. oxydans*, are particularly known for producing bisulfite-binding carbonyl compounds, including metabolites such as gluconic acid, 5-oxofructose, and dihydroxyacetone (Carrascosa et al., 2011; Du Toit and Pretorius, 2002). This means that the activity of the antimicrobials may depend on the microbial load and activity of microbes present. There may be some adaptation of the microbes to KMS after more than one spray, for example, by producing more binding molecules. To test the dynamics between the formulations and the microorganisms in the vineyard, VA, pH, and microbial samples could be taken before and after successive spray treatments in sour rot-infected clusters. Weather conditions may also change spray efficacy, with precipitation having a

washing effect over the microorganisms (Gonzalez et al., 2005), yet diluting the treatments. An experiment could be initiated with sprays specifically targeted after precipitation events, versus a weekly or bi-weekly schedule.

To further understand the efficacy of KMS and Milstop to limit sour rot microorganisms, these treatments were applied to *G. oxydans* and *H. uvarum in vitro*. In the disk diffusion assay, treatment-saturated disks resulted in microbial clearance from YPD plates, with an ANOVA-calculated *p*-value of <0.0001 for each microbe (Figure 5.5). The null hypothesis that the products would not clear the growth of the sour rot organisms compared to an untreated control, could be rejected. Only KMS at a concentration of 10 g/L worked against *H. uvarum*, clearing a radius of 1.0 mm beyond the disk. KMS 10 g/L also cleared the greatest radius of *G. oxydans*, 9.7 mm beyond the disk. KMS at all concentrations was able to inhibit *G. oxydans* growth, in contrast to Milstop (Figure 5.5). KMS 10 kg/1000L was also able to reduce sour rot to the lowest mean severity in the field in 2011, 12.54%, although in 2010 and in 2011 KMS 10 kg/1000L was not significantly different compared to KMS 5 kg/1000L (Figure 5.1 and 5.2). Based on the disk assay, there was some indication that higher concentrations of KMS work better to inhibit microbial growth. Although, in the complex chemical environment of the grape, it seemed the difference in a double concentrated spray was not always significant. Higher concentrations of KMS could be tested in field trials.

Milstop showed no activity against *G. oxydans* and *H. uvarum* in the disk diffusion assay. Activity in the field may be due to chemical neutralization of the acidic diseased grape, or may be due to its activity against other pathogens that could weaken the grape and provide entrance for the sour rot microbes (Gadoury et al., 2007; Marchetti et al., 1984). This protective quality could be exhibited especially since Milstop was applied in a full-season schedule starting at veraison (Table 5.1 and 5.2). Similarly, bicarbonate was applied 90 days and 30 days before grape harvest in Italy (Nigro et al., 2006). Bicarbonate could reduce *Botrytis* bunch rot to 57.7% of the untreated plots, and sour rot to 51.4% (Nigro et al., 2006). To test the efficacy of Milstop

against sour rot microbes in the field, compared to the protective effect against wounding pathogens, Milstop sprays could be initiated closer and closer to harvest.

To further understand the activity of Milstop and KMS in the grape environment, treatments were applied to wounded table grapes *in vitro*. When treatments were applied before inoculation and 24 hours after inoculation with *G. oxydans* and *H. uvarum,* the null hypothesis was that the treatments would not reduce the symptoms of sour rot compared to an untreated control. With treatment before inoculation, both inocula resulted in an ANOVA-calculated *p*-value of <0.0001. Therefore the null hypothesis could be rejected. With treatment after inoculation, only the *G. oxydans* trial resulted in a significant *p*-value of <0.0001, leading to the rejection of the null hypothesis. *H. uvarum* tests resulted in a *p*-value of 0.268 (n.s.), leading to the acceptance of the null hypothesis. KMS 5 g/L and 10 g/L were the only treatments to reduce symptoms caused by *G. oxydans,* and only KMS 5 g/L reduced symptoms caused by *H. uvarum* when applied before inoculation (Figure 5.6). KMS 10 g/L and acidified KMS reduced symptoms caused by *G. oxydans* when applied 24 hours after inoculation, while none of the treatments reduced the symptoms of *H. uvarum* when applied after inoculation (Figure 5.7).

Yeasts can produce compounds that bind bisulfite, such as acetaldehyde, pyruvic acid and 2-oxoglutaric acid (Barbe et al., 2001; Carrascosa et al., 2011). This could explain how the established population of *H. uvarum* was better able to resist treatment 24 hours after inoculation, compared to the inoculum applied over the pre-dipped treatment (Figure 5.6 and 5.7). *G. oxydans* was more susceptible to acidified KMS 24 hours after inoculation in the *in vitro* experiments (Figure 5.7), but these results did not translate in the field (Figure 5.2). This could be due to the field environment neutralizing the solution, the effect of successive treatments, or the microbial load present during treatment. The grape assay could be repeated with a longer incubation time and therefore more establishment of disease symptoms before the application of treatments. To understand the dynamics between microorganisms and treatments in the field, inoculum could be sprayed in the vineyard to whole clusters. Experimental designs would

include replicated plots with and without wounding, using *G. oxydans* and *H. uvarum* inoculation before and after treatments, to consider protective effects of the products and the ability for established microbial populations to resist the products.

KMS is not registered for spraying in grapes (Ontario Ministry of Agriculture and Food, 2014). Milstop is registered for powdery mildew in grapes (Ontario Ministry of Agriculture and Food, 2014), yet may not be the best treatment for sour rot. Although it showed reduction of sour rot in the field, agreeing with previous literature (Nigro et al., 2006), this may be through integrated effects, reducing grape damage caused by fungal pathogens (Bisiach et al., 1986). Milstop did not show great efficacy in laboratory trials limiting *G. oxydans* or *H. uvarum* (Figure 5.5, 5.6, and 5.7). Further studies are necessary to test more products for activity against the sour rot organisms.

CHAPTER 6: DISCUSSION AND FUTURE RESEARCH

Sour rot is a late-season bunch rot of economic importance to the wine grape growers in Ontario, particularly in warm years with high precipitation. There is a limited field of research papers associating sour rot with etiological organisms (Gravot et al., 2001), environmental factors (Gadoury et al., 2007), and studying treatments for the agricultural disease (Bisiach et al., 1986; Nigro et al., 2006).

Sour rot has been reported in other areas of the world to be caused by an array of microorganisms, particularly apiculate and fermentative yeasts and acetic acid bacteria (Barata et al., 2012a). These microbes are reported as a part of the normal epiphytic microflora of grapes during ripening (Marchetti et al., 1984). However, when they enter the berry, they can survive in the anaerobic conditions and cause chemical changes to the grape, producing high levels of acetic acid and ethyl acetate, which are wine spoilage compounds (Andorra et al., 2010; Clemente-Jimenez et al., 2004; Du Toit and Pretorius, 2002). In the present research, microorganisms were cultured from sour rotted clusters, subjected to genetic identification, and were reintroduced to the grape in pathogenicity assays to ascertain which organisms were closely associated with the disease and which were responsible for symptom production. It was reported that the organisms associated sour rot symptoms were acetic acid bacteria, particularly *Gluconobacter oxydans*, *Gluconobacter cerinus*, and *Acetobacter malorum* (Section 2.3.1) and these organisms were responsible for the greatest acetic acid production in the grape environment (Section 2.3.2). The ecologically dominant apiculate yeast, *Hanseniaspora uvarum* (Section 2.3.1), was also capable of causing symptoms but had significantly lower acetic acid production (Section 2.3.2). The fermentative yeast, *Candida zemplinina*, was found frequently associated with sour rotted grapes (Section 2.3.1), but was not responsible for high disease symptom ratings or acetic acid production (Section 2.3.2). Future research could utilize gas chromatography-olfactometry-mass spectrometry (GC-O-MS) to quantify levels of ethyl acetate formed in symptomatic grapes by these microorganisms. It would also be of interest to study

more strains of these species, since there are strain-dependent qualities in enzyme activity and metabolite production (Drysdale and Fleet, 1988; Romano et al., 1997; Strauss et al., 2001). Direct DNA sampling, such as through the use of microarray analysis (Zhou, 2003), or pyrosequencing (Will et al., 2010) could result in more sensitive detection of microorganisms present in lower numbers or existing in a viable but not culturable (VBNC) state.

Sour rot is mainly associated with grape injury (Barata et al., 2012a). This has led to some confusion about the causal agents of sour rot. For example, other pests such as the fungal pathogens *Botrytis cinerea* (Marchetti et al., 1984) and *Erisiphe necator* (Gadoury et al., 2007) can perforate grape skin leading to entry points for sour rot yeasts and bacteria. In the present study, the yeasts *H. uvarum* and *C. zemplinina* could not infect an intact grape, requiring an injury point for entry, while the acetic acid bacterium, *G. oxydans,* was able to penetrate grapes through the pedicel attachment site (Figure 2.3). Too much water can lead to grape swelling when there is a period of high precipitation (Gravot et al., 2001). This can cause microfissures in the skin, or can lead to weaknesses at the pedicel attachment site. Cultivars with thin skins and tightly packed clusters, such as Riesling and Pinot Noir, were specifically targeted for the present research due to their susceptibility to such injury, associated with sour rot.

Infected Riesling and Pinot Noir clusters were sampled for microbial populations from across the Niagara Peninsula, with a higher relative frequency of *H. uvarum* being recovered from Riesling (Table 3.1). Acetic acid bacteria were found in highest dominance at sites in the Niagara Lakeshore sub-appellation (Figure 3.2), which has a wetter climate than other areas in the region (Schlosser et al., 2005). Riesling and Pinot Noir cultivars were sampled over time. Organisms not associated with sour rot symptoms dominated in the period after veraison before sour rot symptoms were detected, an oxidative yeast, *A. pullulans*, a filamentous fungus, *A. alternata*, and a bacterium, *P. fluorescens* (Figure 3.3). On the sampling day of sour rot detection, *H. uvarum* and acetic acid bacteria became dominant in the population and it was not until the next sampling date that *C. zemplinina* was observed in succession (Figure 3.3). Since *C.*

zemplinina is a fructophilic yeast (Andorra et al., 2010), and a low producer of acetic acid (Magyar and Toth, 2011), it is possible that this yeast was an opportunist of the sour rot environment.

To test the effect of ecological succession on the symptoms of sour rot, sequential inoculations were performed using *G. oxydans*, *H. uvarum*, and *C. zemplinina* in *in vitro* grapes. Although *H. uvarum* could cause symptoms alone, symptoms were less when this yeast was paired with *C. zemplinina* or when *C. zemplinina* was inoculated alone, compared to sequential inoculations containing *G. oxydans* (Figure 3.4). Incubations containing *G. oxydans* had more acetic acid than incubations without (Figure 3.5). *C. zemplinina* seemed to mitigate disease. This could be further tested by looking at different inoculation concentrations, for example, the mitigation of disease symptoms could be tested when *G. oxydans* was present but out-competed by a much higher concentration of *C. zemplinina*.

