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New insights into sour rot: a complex interaction between the microbial community, vinegar flies and weather

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Abstract: Sour rot is a disease that affects grape berries once they reach ripeness and involves pre-harvest cluster decay accompanied by a smell of vinegar. It is caused by yeasts and acetic acid bacteria who are frequently vectored by vinegar flies and are all regulated by the actual weather conditions. For getting new insights in the etiology of sour rot we studied and manipulated concurrently the microbial community, transmission patterns as well as meteorological conditions. A co-inoculation experiment indicated that the two yeast genera *Candida* and *Hanseniaspora* contribute to the development of sour rot symptoms and acetic acid production and that they might well be able to provoke sour rot on their own. When studying the vectoring capacity of vinegar flies, females of *Drosophila suzukii* induced more severe sour rot damage than their males or *D. melanogaster*. This might indicate that egg laying and/or larval development favour microbial colonisation and/or sour rot development. Yet, we were also able to show that direct fruit to fruit transmission is common when there is physical contact between diseased grapes and healthy but injured berries. This mechanism is probably responsible for the propagation of sour rot within grape clusters as frequently observed in the vineyards by the direct aggregation of several diseased berries. Moreover, we were able to demonstrate in the laboratory that the optimal temperature for the development of sour rot lays around 30 °C. Above and below this temperature, acetic acid production was reduced with being almost absent at 20 °C and below. Finally, a greenhouse experiment revealed that temperature and precipitation affected the number of eggs laid by *D. suzukii* females in the berries of caged grapevines, which thereafter determined the severity of sour rot acetic and consequently acid concentration within grapes. Overall, our study provides additional insights in the etiology of sour rot and contribute to a better understanding of the complex interaction between microbial communities, sour rot transmission, assumed vectors and favourable weather conditions in vineyards.

Key words: *Vitis vinifera*, Drosophilidae, spotted wing drosophila, microbial diversity inoculation experiment

Introduction

Sour rot is a syndrome that involves pre-harvest cluster decay accompanied by the smell of vinegar (Hall et al., 2018) and the disease affects grape berries once they reach ripeness. It is caused by yeasts and acetic acid bacteria whom are frequently vectored by vinegar flies and are all regulated by the actual weather conditions. It is commonly assumed that yeasts are the primary component of sour rot disease since they ferment in a first step sugars into alcohol. In

a next step acetic acid bacteria transform the fermented alcohol into acetic acid and/or gluconic acid that initiate the pungent vinegar smell in sour rot diseased grapes (Barata et al., 2012).

For getting new insights in the etiology of sour rot we studied and manipulated concurrently the microbial community, transmission patterns as well as meteorological conditions.

Materials and methods

Microbial identification

Sour rot diseased grapes of the variety Chasselas were collected around Nyon (Switzerland) at the end of September 2021. The berries were placed in airtight bags and macerated for two weeks. Thereafter berries were crushed in the bag and microbes were allowed to develop on growing media. Yeasts were grown in petri dishes using Potato Dextrose Agar (Millipore®, Burlington, USA) and identified by sequencing the ITS region (Gardes and Bruns, 1993). Bacteria were cultivated on GY agar plates and identified by sequencing the 16S RNA gene.

Co-inoculation experiment

Different solutions containing a combination of each of the 6 bacteria (*Komagataeibacter xylinus*, *K. oboediens*, *K. rhaeticus*, *K. saccharivorans*, *Gluconobacter oxydans* and *G. sphaericus*) with each of the 5 yeasts (*Candida californica*, *Saccharomycopsis vini*, *Pichia occidentalis*, *P. membranifaciens* and *Hanseniaspora uvarum*) were prepared at a concentration of 10^6 cells/ml. Sterilised grape berries were injected with 20 µl of the prepared solution. Each berry was stored individually in a closed box (3.6 cm height, 3 cm diameter) at 25 °C for 10 days. Each treatment was replicated 5-times. To estimate sour rot development, individual grape berries were pressed in a domestic garlic press and the obtained juice was than centrifugated to measure the acetic acid concentration using the commercial kit Acetic acid Enzytec Liquid from R-biopharm AG, Darmstadt, Germany.

Contact transmission experiment

Single grape berries of the table grape variety Vittoria were injected with 20 µl of a solution containing 10^6 cells/ml of a mixture of all the isolated microorganisms retrieved from sour rot infected grape clusters. When they turned brown and showed clear signs of sour rot infection, single grape berries were put into contact with healthy grapes from the same variety previously wounded and sterilized. The position of the healthy and infected berry was manipulated by either bringing into contact an infected wound touching a healthy wound (IW-HW), an infected wound touching healthy skin (IW-HS), an infected skin touching a healthy wound (IS-HW) and an infected skin touching healthy skin (IS-HS). Thereafter, they were kept at 25 °C for 14 days in small individual boxes (3.6 cm height, 3 cm diameter) before symptoms on healthy berries was assessed visually. Each of the four treatments was replicated 8-times.

Experiment on the vectoring capacities of vinegar flies

In the laboratory reared adults of *Drosophila suzukii* and *D. melanogaster* were kept for at least 12 h inside a box together with the spores of microorganisms provoking sour rot. Two *D. suzukii* females, two *D. suzukii* males or two *D. melanogaster* females were placed in a small box (3.6 cm height, 3 cm diameter) containing a single Vittoria grape berry. Half of the grapes were sound whereas the other half was wounded. Boxes were stored for 10 days in a climate chamber (22 °C, 65-75 % RH, L/D 16 h/8 h). As a positive control, a wounded grape infected with the microbial solution but without any flies was maintained in the same conditions. The acetic acid

concentration was measured using the previously mentioned commercial enzymatic kit. Overall, each treatment was repeated five-times on totally five different and independent berries.

Development of sour rot under different temperatures in the laboratory

Sterile Vittoria grape berries were wounded and injected with 20 µl of a solution containing 10^6 cells/ml of a mixture of microorganisms provoking sour rot. Thereafter, each berry was placed individually in a box (3.6 cm height, 3 cm diameter). Boxes were then placed for 10 days in climatic chambers regulated at 10 °C, 15 °C, 20 °C, 25 °C, 30 °C or 35 °C. Once again, the acetic acid concentration of each berry was measured individually using the mentioned enzymatic kit. Overall, each treatment was repeated five-times on totally five different and independent berries.

Effect of temperature and rain on the development of sour rot in the greenhouse

A total of 10 grapevine plants of the variety Garanoir at the ripening stage (BBCH 81) were placed inside insect cages (75 cm × 75 cm × 115 cm). Always five plants were separated in two different greenhouse compartments, where one was kept at 25 °C and the other one at 35 °C. In each compartment one of the five insect cages served as a control, whereas we released in each of the other four cages 40 *D. suzukii* females beforehand exposed to sour rot provoking spores of the previously mentioned mixture of all the isolated microorganisms.

On each grapevine plant in a cage, half of the clusters were daily sprayed with distilled water to simulate a rain event. After 25 days, all grape clusters were collected (e. g., two to four clusters) and sour rot infection was visually estimated by estimating the percentage of diseases berries. Additionally, the number of eggs laid was counted in 50 randomly collected berries. Thereafter, these 50 berries were pressed and juices' acetic acid concentration was measured with the mentioned enzymatic kit.

Results and discussion

Microbial identification

After their genetic identification, the presumable 11 strains of yeasts were reclassified in the five different species *Candida californica*, *Saccharomycopsis vini*, *Pichia occidentalis*, *P. membranifaciens* and *Hanseniaspora uvarum*, belonging to four different genera. The same was true for the bacteria strains. Similarly, the 10 bacteria strains belonged to the six species *Komagataeibacter xylinus*, *K. oboediens*, *K. rhaeticus*, *K. saccharivorans*, *Gluconobacter oxydans* and *G. sphaericus* covering two genera.

All yeast species belong to the order Saccharomycetales. Many of this species are known to take part in fermentative processes and yeasts of the genus *Candida* and *Pichia* can spoil wines (Fleet, 2003). The bacteria identified belong to the acetous group of the Acetobacteraceae family. The genus *Komagataeibacter* is commonly found in alcoholic and acidic environments as they are usually isolated from wines, vinegars and other products of fermentative processes. Moreover, bacteria from the genus *Gluconobacter* were already isolated from sour rot diseased grapes (Gao et al., 2020).

Co-inoculation experiment

Combinations of bacteria with yeasts of the species *C. californica* or *H. uvarum* provoked acetic acid concentrations (Table 1). Moreover, both yeast species might be able to induce sour rot on their own. This implies that these yeasts are able to first ferment sugar into ethanol and

thereafter into acetic acid. On its own, no bacterium was able to produce a considerable amount of acetic acid (Table 1).

Table 1. Average acetic acid concentration (g/l) of the co-inoculation of each bacterium with each yeast. Different letters indicate different statistical groups according to Tukey post-hoc test (P = 0.05).