In vitro grape inoculations were incubated under different conditions to establish the effect of temperature and grape ripeness on the growth and symptom production of the sour rot microbes, *G. oxydans* and *H. uvarum*. In these laboratory trials, very minor sour rot symptoms could be initiated at temperatures between 5 and 10°C (Figure 4.5), which agrees with the minimum cardinal temperatures of the organisms involved, 8-10°C in *G. oxydans* (Du Toit and Pretorius, 2002) and 4.71°C in *H. uvarum* (Salvado et al., 2011). However, sour rot developed earlier and became more severe at temperatures between 20 and 25°C, the maximum temperature range tested (Figure 4.5). This also agrees with the optimal temperatures of these organisms, 25-30°C in *G. oxydans* (Carrascosa et al., 2011) and 24.51°C in *H. uvarum* (Salvado et al., 2011). It would be interesting to add one more temperature range to the design of the present research, to test the growth of the sour rot organisms between 25 and 30°C.

Berries of different ripeness, measured in soluble solids, were incubated at 22°C with a mix of *H. uvarum*, *C. zemplinina*, *A. malorum* and *G. oxydans*. Sour rot symptoms tripled in grapes between 13.4°Brix and 15.5°Brix (Figure 4.5), while volatile acidity increased by a factor

of seven (Figure 4.6). If berries of different ripeness could be incubated at different temperatures, the factors of temperature and ripeness could be compared, for example, the organisms may be able to more effectively infect less ripe grapes at higher temperatures. It would also be useful to evaluate the effect of rainfall on microbial populations. Sampling could be performed before and after simulated rain events in the field. Rain events may be correlated with increased growth in higher humidity, or decreased microbial activity due to a washing effect (Gonzalez et al., 2005).

Total daily rainfall, daily mean temperature, grape ripeness, and sour rot disease progress were monitored in a Riesling vineyard over four years. The highest sour rot severity was observed at harvest in mid-October 2011 at 55% (Figure 4.3). 2010 and 2012 were harvested with similar severities of 12% in early October (Figure 4.2 and 4.4). Although it could be hypothesized that the higher severity in 2011 was due to a later harvest, the lowest sour rot severity was less than 1% during a late October harvest in 2009 (Figure 4.1). Sour rot was first detected in early September in 2011 and 2012, but at a much higher ripeness in 2012, at 18°Brix compared to 15°Brix in 2011 (Table 4.1). Sour rot was detected later, in mid-September in 2010, with the same ripeness as observed in 2012, 18°Brix. Sour rot was detected very late, in mid-October, at a ripeness of 19°Brix in 2009 (Table 4.1). In 2011, which was the year with the greatest disease severity, sour rot was observed to begin development at the lowest ripeness in the berries. This year showed higher daily mean temperatures and total daily rainfall, compared to 2009, 2010, and 2012 (Section 4.3.1). Higher daily mean temperatures and total daily rainfall were correlated with higher sour rot severity at harvest. To further study this correlation, disease modeling could be employed using multiple regression analysis (Smith et al., 2007).

Treatments for sour rot are limited due to the nature of the etiological agents, and the late appearance of symptoms in the field approaching harvest. It would be impossible to eliminate the grape yeast and bacterial microflora without the growth of unwanted organisms and loss of wanted organisms. For example, bacteria resistant to antibiotics may overgrow when antibiotics are applied, or there may be a loss of fermentative activity during winemaking when fungicides

targeting fermentative yeasts are applied too close to harvest (Bisson, 1999). However, when spoilage microbes overgrow, farmers require a way to manage symptoms of sour rot to preserve their crop. Potential treatments for sour rot were tested in the present study in the field and under *in vitro* conditions. KMS, potassium metabisulphite, though not registered for agricultural applications, is a food-grade preservative used in wine manufacturing practices. Milstop is a registered organic fungicide composed of potassium bicarbonate, which may lead to the dehydration of yeast and bacterial cells, or at least reduce wounding pathogens (Chapter 5).

In vivo testing showed that KMS at concentrations above 5 kg/1000L and Milstop sprayed at the label concentration of 5.6 kg/1000L were able to reduce the severity of sour rot compared to untreated control plots, even in years where severity in untreated plots exceeded 50% (Figure 5.1 and 5.2). KMS was able to reduce the volatile acidity in sour rot-infected vineyard plots to below the winery rejection threshold of 0.24 g acetic acid/L (Grape Growers of Ontario, 2010), in a year when sour rot severity reached 12% in untreated plots (Figure 5.1). KMS did not necessarily lead to greater reduction in sour rot severity or volatile acidity when more sprays were conducted and initiated earlier in the season (Figure 5.3 and 5.4). It may be of worth to test the timing of Milstop sprays similarly, to note its efficacy after fewer sprays.

In vitro testing showed that KMS applied by disk at a concentration as low as 5 g/L could clear a radius in a plated culture of *G. oxydans*, but a concentration of 10 g/L was necessary to clear a radius in *H. uvarum* (Figure 5.5). In an *in vitro* grape assay, only KMS 5 g/L could reduce sour rot symptoms produced by *H. uvarum*, when applied directly before inoculation (Figure 5.6), while none of the treatment formulations worked 24 hours after inoculation (Figure 5.7). Since yeasts produce compounds that can bind to bisulfite (Barbe et al., 2001), it is possible for these organisms to establish a population with some chemical resistance to the treatment. The symptoms produced by *G. oxydans* were reduced by the application of KMS 5 g/L and 10 g/L before inoculation (Figure 5.6), and KMS 10 g/L and acidified KMS 24 hours after inoculation (Figure 5.7). In contrast, acidified KMS did not show efficacy in the field (Figure 5.2). It is

possible that under vineyard conditions, the solution was neutralized or subject to a greater microbial load. The dynamic between microbial establishment and treatment efficacy could be tested in the vineyard by spraying microbial inoculum before and after initiating different treatment schedules.

The established wisdom states that sour rot is best controlled using integrated pest management strategies, to improve overall grape health and reduce injury caused by other pests and pathogens (Oliva et al., 1999). This includes the maintenance of an aerated canopy, vine balance and nutrition, and the application of registered pesticides (Duncan et al., 1995).

An important factor in sour rot management is the emergence of the knowledge that *Drosophila melanogaster*, the common vinegar fly, is a vector of sour rot microbes (Barata et al., 2012c). Future research may look into the phenology of the fly on the grape host, as well as aligning the taxonomy of microbes found in and on the fly with the taxonomy of grape microbes. Management of this fly in vineyards could further reduce sour rot, achieved through the use of insecticides, trapping, physical exclusion, or pheromone disruption by finding a novel target specific to the mating behaviour of the species. Deltamethrin is an insecticide active against *D. melanogaster* which reduced sour rot by 50% in previous research (Bisiach et al., 1986). However, in years of high precipitation, water itself acted to disseminate sour rot organisms, indicating that an integrated approach to disease management is still necessary.

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APPENDIX I

Media preparation standard operating procedures

a. Two types of microbiological media are routinely used: GYC (glucose, yeast, calcium carbonate agar) amended with cycloheximide is used to select for acid-producing bacteria, and is also useful in the isolation of cycloheximide-resistant yeasts such as *Hanseniaspora uvarum*; YPD (yeast, peptone, dextrose agar) amended with streptomycin sulfate and chloramphenicol is used to select for a wide range of yeasts and fungi.

The recipe for GYC includes:	The recipe for YPD includes:
20 g dextrose	4 g yeast
4 g yeast extract	4 g peptone
12 g $CaCO_3$	8 g dextrose
10 g agar	10 g agar
/ 400 mL dH_2O	/ 400 mL dH_2O

b. Powder is weighed using plastic weigh boats and is added to a 1L schott bottle. 400mL dH_2O, measured using a graduated cylinder, is added slowly while swirling to dissolve the powder. A magnetic stir bar may be added, to aid in mixing before and after autoclaving.
c. The bottles are loosely capped (not sealed- so that pressure can be released) and autoclave indicator tape is applied (stripes turn black to indicate an adequate heat is reached).
d. The media is autoclaved on a slow exhaust (liquid cycle) with 15-20 minutes of sterilization. This will completely liquefy the agar and sterilize any contaminants. When removing the bottles from the autoclave, wear heat protective gloves, seal the caps completely to avoid spills, and swirl the bottles gently to avoid settling of the agar. If the bottle was autoclaved with a magnetic stir bar, it may be placed gently on a stir plate.
e. While the media is being autoclaved, the amendments can be prepared. Weigh the powdered antibiotics using an analytical balance, directly into a 15mL plastic culture tube. All amendments are dissolved to a concentration of 100 mg/mL in the initial solution.
f. The amendments utilize different solvents and can withstand different temperature extremes, which can be confirmed by referencing the Merck Chemical Index.

Cycloheximide is dissolved in:	Streptomycin Sulfate is dissolved in:	Chloramphenicol is dissolved in:
acetone	dH_2O	95% ethanol

g. As a rule, none of these amendments are subjected to the heat of autoclaving, which is why they are filter-sterilized and should be added to the media just as it is about to be poured.
h. After the powder is dissolved, often requiring the action of a vortex, in a sterile hood or near a flame, add the contents to a sterile plastic syringe, affix a filter disk to the tip of the syringe, and use the plunger to force the solution through the tip and into a second, sterile plastic tube. The sterile antibiotic solution can be stored at 4°C in the long term.
i. The sterile100 mg/mL solutions of antibiotics can be added to the appropriate media once that media reaches a temperature of about 60°C, which may be described as the temperature you would drink coffee. 400 μL of each solution is added to 400 mL of media using a micropipette and the media is gently mixed. This yields a final concentration of 0.1 mg/mL of each amendment.
j. 400 mL of media is a generous amount to pour 25 100 mm plates (1 sleeve). Plates should be poured in a sterile hood or near a flame. Swirl gently to avoid bubbles, and pour by hand until