Yeast / Bacterium	*Candida californica*	*Saccharomycopsis vini*	*Pichia occidentalis*	*Pichia membranifaciens*	*Hanseniaspora uvarum*	*Water*
Komagataeibacter xylinus	3.8 abc	0.02 e	0.01 e	0.03 e	0.19 e	0.01 e
K. oboediens	2.99 abcd	0.01 e	0.03 e	0.00 e	1.36 bcde	0.06 e
K. rhaeticus	1.88 bcde	0.031 e	0.02 e	0.00 e	0.94 cde	0.06 e
K. saccharivorans	5.48 a	0.02 e	0.09 e	0.03 e	5.32 a	0.02 e
Gluconobacter oxydans	3.68 ab	0.00 e	0.13 e	0.01 e	2.56 bcde	0.17 e
G. sphaericus	3.49 abc	0.01 e	0.01 e	0.01 e	2.68 abcde	0.21 e
Water	1.54 bcde	0.01 e	0.05 e	0.01 e	0.25 de	0.01 e

Under field conditions grapes are usually colonised by a complex community of microorganisms and it is unlikely to encounter a single species on its own (Renouf et al., 2005). The differences observed among the possible combinations indicates that the severity of sour rot disease most likely depends on the microbiome present.

Contact transmission experiment

All healthy berries got infected by sour rot when their wound directly touched a sour rot infected berry (Figure 1). None or the healthy berries got infected when the intact skin touched a sour rot infected berry.

This shows that the transmission of sour rot causal agents from one grape to another is possible under laboratory conditions without insect vectors if the wound of a healthy berry touches a sour rot infected one. However, the microbial agents are not able to penetrate the skin of healthy berries and can only colonize it if there is an opening. This mechanism is probably responsible for the propagation of sour rot within grape clusters frequently as characterised by a direct aggregation of diseased berries.

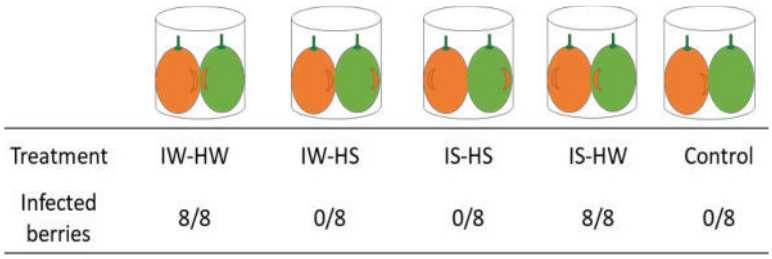

Figure 1. Sour rot symptomatic grapes out of the total amount of replicas after 14 days. IW = infected wound, HW = healthy wound, IS = infected skin, HS = healthy skin

Experiment on the vectoring capacities of vinegar flies

Besides the positive control, acetic acid was only found on wounded berries exposed to *D. suzukii* or *D. melanogaster* females (Figure 2). However, the acetic acid concentration was higher in berries exposed to females of *D. suzukii* than *D. melanogaster*. No acetic acid production was measured in wounded berries exposed to *D. suzukii* males and in unwounded berries exposed to vinegar fly males or females.

Our data indicate differences in the vectoring capability of *D. suzukii* and *D. melanogaster*. Contrary to expectations, *D. suzukii* females were not able to provoke sour rot in unwounded berries. This might be due to the hardness of the table grape's skin, which might have prevented them from laying their eggs. Using a thinner skinned and more sensitive grape variety, such as Vernatch, *D. suzukii* females might, however, well have been able to lay their eggs also in intact and sound berries, something improbable for *D. melanogaster* females.

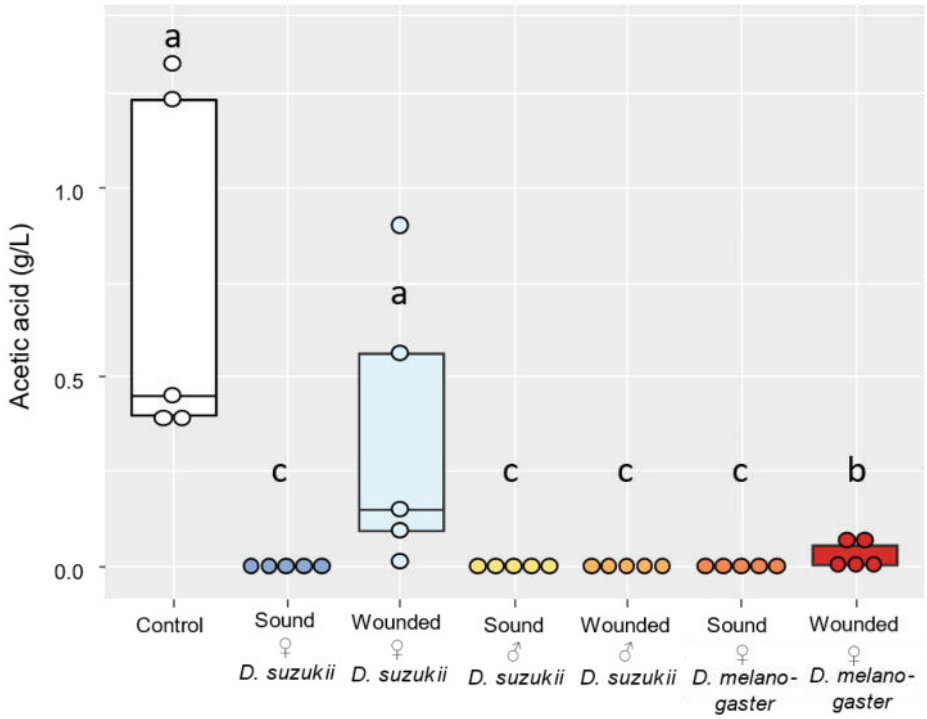

Figure 2. Acetic acid concertation (g/l) after 10 days in sound or wounded grape berries exposed to adults of *D. suzukii* or *D. melanogaster*. Different letters indicate statistical differences according to a Kruskal-Wallis test ($\chi^2 = 31.9$, $P < 0.001$).

Development of sour rot under different temperatures

No acetic acid was produced at a temperature of 10 °C (Figure 3). Starting at day 6 small amounts of acetic acid were detected at 15 °C and 20 °C, while at 25 °C, 30 °C and 35 °C it was detectable from day 3 on (data not shown). Significant amounts of acetic acid were detected between 25 °C and 35 °C (p-value < 0.001) at the end of the experiment (Figure 3). 30 °C is the most suitable temperature for acetic acid production.

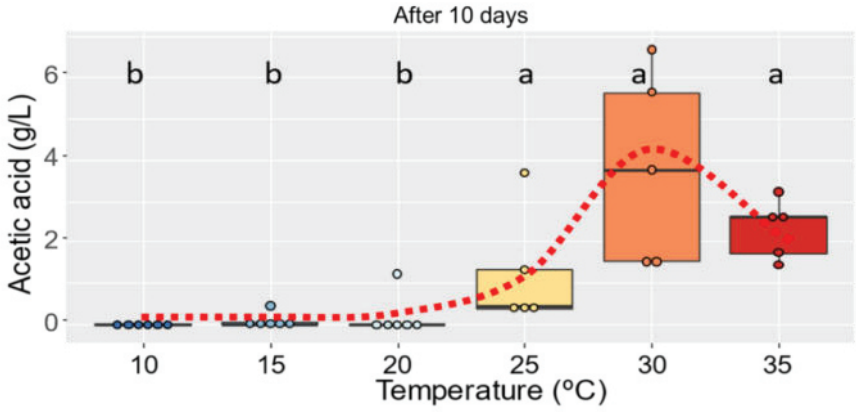

Figure 3. Boxplot of the acetic acid concentration (g/l) after 10 days at temperature regimes between 10 °C to 35 °C. Different letters indicate statistical differences according to a Kruskal-Wallis test ($\chi 2 = 26.4$, $P < 0.001$). The dotted red line represents the best polynomic fit of the acetic acid development over the temperature regime.

Our data show that the production of acetic acid and thus the development of sour rot disease depends on temperature. The optimum seems to be around 30 °C. At 35 °C acetic acid production was reduced and it can be assumed that with increasing temperature the incidence of sour rot would decrease. Moreover, nearly no acetic acid production was measured at 20 °C and lower temperatures. This is accordance with observations in the field, since it is generally assumed that autumnal temperature must reach 20 °C for the development of sour rot in vineyards (Viret, 2014).

Effect of temperature and rain on the development of sour rot in the greenhouse

After 25 days significantly more eggs were laid at 25 °C than 35 °C and simulated rain tended to increase oviposition (Figure 4 a). No development of sour rot was recorded in the two control treatments as well as at 35 °C independent of simulated rain (Figure 4 b). At 25 °C sour rot development was strongly favoured by the simulated rain and the percentage of sour rot diseased grapes increased with the number of *D. suzukii* eggs laid. The highest acetic acid concentration was measured at 25 °C with simulated rain (Figure 4 c). Once again acetic acid concentration increased with the number of eggs laid. Overall, the acetic acid concentration was much lower in our greenhouse experiment than the previous laboratory experiments. This is most likely explained by the much less favourable physical conditions in the greenhouse and reduced spore transmission by *D. suzukii* females in these larger cages.