the flow of media *nearly* covers the base of the dish. Replace the lid that covers the base, swirl the plate, and stack the plates to dry for 24-48 hours depending upon condensation.
k. Plates should be used as soon as possible within the week. If you must, it is best to routinely store poured plates at room temperature to avoid condensation. Plates are stored upside-down and sealed in the plastic sleeve from which they were purchased. However, plates that contain amendments must be stored at 4°C in the longer term to preserve the antibiotics. Perhaps remove these a day early from the fridge, unwrap and leave stacked overnight in the hood to allow excessive condensation to evaporate.
l. Peptone water is used to dilute the grape pulp for plating. The solution is prepared with 1 g peptone in 1000 mL dH_2O, mixed with 100 µL Tween-20. The Tween is extremely viscous and must be pipetted very slowly. The solution can be aliquoted into several Schott bottles, or flasks covered with tin foil, to be autoclaved. The peptone is prone to spoilage if it is not sterile.
m. Grapes are routinely considered to have a volume of 1 mL each in microbiology. When crushing grapes or simply agitating them to remove the surface microbes, it is best to add one grape per mL of peptone water. For example, to get a good sample of surface organisms from a cluster, prepare a sterile flask with 50 mL of peptone water, then use a pair of flamed scissors to cut the pedicel of 50 grapes, letting them fall into the flask. Agitate these for at least 5 minutes.
n. Alternately, if grapes bear internal organisms as in sour rot, they can be surface-sterilized by dipping in 95% ethanol and exposing briefly to flame. The flesh may then be crushed in 1 mL peptone water per grape, and the resulting mixture would be a two times dilution.
o. Experiments have been performed proving that freezing grapes for one to two months should not affect the distribution of microorganisms recovered from them.
p. Rinses from fresh or frozen grapes, crushed or intact, or a fresh juice sample can be plated directly onto the YPD or GYC media. Simply mix the sample very well by vortexing, remove 100 µL using a pipette under sterile conditions, deposit onto a plate, and use the hockey stick spreader to push the fluid back and forth while you turn the plate with your other hand. The plate can be sealed with Parafilm, and is usually incubated upside-down for 3-5 days at room temperature (or alternately in a 25-30°C incubator).
q. Many samples will need to be diluted in order to attain a spread plate with 30-300 colonies (an adequate number to distinguish and count). For example, up to 10^5 colonies/grape have been reported. To prepare a dilution series, add 900 µL of peptone water to a number of Eppendorf tubes. Add 100 µL of your sample to the first tube, vortex it, use a fresh tip, and remove 100µL from that tube to add to the next in a series. Each will be ten times more dilute than the last. For the sour rot project, the best plates were often taken from 10^1-10^3 times dilutions. Plates of each dilution should be prepared at least in duplicate, but ideally in triplicate, to ensure there is precision in the plating methods.

APPENDIX II

Electrophoresis, DNA purification and sequencing standard operating procedures

a. PCR products are run towards a positive electrode through a 1% agarose gel. The negative charge of the nucleotides provides motive force in the electrical gradient and the agarose polymer will separate the nucleotide strands based on size.
b. 0.5-1x tris-borate-EDTA (TBE) buffer is used to prepare the agarose gel and to act as a running buffer. The recipe for 5x TBE is as follows:

54 g Trizma (Tris Base)
27.5 g boric acid
20 mL 0.5 M EDTA (pH=8)
/fill to 1 L with dH_2O

The 0.5 M pH 8 ethylenediaminetetraacetic acid (EDTA) is prepared by dissolving 18.61 g of disodium EDTA·$2H_2O$ in 80 mL distilled water. Adjust the solution to pH 8 using approximately 2 g NaOH pellets. EDTA will only dissolve at an alkaline pH. Once the 5x TBE stock solution is prepared, it can be diluted by five or ten times to the appropriate working concentration. The solutions keep longer in the fridge, but should be fine on the bench top for approximately 6 months.
c. Prepare the agarose gel depending upon the size of your mould. For example, for a small 1% gel, add 50 mL TBE buffer to 0.5 g agarose powder in a flask.
d. Boil the mixture prepared in (c.) over a hotplate or in the microwave for just over a minute to dissolve the agarose. The solution can be cooled at room temperature or for about thirty seconds under running water. It should be around 60°C.
e. Add 1-2 µL Ethidium Bromide (EtBr) and swirl before you pour the solution into the mould, then add the comb which will form the loading wells.
f. Cover your agarose gel with tinfoil as it sets for at least a half hour. The EtBr is light sensitive.
g. Once the gel is set and the agarose has polymerized, remove the comb, and just cover the gel with TBE running buffer in the electrophoresis apparatus.
h. Use a square of Parafilm to mix your PCR product with the loading dye. Apply 1 µL drops of loading dye to the Parafilm, then using a fresh pipette tip, mix in 5 µL of PCR product by plunging the pipette up and down to the first stop (not beyond, or you will release air bubbles). Aspirate all 6 µL by resetting the pipette volume or by beginning your aspiration past the first stop when set to 5 µL.
i. Apply the 6 µL of PCR product and loading dye to a well by inserting the tip into the TBE buffer, but do not drive the tip through the bottom of the well. Do not dispense all the way to the second stop or you may form air bubbles.
j. Apply 10 µL of the standard ladder (Norgen Highranger, Norgen Biotek, Canada) as recommended to a well. It does not need to be mixed with loading dye since it comes pre-prepared.
k. Once all the wells are loaded, cover the apparatus making sure the red (positive) electrode is at the end furthest from the wells.
l. Set the voltage typically from 80-100 V and once you see the loading dye travelling in the right direction, let the gel run for approximately 45 minutes.
m. Using your gel electrophoresis results, determine which PCR reactions were successful and only purify those products using the QIAquick PCR purification kit (Qiagen, Netherlands). Follow the kit's directions but briefly:
n. Add 95-100% ethanol to the PE buffer as directed when you open the kit.
o. Add 5 volumes of PB buffer directly to your PCR reaction tube. For example, add 225 µL PB to 45µL PCR product (50 µL reaction less 5 µL for screening products by electrophoresis).
p. Apply the contents of step b to labeled QIAquick spin columns. BIND.

q. Spin using a microcentrifuge at maximum speed for 1 minute.
r. Discard flowthrough.
s. Add 750 µL PE buffer to the spin column. WASH.
t. Spin at maximum speed for 1 minute.
u. Discard flowthrough.
v. Spin the empty column at maximum speed for 2 minutes. DRY.
w. Discard flowthrough and repeat step (v.) Ethanol will interfere with all downstream processes.
x. Place column in a sterile, pre-labeled Eppendorf tube, then add 30 µL EB buffer. ELUTE.
y. Elution efficiency can be improved by allowing the elution buffer to incubate in the column at room temperature. For example, allow it to sit for 30 minutes while you prepare another 1% electrophoresis gel.
z. Spin at a low speed for 1 minute, then increase to maximum speed for 1 minute.
aa. Products are prepared to be shipped to Robarts Research Institute in London, Ontario. Robarts specifies that all samples should be sent in 1.5 mL Eppendorf tubes, or alternately in a 96 well plate. Tubes should be labeled with the reaction name (usually the template followed by the primer used) and requestor's initials. 10 µL purified PCR product is added to the tube with 5µL forward primer diluted to 2µM. Forms can be obtained from http://www.robarts.ca/request-form. First you must determine the concentration of your purified PCR product, since Robarts requires that samples be sent within a concentration range depending upon their size. If the PCR product is 200-500 bp (such as the bacterial product which should be around 450 bp when using primers 968f and 1401r), the concentration should be between 3-10 ng/µL. If the PCR product is 500-1000 bp (such as some of the yeast ITS products, although they vary in size depending upon the species), the concentration should be between 5-20 ng/µL.
bb. The concentration of DNA present in your purified PCR reactions is most easily determined by repeating gel electrophoresis. Prepare another 1% gel as directed in steps (a.-k.). Be very careful when loading the gel to make sure pipetting is accurate into the wells, since band intensity will be compared to quantify the concentration of each sample.
cc. Run the electrophoresis at a low voltage for as long as possible to get good separation of the bands in the Norgen Highranger DNA standard ladder (Norgen Biotek, Canada).
dd. Compare the intensity of the band representing each sample to the band in the ladder with the most similar intensity.
ee. The Norgen Highranger is prepared with each standard at a known concentration, so based on band similarity, you can estimate the concentration of your samples. Note that you loaded 10 µL of the ladder but only 5 µL of each sample. So, for example, if the sample band bears the most similarity to the intensity of the ladder band known to have 29 ng / 10 µL, then you know you have 29 ng / 5 µL, or approximately 6 ng / µL in your sample.
ff. Dilute your samples to fall most securely within the concentration range specified by Robarts. When samples are too concentrated, the sequencing results will have very intense peaks that are not easy to read, and samples that are too dilute will have weak peaks.
gg. Dilute the 100 µM forward primer to 2 µM with a 10 then 5 times dilution in a series using ddH_2O.
hh. Add 5 µL of the 2 µM primer to your reaction tube with 10 µL of the appropriately diluted purified PCR product. Fill out the form, pack the Eppendorfs in bubble wrap and ship them to Robarts Research Institute. The address is on the header of the order form. Results will be sent back by e-mail within the week.
ii. Sequencing is performed using a dye terminator method (big dye terminator sequencing). The result is a chromatogram with a series of coloured peaks that represent each nucleotide, in sequential order determined by the size of each terminated chain. The chromatogram can be opened by using a simple free software called Chromas available at http://www.technelysium.com.au/chromas_lite.html. Open each sequencing result in Chromas. Chromas will automatically annotate the chromatogram, that is, a series of letters appears across

the top of the screen with each nucleotide letter corresponding to a peak of the appropriate dye. Red=T (thymine), green=A (adenine), black=G (guanine), blue=C (cytosine). However, if peaks overlap or a strange pattern occurs, the computer may annotate with the general nucleotide N, which is not meaningful for the BLAST (basic local alignment search tool) program. Therefore, the annotations must be edited by hand, and large dye peaks at the beginning of the reaction can be entirely disregarded in the sequence, while annotations of very small peaks near the end of the reaction may also be deleted.

jj. Once the sequence is annotated using only reliable peaks, use the edit menu to copy the sequence in FASTA format. Save your annotated sequence.

kk. Search for the annotated sequence in the comprehensively large NCBI nucleotide database. BLAST can be accessed at http://blast.ncbi.nlm.nih.gov/Blast.cgi and the 'nucleotide blast' link is chosen.

ll. Paste the FASTA sequence into the appropriate box, which will automatically include the title. Choose the 'others (nr)' database and exclude 'uncultured/environmental samples'. Press the link to BLAST.

mm. The result will be a table with the identifications of all similar sequences. They will appear in order of their similarity, and measures such as query coverage, maximum identity, and the E-value can be used to determine which identifications are the most reliable. If there is more than one species with a similar E-value, the next strategy is to look up the most recent taxonomy papers on the related species, and align your sequence directly with the type cultures used in the literature.