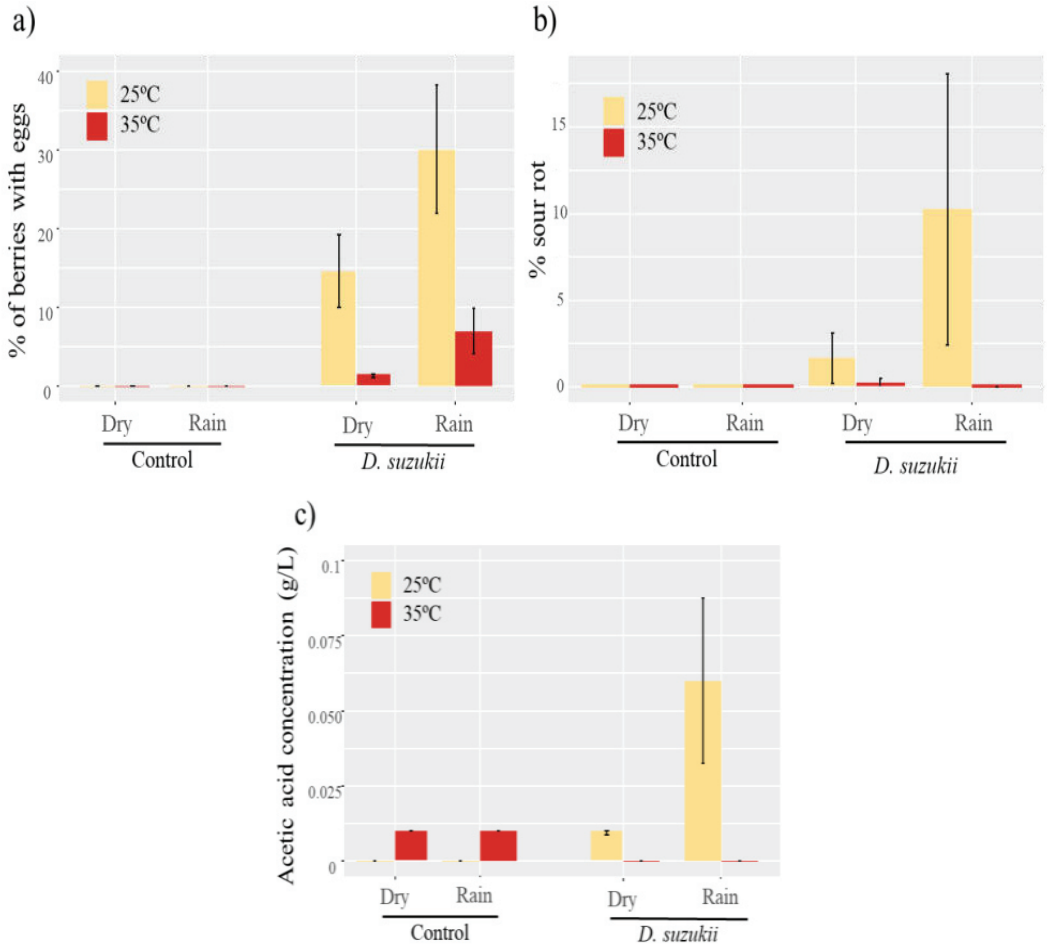

Figure 4. Average (a) percentage of berries with *D. suzukii* eggs, (b) percentage of sour rot infected berries and (c) acetic acid concertation at 25 °C and 35 °C with or without simulated rain (bars = 1 SE).

Conclusions

The interactions of all the factors involved in the development of sour rot are so far only partly understood. Here we confirmed and gained novel insight on the microbial community in sour rot diseased grapes. Moreover, we were able to show that transmission is possible without vectors when a direct physical contact exists between a wounded healthy berry and an inoculum. It will be interesting to see if other studies can confirm this higher vectoring capacity of *D. suzukii* females compared to *D. melanogaster* females. Our data also indicate that more than physical contact might be necessary to transmit sour rot and that the act of oviposition and/or larval development are promoting sour rot development. Moreover, the development of sour rot needs a temperature regime over 20 °C and benefits strongly from rain as indicated by our laboratory and greenhouse experiments manipulating weather conditions. In the greenhouse, greater oviposition translated to a higher severity of sour rot at 25 °C. Overall, our experiments provide additional insights in the etiology of sour rot and contribute to a better understanding of the complex interaction between microbial communities, sour rot transmission, assumed vectors and favourable weather conditions in vineyards

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