APPENDIX III

Bacterial sequences and accession reports

Submitted to the National Center for Biotechnology Information (NCBI) Nucleotide database

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  VERSION
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  Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Acetobacter.
  REFERENCE 1 (bases 1 to 387)
  AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
  TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
  JOURNAL Unpublished
  REFERENCE 2 (bases 1 to 387)
  AUTHORS Huber,C.
  TITLE Direct Submission
  JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
  COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
  Bankit Comment: TOTAL # OF SEQS:12.
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  SOURCE Bacillus subtilis
  ORGANISM Bacillus subtilis
  Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
  REFERENCE 1 (bases 1 to 400)
  AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
  TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
  JOURNAL Unpublished
  REFERENCE 2 (bases 1 to 400)
  AUTHORS Huber,C.
  TITLE Direct Submission
  JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
  COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
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  REFERENCE 1 (bases 1 to 389)
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  TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
  JOURNAL Unpublished
  REFERENCE 2 (bases 1 to 389)
  AUTHORS Huber,C.
  TITLE Direct Submission
  JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
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  REFERENCE 1 (bases 1 to 393)
  AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
  TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
  JOURNAL Unpublished
  REFERENCE 2 (bases 1 to 393)
  AUTHORS Huber,C.
  TITLE Direct Submission
  JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
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REFERENCE 1 (bases 1 to 373)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 373)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
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REFERENCE 1 (bases 1 to 383)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 383)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
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361 cggtgaatac gttcccgggt ctg

- LOCUS Seq7 383 bp DNA linear BCT 20-OCT-2014
  DEFINITION [Gluconobacter oxydans] >56-47.BWRC1_968f sequence exported from Gluconobacter oxydans III.ab1.
  ACCESSION KP010386
  VERSION
  KEYWORDS .
  SOURCE Gluconobacter oxydans
  ORGANISM Gluconobacter oxydans
  Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Gluconobacter.
  REFERENCE 1 (bases 1 to 383)
  AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
  TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
  JOURNAL Unpublished
  REFERENCE 2 (bases 1 to 383)
  AUTHORS Huber,C.
  TITLE Direct Submission
  JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
  COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
  Bankit Comment: TOTAL # OF SEQS:12.
  Bankit Comment: TOTAL # OF SETS:2.

  ##Assembly-Data-START##
  Sequencing Technology :: Sanger dideoxy sequencing
  ##Assembly-Data-END##
  FEATURES Location/Qualifiers
  source 1..383
  /organism="Gluconobacter oxydans"
  /mol_type="genomic DNA"
  /strain="III"
  /isolate="III"
  /isolation_source="Grape berry"
  /host="Vitis vinifera"
  /db_xref="taxon:442"
  /country="Canada"
  /collection_date="2010"
  /note="[cultured bacterial source]"
  gene <1..>383
  /gene="rRNA"
  BASE COUNT 80 a 93 c 116 g 94 t
  ORIGIN
  1 tactcagaga tgggtatttc ttcggacctc ccgcacaggt gctgcatggc tgtcgtcagc
  61 tcgtgtcgtg agatgttggg ttaagtcccg caacgagcgc aacccttgtc tttagttgcc
  121 agcactttca ggtgggcact ctagagagac tgccggtgac aagccggagg aaggtgggga
  181 tgacgtcaag tcctcatggc ccttatgtcc tgggctacac acgtgctaca atggcggtga
  241 cagtgggaag ctatgtggtg acacagtgct gatctctaaa agccgtctca gttcggattg
  301 tactctgcaa ctcgagtaca tgaaggtgga atcgctagta atcgcggatc agcatgccgc
  361 ggtgaatacg ttcccgggtc ttg

- LOCUS Seq8 376 bp DNA linear BCT 20-OCT-2014
DEFINITION [Gluconobacter oxydans] >91-CM20-968f_968f sequence exported from Gluconobacter oxydans IV.ab1.
ACCESSION KP010387
VERSION
KEYWORDS .
SOURCE Gluconobacter oxydans
ORGANISM Gluconobacter oxydans
Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Gluconobacter.
REFERENCE 1 (bases 1 to 376)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 376)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:12.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
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/mol_type="genomic DNA"
/strain="IV"
/isolate="IV"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:442"
/country="Canada"
/collection_date="2011"
/note="[cultured bacterial source]"
gene <1..>376
/gene="rRNA"
BASE COUNT 78 a 93 c 114 g 91 t
ORIGIN
1 agatggacct ttcttcggac ctcccgcaca ggtgctgcat ggctgtcgtc agctcgtgtc
61 gtgagatgtt gggttaagtc ccgcaacgag cgcaaccctt gtctttagtt gccagcactt
121 tcaggtgggc actctagaga gactgccggt gacaagccgg aggaaggtgg ggatgacgtc
181 aagtcctcat ggcccttatg tcctgggcta cacacgtgct acaatggcgg tgacagtggg
241 aagctacatg gtgacatggt gctgatctct aaaagccgtc tcagttcgga ttgtactctg
301 caactcgagt acatgaaggt ggaatcgcta gtaatcgcgg atcagcatgc cgcggtgaat
361 acgttcccgg gtcttg

- LOCUS Seq9 382 bp DNA linear BCT 20-OCT-2014
DEFINITION [Gluconobacter oxydans] >02-CM26-968F_968f sequence exported from Gluconobacter oxydans V.ab1.
ACCESSION KP010388
VERSION
KEYWORDS .
SOURCE Gluconobacter oxydans
ORGANISM Gluconobacter oxydans
Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Gluconobacter.
REFERENCE 1 (bases 1 to 382)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 382)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:12.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..382
/organism="Gluconobacter oxydans"
/mol_type="genomic DNA"
/strain="V"
/isolate="V"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:442"
/country="Canada"
/collection_date="2011"
/note="[cultured bacterial source]"
gene <1..>382
/gene="rRNA"
BASE COUNT 79 a 94 c 117 g 92 t
ORIGIN
1 ggttcagaga tggacctttc ttcggacctc ccgcacaggt gctgcatggc tgtcgtcagc
61 tcgtgtcgtg agatgttggg ttaagtcccg caacgagcgc aacccttgtc tttagttgcc
121 agcactttca ggtgggcact ctagagagac tgccggtgac aagccggagg aaggtgggga
181 tgacgtcaag tcctcatggc ccttatgtcc tgggctacac acgtgctaca atggcggtga
241 cagtgggaag ctacatggtg acatggtgct gatctctaaa agccgtctca gttcggattg
301 tactctgcaa ctcgagtaca tgaaggtgga atcgctagta atcgcggatc agcatgccgc
361 ggtgaatacg ttcccgggtc tg

- LOCUS Seq10 364 bp DNA linear BCT 20-OCT-2014
  DEFINITION [Gluconobacter oxydans] >03-CM27-968F_968f sequence exported from Gluconobacter oxydans VI.ab1.
  ACCESSION KP010389
  VERSION
  KEYWORDS .
  SOURCE Gluconobacter oxydans
  ORGANISM Gluconobacter oxydans
  Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Gluconobacter.
  REFERENCE 1 (bases 1 to 364)
  AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
  TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
  JOURNAL Unpublished
  REFERENCE 2 (bases 1 to 364)
  AUTHORS Huber,C.
  TITLE Direct Submission
  JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
  COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
  Bankit Comment: TOTAL # OF SEQS:12.
  Bankit Comment: TOTAL # OF SETS:2.

  ##Assembly-Data-START##
  Sequencing Technology :: Sanger dideoxy sequencing
  ##Assembly-Data-END##
  FEATURES Location/Qualifiers
  source 1..364
  /organism="Gluconobacter oxydans"
  /mol_type="genomic DNA"
  /strain="VI"
  /isolate="VI"
  /isolation_source="Grape berry"
  /host="Vitis vinifera"
  /db_xref="taxon:442"
  /country="Canada"
  /collection_date="2011"
  /note="[cultured bacterial source]"
  gene <1..>364
  /gene="rRNA"
  BASE COUNT 78 a 90 c 108 g 88 t
  ORIGIN
  1 agatgacctt tcttcggacc tcccgcacag gtgctgcatg gctgtcgtca gctcgtgtcg
  61 tgagatgttg ggttaagtcc cgcaacgagc gcaacccttg tctttagttg ccagcacttt
  121 caggtgggca ctctagagag actgccggtg acaagccgga ggaaggtggg gatgacgtca
  181 agtcctcatg gcccttatgt cctgggctac acacgtgcta caatggcggt gacagtggga
  241 agctacatgg tgacatggtg ctgatctcta aaagccgtct cagttcggat tgtactctgc
  301 aactcgagta catgaaggtg gaatcgctag taatcgcgga tcagcatgcc gcggtgaata
  361 cttc

- LOCUS Seq11 383 bp DNA linear BCT 20-OCT-2014
DEFINITION [Gluconobacter oxydans] >07-CM31-968F_968f sequence exported from Gluconobacter oxydans VII.ab1.
ACCESSION KP010390
VERSION
KEYWORDS .
SOURCE Gluconobacter oxydans
ORGANISM Gluconobacter oxydans
Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Gluconobacter.
REFERENCE 1 (bases 1 to 383)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 383)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:12.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..383
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/mol_type="genomic DNA"
/strain="VII"
/isolate="VII"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:442"
/country="Canada"
/collection_date="2011"
/note="[cultured bacterial source]"
gene <1..>383
/gene="rRNA"
BASE COUNT 79 a 94 c 117 g 93 t
ORIGIN
1 ggttcagaga tggacctttc ttcggacctc ccgcacaggt gctgcatggc tgtcgtcagc
61 tcgtgtcgtg agatgttggg ttaagtcccg caacgagcgc aacccttgtc tttagttgcc
121 agcactttca ggtgggcact ctagagagac tgccggtgac aagccggagg aaggtgggga
181 tgacgtcaag tcctcatggc ccttatgtcc tgggctacac acgtgctaca atggcggtga
241 cagtgggaag ctacatggtg acatggtgct gatctctaaa agccgtctca gttcggattg
301 tactctgcaa ctcgagtaca tgaaggtgga atcgctagta atcgcggatc agcatgccgc
361 ggtgaatacg ttcccgggtc ttg

- LOCUS Seq12 363 bp DNA linear BCT 20-OCT-2014
DEFINITION [Pseudomonas fluorescens] >78-C5_968F sequence exported from Pseudomonas fluorescens.ab1.
ACCESSION KP010391
VERSION
KEYWORDS .
SOURCE Pseudomonas fluorescens
ORGANISM Pseudomonas fluorescens
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
REFERENCE 1 (bases 1 to 363)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 363)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:12.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..363
/organism="Pseudomonas fluorescens"
/mol_type="genomic DNA"
/strain="I"
/isolate="I"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:294"
/country="Canada"
/collection_date="2009"
/note="[cultured bacterial source]"
gene <1..>363
/gene="rRNA"
BASE COUNT 86 a 89 c 102 g 86 t
ORIGIN
1 tgactttcag agatggattg gtgccttcgg gaacattgag acaggtgctg catggctgtc
61 gtcagctcgt gtcgtgagat gttgggttaa gtcccgtaac gagcgcaacc cttgtcctta
121 gttaccagca cgttatggtg ggcactctaa ggagactgcc ggtgacaaac cggaggaagg
181 tggggatgac gtcgagtcat catggccctt accgcctggg ctacacacgt gctacaatgg
241 tcggtacgaa gggttgccaa gccgctaggt ggatctaatc ccaaaaaacc gatcgtagtc
301 cagatcgcac tctgcaactc tgctgcatgg attccgaatc cctagtaatc tctaatcaaa
361 atg

Yeast sequences and accession reports

Submitted to the National Center for Biotechnology Information (NCBI) Nucleotide database

- LOCUS Seq1 487 bp DNA linear PLN 20-OCT-2014
  DEFINITION [Aureobasidium pullulans] >80-C7_ITS1 sequence exported from Aureobasidium pullulans.ab1.
  ACCESSION KP010392
  VERSION
  KEYWORDS .
  SOURCE Aureobasidium pullulans
  ORGANISM Aureobasidium pullulans
  Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetidae; Dothideales; Dothioraceae; mitosporic Dothioraceae; Aureobasidium.
  REFERENCE 1 (bases 1 to 487)
  AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
  TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
  JOURNAL Unpublished
  REFERENCE 2 (bases 1 to 487)
  AUTHORS Huber,C.
  TITLE Direct Submission
  JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
  COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
  Bankit Comment: TOTAL # OF SEQS:20.
  Bankit Comment: TOTAL # OF SETS:2.

  ##Assembly-Data-START##
  Sequencing Technology :: Sanger dideoxy sequencing
  ##Assembly-Data-END##
  FEATURES Location/Qualifiers
  source 1..487
  /organism="Aureobasidium pullulans"
  /mol_type="genomic DNA"
  /strain="I"
  /isolate="I"
  /isolation_source="Grape berry"
  /host="Vitis vinifera"
  /db_xref="taxon:5580"
  /country="Canada"
  /collection_date="2009"
  gene <1..>487
  /gene="rRNA"
  BASE COUNT 115 a 119 c 118 g 135 t
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  1 tgctcgcgcc cgacctccaa ccctttgttg ttaaaactac cttgttgctt tggcgggacc
  61 gctcgagtct cgagccgctg gggattcgtc ccaggcgagc gcccgccaga gttaaaccaa
  121 actcgttgtt atttaaccgg tcgtctgagt taaaattttg aataaatcaa gagctttata
  181 gcacacgtga tctcttggtt ctcgcatcga atgaagaagg cagcgaagtg cgataagtaa
  241 tgtgaattgc agaattcatt gaatcatcga atctttgaac gcacattgcg ccccttggta
  301 ttcctgaggg gcatgcctgt tcgagcgtca ttacaccact caagctatgc ttggtattgg
  361 gcatcatcct tagttgggcg cggcttagag acctctgcga ggccactccg gctttagagc
  421 gatactaaat ttattcgaac gtctgtcaaa ggagaggaac tctgccgact gaaaccttta
  481 tttgtct

- LOCUS Seq2 648 bp DNA linear PLN 20-OCT-2014
DEFINITION [Candida zemplinina] >45-36.YGNC2_ITS1 sequence exported from Candida zemplinina I.ab1.
ACCESSION KP010393
VERSION
KEYWORDS .
SOURCE Starmerella bacillaris
ORGANISM Starmerella bacillaris
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetales incertae sedis; Starmerella.
REFERENCE 1 (bases 1 to 648)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 648)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..648
/organism="Starmerella bacillaris"
/mol_type="genomic DNA"
/strain="I"
/isolate="I"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:1247836"
/country="Canada"
/collection_date="2010"
gene <1..>648
/gene="rRNA"
BASE COUNT 189 a 124 c 131 g 203 t 1 others
ORIGIN
1 gcaaaccact gtgacagctt agacttcggt ctttgcaatt gcttgggtgt cgaaaggcgc
61 ccaatcttta aaacttttat atttgttctg aaacaatgaa aatttaaaac tttcaacaac
121 ggatctcttg gttctcgtat cgatgaagaa cgcagcaaag cgcgataggt aatgcgaatt
181 gcagacgtga gtcattgaat ttttgaacgc atattgcgct attagtttgt ctaatagcat
241 gcttgttgga gtgataatct tcctctcaac catttttggt atgaggtctt gctccttta
301 ggagttaaaa tcatggaagt gcacacgtta attaactctg tgcagttata cacttttcat
361 cctccaatca agcaaggtta cccgctgaac ttaagcatat caataancgg aggaaagagt
421 catttctgaa ggctttttgc caaaaccact gtgaacagct tagacttcgg tctttgcaat
481 tgcttgggtg tcgaaaggcg cccaatcttt aaaactttta tatttgttct gaaacaatga
541 aaatttaaaa ctttcaacaa cggatctctt ggttctcgta tcgatgaaga acgcacaagc
601 gcgataggta atgcgaattg cagactgagt cattgaattt ttgaacgc

- LOCUS Seq3 599 bp DNA linear PLN 20-OCT-2014
DEFINITION [Candida zemplinina] >43-34.YCGC2_ITS1 sequence exported from Candida zemplinina II.ab1.
ACCESSION KP010394
VERSION
KEYWORDS .
SOURCE Starmerella bacillaris
ORGANISM Starmerella bacillaris
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetales incertae sedis; Starmerella.
REFERENCE 1 (bases 1 to 599)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 599)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..599
/organism="Starmerella bacillaris"
/mol_type="genomic DNA"
/strain="II"
/isolate="II"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:1247836"
/country="Canada"
/collection_date="2010"
gene <1..>599
/gene="rRNA"
BASE COUNT 173 a 117 c 117 g 189 t 3 others
ORIGIN
1 ccactgtgac agcttagact tcggtctttg caattgcttg ggtgtcgaaa ggcgcccaat
61 ctttaaaact tttatatttg ttctgaaaca atgaaaattt aaaactttca acaacggatc
121 tcttggttct cgtatcgatg aagaacgcag caaagcgcga taggtaatgc gaattgcaga
181 cgtgagtcat tgaatttttg aacgcatatt gcgctattag tttgtctaat agcatgcttg
241 ttggagtgat aatcttcctc tcaaccattt ttggtatgag gtcttgctcc ttttaggagt
301 taaaatcatg gaagtgcaca cgttaattaa ctctgtgcag ttatacactt ttcatcctcc
361 aatcaagcaa ggttacccgc tgaacttaag catatcaata agcggaggaa agagatcatt
421 tctgaaggct ttttgccaaa accactgtga acagcttana cttcngtctt tgcaattgct
481 tgggtgtcna aaggcgccca atctttaaaa cttttatatt tgttctgaaa caatgaaaat
541 ttaaaacttt caacaacgga tctcttggtt ctcgtatcga tgaagaacgc agcaagcgc

- LOCUS Seq4 728 bp DNA linear PLN 20-OCT-2014
DEFINITION [Candida zemplinina] >50-41.YCrSBC2_ITS1 sequence exported from Candida zemplinina III.ab1.
ACCESSION KP010395
VERSION
KEYWORDS .
SOURCE Starmerella bacillaris
ORGANISM Starmerella bacillaris
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetales incertae sedis; Starmerella.
REFERENCE 1 (bases 1 to 728)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 728)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.
##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..728
/organism="Starmerella bacillaris"
/mol_type="genomic DNA"
/strain="III"
/isolate="III"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:1247836"
/country="Canada"
/collection_date="2010"
gene <1..>728
/gene="rRNA"
BASE COUNT 206 a 135 c 149 g 238 t
ORIGIN
1 agcttttgca aaccactgtg acagcttaga cttcggtctt tgcaattgct tgggtgtcga
61 aaggcgccca atctttaaaa cttttatatt tgttctgaaa caatgaaaat ttaaaacttt
121 caacaacgga tctcttggtt ctcgtatcga tgaagaacgc agcaaagcgc gataggtaat
181 gcgaattgca gacgtgagtc attgaatttt tgaacgcata ttgcgctatt agtttgtcta
241 atagcatgct tgttggagtg ataatcttcc tctcaaccat ttttggtatg aggtcttgct
301 ccttttagga gttaaaatca tggaagtgca cacgttaatt aactctgtgc agttatacac
361 ttttcatcct ccaatcaagc aaggttaccc gctgaactta agcatatcaa taagcggagg
421 aagagatcat ttctgaaggc tttttgccaa aaccactgtg aacagcttag acttcggtct
481 ttgcaattgc ttgggtgtcg aaaggcgccc aatctttaaa acttttatat ttgttctgaa
541 acaatgaaaa tttaaaactt tcaacaacgg atctcttggt tctcgtatcg atgaagaacg
601 cagcaaagcg cgataggtaa tgcgaattgc agacgtgagt cattgaattt ttgaacgcat
661 attgcgctat tagtttgtct aatagcatgc ttgttggagt gataatcttc tctcacattt
721 ttggtatg

- LOCUS Seq5 655 bp DNA linear PLN 20-OCT-2014
DEFINITION [Candida zemplinina] >46-37.YKRC2_ITS1 sequence exported from Candida zemplinina IV.ab1.
ACCESSION KP010396
VERSION
KEYWORDS .
SOURCE Starmerella bacillaris
ORGANISM Starmerella bacillaris
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetales incertae sedis; Starmerella.
REFERENCE 1 (bases 1 to 655)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 655)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..655
/organism="Starmerella bacillaris"
/mol_type="genomic DNA"
/strain="IV"
/isolate="IV"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:1247836"
/country="Canada"
/collection_date="2010"
gene <1..>655
/gene="rRNA"
BASE COUNT 193 a 124 c 132 g 205 t 1 others
ORIGIN
1 gcaaaccact gtgacagctt agacttcggt ctttgcaatt gcttgggtgt cgaaaggcgc
61 ccaatcttta aaacttttat atttgttctg aaacaatgaa aatttaaaac tttcaacaac
121 ggatctcttg gttctcgtat cgatgaagaa cgcagcaaag cgcgataggt aatgcgaatt
181 gcagacgtga gtcattgaat ttttgaacgc atattgcgct attagtttgt ctaatagcat
241 gcttgttgga gtgataatct tcctctcaac catttttggt atgaggtctt gctccttta
301 ggagttaaaa tcatggaagt gcacacgtta attaactctg tgcagttata cacttttcat
361 cctccaatca agcaaggtta cccgctgaac ttaagcatat caataancgg aggaaagaga
421 tcatttctga aggctttttg ccaaaaccac tgtgaacagc ttagacttcg gtctttgcaa
481 ttgcttgggt gtcgaaaggc gcccaatctt taaaactttt atatttgttc tgaaacaatg
541 aaaatttaaa actttcaaca acggatctct tggttctcgt atcgatgaag aacgcagcaa
601 acgcgatagg taatgcgaat tgcagacgtg agtcattgaa tttttgaacg catat

- LOCUS Seq6 664 bp DNA linear PLN 20-OCT-2014
DEFINITION [Candida zemplinina] >47-38.YGRC2_ITS1 sequence exported from Candida zemplinina V.ab1.
ACCESSION KP010397
VERSION
KEYWORDS .
SOURCE Starmerella bacillaris
ORGANISM Starmerella bacillaris
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetales incertae sedis; Starmerella.
REFERENCE 1 (bases 1 to 664)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 664)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..664
/organism="Starmerella bacillaris"
/mol_type="genomic DNA"
/strain="V"
/isolate="V"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:1247836"
/country="Canada"
/collection_date="2010"
gene <1..>664
/gene="rRNA"
BASE COUNT 193 a 126 c 134 g 209 t 2 others
ORIGIN
1 gcaaccactg tgacagctta gacttcggtc tttgcaattg cttgggtgtc gaaaggcgcc
61 caatctttaa aacttttata tttgttctga aacaatgaaa atttaaaact ttcaacaacg
121 gatctcttgg ttctcgtatc gatgaagaac gcagcaaagc gcgataggta atgcgaattg
181 cagacgtgag tcattgaatt tttgaacgca tattgcgcta ttagtttgtc taatagcatg
241 cttgttggag tgataatctt cctctcaacc atttttggta tgaggtcttg ctccttttag
301 gagttaaaat catggaagtg cacacgttaa ttaactctgt gcagttatac acttttcatc
361 ctccaatcaa gcaaggttac ccgctgaact taagcatatc aataagcgga ggaaagagtc
421 atttctgaag gctttttgcc aaaaccactg tgaacagctt agacttcggt ctttgcaatt
481 gcttgggtgt cgaaaggcgc ccaatcttta aaacttttat atttgttctg aaacaatgaa
541 aatttaaaac tttcaacaac ggatctcttg gttctcntat cnatgaagaa cgcagcaaag
601 cgcgataggt aatgcgaatt gcagacgtga gtcattgaat ttttgaacgc atattgcgct
661 atta

- LOCUS Seq7 610 bp DNA linear PLN 20-OCT-2014
DEFINITION [Candida zemplinina] >44-35.YWRC2_ITS1 sequence exported from Candida zemplinina VI.ab1.
ACCESSION KP010398
VERSION
KEYWORDS .
SOURCE Starmerella bacillaris
ORGANISM Starmerella bacillaris
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetales incertae sedis; Starmerella.
REFERENCE 1 (bases 1 to 610)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 610)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..610
/organism="Starmerella bacillaris"
/mol_type="genomic DNA"
/strain="VI"
/isolate="VI"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:1247836"
/country="Canada"
/collection_date="2010"
gene <1..>610
/gene="rRNA"
BASE COUNT 177 a 117 c 123 g 192 t 1 others
ORIGIN
1 ccactgtgac agcttagact tcggtctttg caattgcttg ggtgtcgaaa ggcgcccaat
61 ctttaaaact tttatatttg ttctgaaaca atgaaaattt aaaactttca acaacggatc
121 tcttggttct cgtatcgatg aagaacgcag caaagcgcga taggtaatgc gaattgcaga
181 cgtgagtcat tgaatttttg aacgcatatt gcgctattag tttgtctaat agcatgcttg
241 ttggagtgat aatcttcctc tcaaccattt ttggtatgag gtcttgctcc ttttaggagt
301 taaaatcatg gaagtgcaca cgttaattaa ctctgtgcag ttatacactt ttcatcctcc
361 aatcaagcaa ggttacccgc tgaacttaag catatcaata ancggaggaa agagatcatt
421 tctgaaggct ttttgccaaa accactgtga acagcttaga cttcggtctt tgcaattgct
481 tgggtgtcga aaggcgccca atctttaaaa cttttatatt tgttctgaaa caatgaaaat
541 ttaaaacttt caacaacgga tctcttggtt ctcgtatcga tgaagaacgc agcaagcgcg
601 ataggtaatg

- LOCUS Seq8 628 bp DNA linear PLN 20-OCT-2014
DEFINITION [Candida zemplinina] >48-39.YIRC2_ITS1 sequence exported from Candida zemplinina VII.ab1.
ACCESSION KP010399
VERSION
KEYWORDS .
SOURCE Starmerella bacillaris
ORGANISM Starmerella bacillaris
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetales incertae sedis; Starmerella.
REFERENCE 1 (bases 1 to 628)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 628)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..628
/organism="Starmerella bacillaris"
/mol_type="genomic DNA"
/strain="VII"
/isolate="VII"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:1247836"
/country="Canada"
/collection_date="2010"
gene <1..>628
/gene="rRNA"
BASE COUNT 184 a 121 c 129 g 194 t
ORIGIN
1 gcaaccactg tgacagctta gacttcggtc tttgcaattg cttgggtgtc gaaaggcgcc
61 caatctttaa aacttttata tttgttctga aacaatgaaa atttaaaact ttcaacaacg
121 gatctcttgg ttctcgtatc gatgaagaac gcagcaaagc gcgataggta atgcgaattg
181 cagacgtgag tcattgaatt tttgaacgca tattgcgcta ttagtttgtc taatagcatg
241 cttgttggag tgataatctt cctctcaacc atttttggta tgaggtcttg ctccttttag
301 gagttaaaat catggaagtg cacacgttaa ttaactctgt gcagttatac acttttcatc
361 ctccaatcaa gcaaggttac ccgctgaact taagcatatc aataagcgga ggaaagagat
421 catttctgaa ggctttttgc caaaaccact gtgaacagct tagacttcgg tctttgcaat
481 tgcttgggtg tcgaaaggcg cccaatcttt aaaactttta tatttgttct gaaacaatga
541 aaatttaaaa ctttcaacaa cggatctctt ggttctcgta tcgatgaaga acgcagcaaa
601 gcgcgatagg taatgcgaat tgcagacg

- LOCUS Seq9 311 bp DNA linear PLN 20-OCT-2014
DEFINITION [Hanseniaspora uvarum] >16-7.YHBC3a_ITS1 sequence exported from Hanseniaspora uvarum I.ab1.
ACCESSION KP010400
VERSION
KEYWORDS .
SOURCE Hanseniaspora uvarum
ORGANISM Hanseniaspora uvarum
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycodaceae; Hanseniaspora.
REFERENCE 1 (bases 1 to 311)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 311)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..311
/organism="Hanseniaspora uvarum"
/mol_type="genomic DNA"
/strain="I"
/isolate="I"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:29833"
/country="Canada"
/collection_date="2010"
gene <1..>311
/gene="rRNA"
BASE COUNT 95 a 53 c 44 g 119 t
ORIGIN
1 cttgttgctc gagttcttgt ttagatcttt tacaataatg tgtatcttta ttgaagatgt
61 gcgcttaatt gcgctgcttc tttaaagtgt cgcagtgaaa gtagtcttgc ttgaatctca
121 gtcaacgcta cacacattgg agtttttta ctttaattta attctttctg ctttgaatcg
181 aaaggttcaa ggcaaaaaac aaacccaaac aattttattt tattataatt ttttaaacta
241 aaccaaaatt cctaacggaa attttaaaat aatttaaaac tttcaacaac ggatctcttg
301 gttctcgcat c

- LOCUS Seq10 567 bp DNA linear PLN 20-OCT-2014
DEFINITION [Hanseniaspora uvarum] >26-17.YSPC3a_ITS1 sequence exported from Hanseniaspora uvarum II.ab1.
ACCESSION KP010401
VERSION
KEYWORDS .
SOURCE Hanseniaspora uvarum
ORGANISM Hanseniaspora uvarum
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycodaceae; Hanseniaspora.
REFERENCE 1 (bases 1 to 567)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 567)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..567
/organism="Hanseniaspora uvarum"
/mol_type="genomic DNA"
/strain="II"
/isolate="II"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:29833"
/country="Canada"
/collection_date="2010"
gene <1..>567
/gene="rRNA"
BASE COUNT 166 a 132 c 91 g 178 t
ORIGIN
1 ttgttgctcg agttcttgtt tagatctttt acaataatgt gtatctttac tgaagatgtg
61 cgcttaattg cgctgcttct ttagagtgtc gcagtgaaag tagtcttgct tgaatctcag
121 tcaacgctac acacattgga gtttttttac tttaatttaa ttctttcggc tttaaaccaa
181 agggtccagg gcaaaaaaca aacccaaaca attttatttt attataattt tttaaactaa
241 cccaaaattc caaacgaaaa ttttaaaata atttaaaact ttcaccaccg aacccctggg
301 tccccccaca ataaaaaacg aaccaaattg caataattaa ggggaattgc aaatccccgg
361 gaaccattga atttttaaac ccccattgcc cccttgacca ttcccagggg caggccggtt
421 taaccgcctt ttccttccca aaaaataatt tataattttt gggtgggggg ccaatcccca
481 gggttacctg gaaattgaaa acggttccat ccttttaat tcacccctta cctcctttgg
541 aaaccctgtc ccccctggga ggaattt

- LOCUS Seq11 364 bp DNA linear PLN 20-OCT-2014
DEFINITION [Hanseniaspora uvarum] >23-14.YGRC3a_ITS1 sequence exported from Hanseniaspora uvarum III.ab1.
ACCESSION KP010402
VERSION
KEYWORDS .
SOURCE Hanseniaspora uvarum
ORGANISM Hanseniaspora uvarum
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycodaceae; Hanseniaspora.
REFERENCE 1 (bases 1 to 364)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 364)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..364
/organism="Hanseniaspora uvarum"
/mol_type="genomic DNA"
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/isolate="III"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:29833"
/country="Canada"
/collection_date="2010"
gene <1..>364
/gene="rRNA"
BASE COUNT 121 a 69 c 58 g 115 t 1 others
ORIGIN
1 tgatacattg ttgctcgagt tcttgtttag atcttttaca ataatgtgta tctttattga
61 agatgtgcgc ttaattgcgc tgcttcttta aagtgtcgca gtgaaagtag tcttgcttga
121 atctcagtca acgctacaca cattggagtt ttttacttta atttaattcc ttccggtttg
181 aatccaaagg gtccagggaa aaaaccaacc ccaaccattt taatttaata aaatttttta
241 aaccaaaccc aaattcctaa aggaaaattt aaaaaaattt naaactttcc accacggaac
301 ccttgggttc tcccatccaa gaaaaaacta acgaaatggc aaaaataatg gggattggcg
361 aaac

- LOCUS Seq12 703 bp DNA linear PLN 20-OCT-2014
DEFINITION [Hanseniaspora uvarum] >22-13.YCRC3a_ITS1 sequence exported from Hanseniaspora uvarum IV.ab1.
ACCESSION KP010403
VERSION
KEYWORDS .
SOURCE Hanseniaspora uvarum
ORGANISM Hanseniaspora uvarum
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycodaceae; Hanseniaspora.
REFERENCE 1 (bases 1 to 703)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 703)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..703
/organism="Hanseniaspora uvarum"
/mol_type="genomic DNA"
/strain="IV"
/isolate="IV"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:29833"
/country="Canada"
/collection_date="2010"
gene <1..>703
/gene="rRNA"
BASE COUNT 210 a 121 c 119 g 253 t
ORIGIN
1 tgatacattg ttgctcgagt tcttgtttag atcttttaca ataatgtgta tctttactga
61 agatgtgcgc ttaattgcgc tgcttcttta gagtgtcgca gtgaaagtag tcttgcttga
121 atctcagtca acgctacaca cattggagtt tttttacttt aatttaattc tttctgcttt
181 gaatccaaag gttcaaggca aaaaacaaac acaaacaatt ttattttatt ataatttttt
241 aaactaaacc aaaattccta acggaaattt taaaataatt taaaactttc aacaacggat
301 ctcttggttc tcgcatccat gaaaaacgta acgaattgcg ataagtaatg tgaattgcag
361 aaactcctga atcattgaat ttttgaacgc acattgcgcc cttgaacatt ctcaggggca
421 tgcctgtttg aacgtcattt ccttctcaaa agaaaattta ttatttttg gttgtgggcg
481 atactcaggg ttagcttgaa attggaaact ggttcagtct tttttaattc aacacttaac
541 ttctttggag acgctgttct cgctgtgatg tatttatgga tttattcctt ttacttttaca
601 agggaaatgg taacgtacct taagcaaagg gttgctttta atattcatca agtttgacct
661 caaatcaggt aggattaccc gctgaactta agcatatcaa taa

- LOCUS Seq13 692 bp DNA linear PLN 20-OCT-2014
DEFINITION [Hanseniaspora uvarum] >10-1.YGNC3a_ITS1 sequence exported from Hanseniaspora uvarum V.ab1.
ACCESSION KP010404
VERSION
KEYWORDS .
SOURCE Hanseniaspora uvarum
ORGANISM Hanseniaspora uvarum
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycodaceae; Hanseniaspora.
REFERENCE 1 (bases 1 to 692)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 692)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..692
/organism="Hanseniaspora uvarum"
/mol_type="genomic DNA"
/strain="V"
/isolate="V"
/isolation_source="Grape berry"
/host="Vitis vinifera"
/db_xref="taxon:29833"
/country="Canada"
/collection_date="2010"
gene <1..>692
/gene="rRNA"
BASE COUNT 196 a 116 c 128 g 252 t
ORIGIN
1 gttgctcgag ttcttgttta gatcttttac aataatgtgt atctttactg aagatgtgcg
61 cttaattgcg ctgcttcttt agagtgtcgc agtgaaagta gtcttgcttg aatctcagtc
121 aacgctacac acattggagt ttttttactt taatttaatt ctttctgctt tgaatcgaaa
181 ggttcaaggc aaaaaacaaa cacaaacaat tttattttat tataattttt taaactaaac
241 caaaattcct aacggaaatt ttaaaataat ttaaaacttt caacaacgga tctcttggtt
301 ctcgcatcga tgaagaacgt agcgaattgc gataagtaat gtgaattgca gatactcgtg
361 aatcattgaa tttttgaacg cacattgcgc ccttgagcat tctcaggggc atgcctgttt
421 gagcgtcatt tccttctcaa aagataattt attatttttt ggttgtgggc gatactcagg
481 gttagcttga aattggagac tgtttcagtc ttttttaatt caacacttag cttctttgga
541 gacgctgttc tcgctgtgat gtatttatgg atttattcgt tttactttac aagggaaatg
601 gtaacgtacc ttaggcaaag ggttgctttt aatattcatc aagtttgacc tcaaatcagg
661 taggattacc cgctgaactt aagcatatca at

- LOCUS Seq14 705 bp DNA linear PLN 20-OCT-2014
DEFINITION [Hanseniaspora uvarum] >25-16.YIRC3a_ITS1 sequence exported from Hanseniaspora uvarum VI.ab1.
ACCESSION KP010405
VERSION
KEYWORDS .
SOURCE Hanseniaspora uvarum
ORGANISM Hanseniaspora uvarum
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycodaceae; Hanseniaspora.
REFERENCE 1 (bases 1 to 705)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 705)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..705
/organism="Hanseniaspora uvarum"
/mol_type="genomic DNA"
/strain="VI"
/isolate="VI"
/isolation_source="Grape berry"
/host="Vitis vinifera"
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ACCESSION KP010406
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REFERENCE 1 (bases 1 to 703)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 703)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

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ACCESSION KP010407
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ORGANISM Hanseniaspora uvarum
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycodaceae; Hanseniaspora.
REFERENCE 1 (bases 1 to 693)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 693)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

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- LOCUS Seq17 693 bp DNA linear PLN 20-OCT-2014
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ACCESSION KP010408
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ORGANISM Hanseniaspora uvarum
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycodaceae; Hanseniaspora.
REFERENCE 1 (bases 1 to 693)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 693)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

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ORGANISM Pichia membranifaciens
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REFERENCE 1 (bases 1 to 404)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 404)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
Bankit Comment: TOTAL # OF SEQS:20.
Bankit Comment: TOTAL # OF SETS:2.

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  REFERENCE 1 (bases 1 to 463)
  AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
  TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
  JOURNAL Unpublished
  REFERENCE 2 (bases 1 to 463)
  AUTHORS Huber,C.
  TITLE Direct Submission
  JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
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- LOCUS Seq20 660 bp DNA linear PLN 20-OCT-2014
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ACCESSION KP010411
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REFERENCE 1 (bases 1 to 660)
AUTHORS Huber,C., Inglis,D. and McFadden-Smith,W.
TITLE The Etiology of Grape Sour Rot in the Niagara Region, Ontario, Canada
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 660)
AUTHORS Huber,C.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-2014) Biological Sciences, Cool Climate Oenology and Viticulture Institute, 500 Glenridge Avenue, St. Catharines, ON L2S 3A1, Canada
COMMENT Bankit Comment: ALT EMAIL:ch04fq@brocku.ca.
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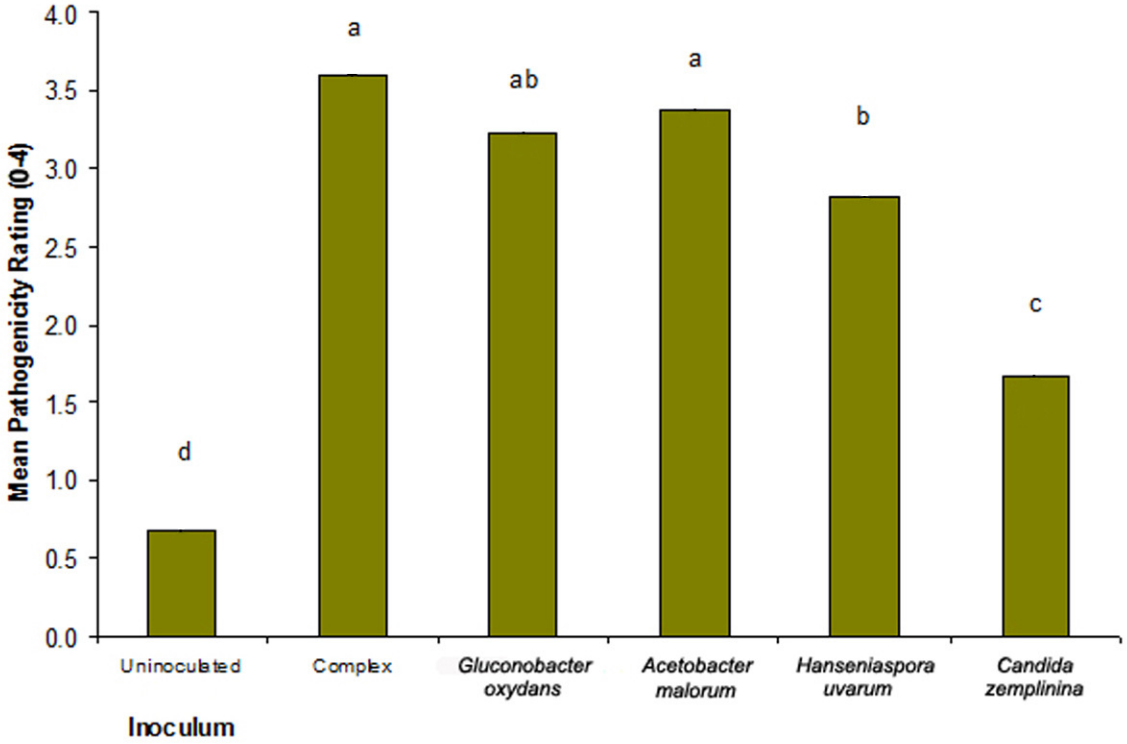
APPENDIX IV

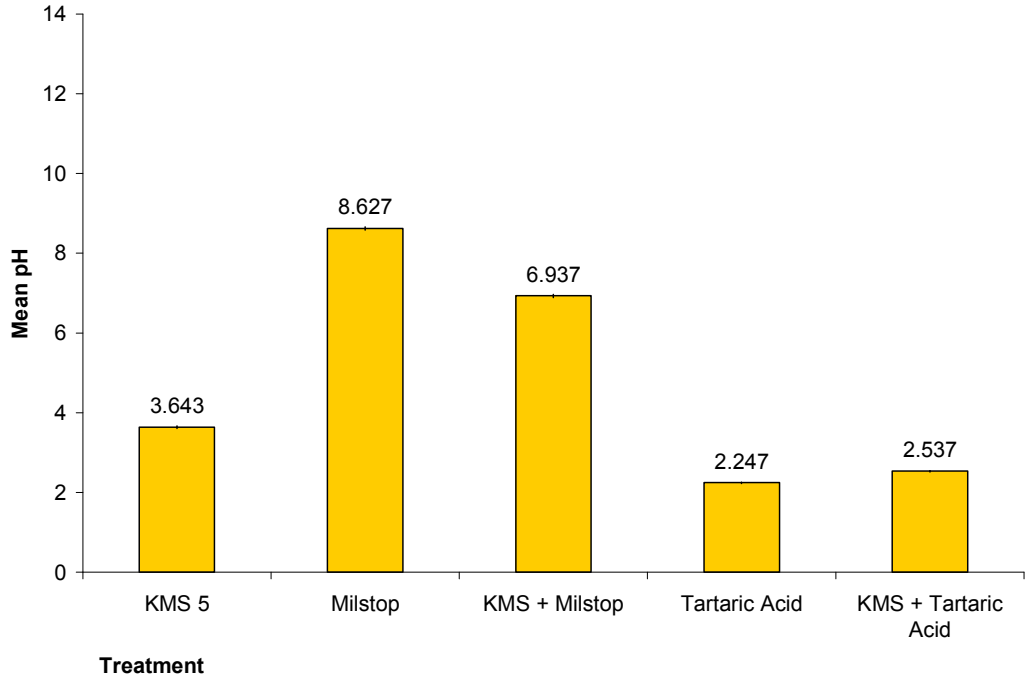
Pathogenicity assay conducted in *Vitis vinifera* cv. Riesling



Appendix IV Figure 1. Mean pathogenicity rating of sour rot-associated yeasts and bacteria incubated 14 days at 22ºC in injured *Vitis vinifera* cv. Riesling. N = 48 berries per inoculum. ANOVA $F(3,5) = 45.00$, $p = <0.0001$. Bars with the same letters are not significantly different according to the Student Newman-Keuls multiple range test.

APPENDIX V

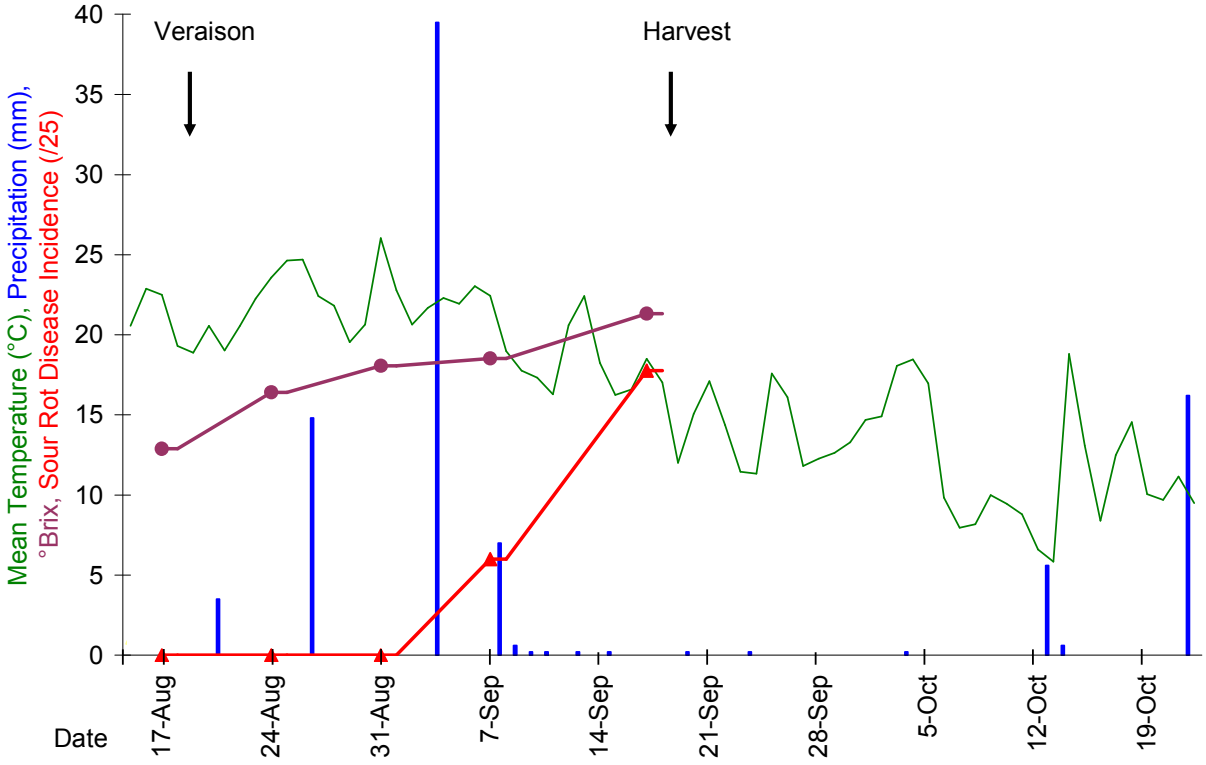
Field treatment pH



Appendix V Figure 1. The mean pH of treatments described in Section 5.2.1, 5.2.2, and 5.2.3. Solutions of 100 mL were prepared in triplicate in dH_2O at the following concentrations and were measured in duplicate using a standardized pH meter: KMS 5 g/L, Milstop 5.6 g/L, KMS 5 g/L + Milstop 5.6 g/L, tartaric acid 5 g/L, and KMS 5 g/L + tartaric acid 5 g/L.

APPENDIX VI

2012 field conditions in *Vitis vinifera* cv. Pinot Noir



Appendix VI Figure 1. Mean incidence of sour rot (red, infected clusters/25), daily mean temperature (green, °C), total daily rainfall (blue, mm), and mean fruit maturity (purple, °Brix) in a commercial Pinot Noir block in the Niagara Peninsula, designated viticultural area, Ontario, Canada, in 2012. Veraison is noted on August 19 and harvest on September 19.

APPENDIX VII

Population sampling raw data and supplementary statistics

Appendix VII Table 1. Raw data from microbial community collections conducted in 2011. Samples were taken from clusters displaying sour rot across the Niagara Peninsula, designated viticultural area, Ontario, Canada. Yeast colonies were enumerated from duplicate YPD plates amended with streptomycin and chloramphenicol. Bacterial colonies were enumerated from duplicate GYC plates amended with cycloheximide.

Site Number	Sub-Appellation	Variety	Collection Date	Colony	Count Plate 1	Count Plate 2	Dilution Factor	Filamentous Fungi
1	Beamsville Bench	Pinot Noir	September	*H. uvarum*	10	14	10	
				C. zemplinina	30	104	10	
				P. membranifaciens	0	2	10	
				Acetic Acid Bacteria	6	15	100	
				B. subtilis	6	5	100	
				A. pullulans	1	0	10	
2	Twenty Mile Bench	Pinot Noir	September	*H. uvarum*	68	23	100	*Botrytis*
				C. zemplinina	18	9	100	
				Acetic Acid Bacteria	56	157	100	
				B. subtilis	90	125	100	
3	Niagara Lakeshore	Pinot Noir	September	*H. uvarum*	9	6	10	
				C. zemplinina	5	9	10	
				Acetic Acid Bacteria	68	16	100	
4	Niagara Lakeshore	Pinot Noir	September	*H. uvarum*	8	9	100	*Botrytis*
				Acetic Acid Bacteria	59	260	100	
				A. pullulans	2	2	1000	
5	Short Hills Bench	Pinot Noir	October	*H. uvarum*	14	16	10	
				C. zemplinina	79	87	10	
				P. membranifaciens	3	4	10	
				Acetic Acid Bacteria	42	42	10	
				B. subtilis	8	8	10	
6	Short Hills Bench	Pinot Noir	September	*H. uvarum*	40	50	100	*Botrytis*
				C. zemplinina	32	56	100	
				P. membranifaciens	35	59	100	
				Acetic Acid Bacteria	38	46	100	
				C14	21	19	100	
7	Niagara Lakeshore	Riesling	October	*H. uvarum*	11	14	1	
				C. zemplinina	54	118	1	
				P. membranifaciens	7	2	1	
				Acetic Acid Bacteria	12	8	100	
8	Niagara Lakeshore	Riesling	September	*H. uvarum*	40	41	100	*Botrytis*
				C. zemplinina	15	12	100	*Penicillium*
				Acetic Acid Bacteria	41	31	100	
9	Four Mile Creek	Riesling	September	*H. uvarum*	130	106	10	
				C. zemplinina	19	17	10	
				P. membranifaciens	0	2	10	
				Acetic Acid Bacteria	27	18	10	
				C10	2	3	10	
10	Niagara River	Riesling	October	*H. uvarum*	5	3	100	*Botrytis*
				Acetic Acid Bacteria	66	62	10	
				B. subtilis	1	0	10	
				C12	3	1	10	
11	Vinemount Ridge	Riesling	September	*H. uvarum*	17	17	100	*Botrytis*
				C. zemplinina	11	11	100	
				Acetic Acid Bacteria	15	13	10	
				A. pullulans	8	4	100	
12	Creek Shores	Riesling	September	*H. uvarum*	13	13	1000	*Botrytis*
				C. zemplinina	5	5	1000	
				P. membranifaciens	2	2	1000	
				Acetic Acid Bacteria	126	81	100	
				C14	0	3	100	
13	Creek Shores	Riesling	September	*H. uvarum*	34	26	1000	*Botrytis*
				C. zemplinina	3	1	1000	
				P. membranifaciens	3	2	1000	
				Acetic Acid Bacteria	123	84	100	
14	Short Hills Bench	Riesling	September	*H. uvarum*	13	3	10	
				C. zemplinina	56	40	10	
				P. membranifaciens	0	2	10	
				Acetic Acid Bacteria	18	21	10	
				C6	1	0	10	

H_0: There are no differences in the mean relative frequencies of the organisms associated with sour rotted grapes according to the date of sampling.

Appendix VII Table 2. Statistical analysis using the Welch statistic, adjusted for differences in variance based on sample sizes, to ascertain differences in the relative frequency of dominant yeasts and bacteria associated with sour rotted grapes according to date of sampling.

Colony	Timing	Mean Relative Frequency	Welch's Statistic
Acetic Acid Bacteria	September	38.69	$F(1,2.96) = 1.07$
	October	59.49	$p = 0.378$
H. uvarum	September	30.58	$F(1,4.28) = 1.15$
	October	16.20	$p = 0.341$
C. zemplinina	September	17.91	$F(1,2.42) = 0.03$
	October	20.86	$p = 0.882$
P. membranifaciens	September	3.47	$F(1,11.62) = 1.29$
	October	0.91	$p = 0.279$
B. subtilis	September	5.66	$F(1,11.99) = 0.76$
	October	1.92	$p = 0.401$
A. pullulans	September	2.53	$F(1,2) = 0.00$
	October	0.00	$p = 1.000$

All *p*-values are greater than 0.05, therefore there the null hypothesis is accepted.

H_0: There are no differences in the mean relative frequencies of the organisms associated with sour rotted grapes according to the cluster's concurrent infection with *Botrytis cinerea*.

Appendix VII Table 3. Statistical analysis using the Welch statistic, adjusted for differences in variance based on sample sizes, to ascertain differences in the relative frequency of dominant yeasts and bacteria associated with sour rotted grapes according to the cluster's concurrent infection with *Botrytis cinerea*.

Colony	***Botrytis cinerea* Detection**	**Mean Relative Frequency**	**Welch's Statistic**
Acetic Acid Bacteria	Yes	38.27	$F(1,8.58) = 0.45$
	No	49.65	$p = 0.519$
H. uvarum	Yes	35.50	$F(1,8.68) = 1.97$
	No	16.83	$p = 0.196$
C. zemplinina	Yes	11.76	$F(1,6.46) = 2.00$
	No	27.58	$p = 0.204$
P. membranifaciens	Yes	4.48	$F(1,7.19) = 1.54$
	No	0.84	$p = 0.254$
B. subtilis	Yes	4.98	$F(1,11.88) = <0.01$
	No	4.69	$p = 0.963$
A. pullulans	Yes	3.45	$F(1,7.00) = 2.14$
	No	0.03	$p = 0.187$

All *p*-values are greater than 0.05, therefore there the null hypothesis is accepted.