Effect of acidity on growth rate and stroma formation of *Monilia fructigena* and *M. polystroma* isolates

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Summary: The effect of acidity (pH) ranges on the mycelial growth and stroma formation of *Monilia fructigena* Pers: Fr. and of *M. polystroma* van Leeuwen was determined on agar plates and apple fruits. Four isolates of each of the brown rot fungi and two apple cultivars, 'James Grieve' and 'Cox's Orange Pippin', were used for the study. For the agar plate study, a range of the initial pH was prepared from 2.5 to 6.5. The dishes were inoculated with a 4 mm plug of each isolate and incubated at 23 °C in darkness. The mycelial growth was measured after 1.5, 4, 7, 10 and 20 days of incubation. After a 30-day incubation, stroma formation was determined by image analysis and weighing of mature stroma. In the fruit experiment, both cultivars were inoculated with one isolate of *M. fructigena* and of *M. polystroma*. The pH changes were determined after 7, 14, 28 and 35 days of incubation in both healthy and inoculated fruits. The fastest mycelial growth was at pH 4.5 for *M. polystroma* and at pH 3.5 for *M. fructigena*. After a 30-day incubation, *M. polystroma* isolates produced twice or three times more stroma compared to *M. fructigena* isolates. For both brown rot fungi, the amount of mature stroma increased from pH 3.5 to 5.5, and then decreased at pH 6.5. Results of the fruit experiment showed that healthy fruits were quite acidic (pH < 3.5), but pH rapidly increased in the inoculated fruits for both cultivars, reaching pH 4.6–5.4 depending on cultivar and fungus isolate. On both cultivars, the stroma developed at a significantly higher pH for *M. polystroma* than for *M. fructigena*. Biological and practical implications of the results are discussed.

Key words: acidity (pH), *in vitro*, *Monilia fructigena*, *Monilia polystroma*, mycelial growth rate, stroma formation

Introduction

Monilia fructigena Pers: Fr. is a common pathogen in apple and pear orchards distributed throughout Europe, Asia, Africa and some countries of South America (*Byrde & Willetts*, 1977; *Holb*, 2003). *Monilia polystroma* van Leeuwen was recently delineated from the Japanese group of *M. fructigena*, which has different characteristics from those of *M. fructigena* in pathogenicity, morphology and genetic material (*Van Leeuwen* et al., 2002). The fungus occurs only in Japan. Both pathogens are important brown rot fungi on several fruit species.

Many factors, such as fruit maturity, nutrient status, acidity and abiotic conditions influence the mycelial growth and mummification process of *Monilia* spp. in fruit. The fastest mycelial growth of *M. fructigena* was obtained when it was grown at 20 to 25 °C in darkness (*Hall*, 1933). The fungus mycelium can colonise Petri dishes after 7–10 days of incubation (*Byrde & Willetts*, 1977). However, the development of stroma formation takes 4 to 8 weeks at 15–20 °C on agar plates (*Harada*,1977; *Najim*, 1987). The biotic and abiotic requirements for mycelial growth are quite similar to those of stroma formation, although stroma formation requires higher water and nutrient contents than those needed for mycelial growth (*Hawker*, 1957). Moreover, *Willetts* (1968b; 1969) observed on another brown rot fungus (*M. fructila*) that when stroma formed, there was a competition for nutrients between macroconidia and stroma. He also noted that moist dark and nutrient abundance enhance the development of stroma formation. Only one study investigated the effect of hydrogen-ion concentration on *Monilia* spp. *Willetts* (1968a) found that the mycelial growth *M. fructicola* after a 4-day incubation was best on potato dextrose agar with an initial pH of 4.0–4.8. In the same study, it was also found that the greatest area of stroma was formed in cultures of an initial pH of 4.8. There was a decrease in the amount at pH 3.5 and only small areas were present at pH 6. At an initial pH of 2.8, no differentiated stroma was present although the mycelium was pigmented. The changes in pH inside an infected fruit have not been investigated yet for *M. fructigena*.

Most requirements for mycelial growth and stroma formation of *M. polystroma* are similar to those of *M. fructigena* (*Van Leeuwen* et al., 2002; *Holb*, 2003); however, its growth rate is faster and abundance of its stroma formation is greater compared to *M. fructigena*. No study has determined the effect of acidity on the biology or morphology of *M. polystroma*, either on agar plates or on infected fruits.

The aim of this study was to determine the effect of different acidity ranges on mycelial growth and stroma formation of four isolates of *M. fructigena* and *M. polystroma*. The study was made on agar plates and the fruits of two apple cultivars. Some of the preliminary results of this study were presented elsewhere (*Holb*, 1999).

Material and method

Fungus species and isolates

Two *Monilinia* species causing brown rot of fruits were studied: *Monilia fructigena* and *M. polystroma* (formerly Japanese *M. fructigena*). A set of four isolates representative of *M. fructigena* species were selected from a culture collection established by the co-operation of several European universities. A corresponding set of four *M. polystroma* isolates were kindly provided by Y. Harada (Faculty of Agriculture and Life Sciences, Hirosaki University). Isolates were cultured on V-8-juice agar slants stored at 10 °C. Sporulation was stimulated by nuv light after every transfer in storage in order to maintain viability of isolates. Isolates were used without single spore culturing. Isolate name, geographical origin and year of isolation of cultures are given in *Table 1*.

Table 1 List of *Monilia fructigena* and *M. polystroma* isolates used in this study

Species	Isolate	Origin	Year of isolation
M. fructigena	PD 8.96	The Netherlands	1996
	HU 1.96	Hungary	1996
	ES 48	Spain	1996
	FR 8	France	1996
M. polystroma	JAP 1145	Japan	1987
	JAP 2315	Japan	1994
	JAP 2316	Japan	1994
	JAP 2317	Japan	1995

Agar plate study

Preparation of acidity ranges. For growing *Monilia* isolates, potato dextrose agar (PDA, Oxoid, Basingstoke) was used as the basic medium. Media were prepared with five ranges of the initial acidity from pH 2.5 to 6.5. The five initial acidities of the cultures were: pH 2.5, 3.5, 4.5, 5.5 and 6.5. For preparing the media, potato dextrose agar (PDA, Oxoid, Basingstoke), Technical Agar (Agar No. 3, Oxoid, Basingstoke), H_3PO_4 acid, KH_2PO_4 and K_2HPO_4 salts were used. The amount of acids and salts was dependent on the necessary acidity that we aimed to reach. During media preparation, pH was measured with a pH meter (CG 840 pH-meter, Schott Gerate GmbH, Germany). After boiling the solution, at 60 °C the pH was checked and corrected with the above mentioned acid or salts. Then, the media with pH 4.5, 5.5 and 6.5 were sterilised at 120 °C for 15 min. The media with pH 2.5 and 3.5 did not harden easily after high temperature sterilization, therefore, sterilisation was made only at 80 °C. Media without acids and salts were also prepared and used as controls.

Assessment of mycelial growth and acidity changes. Cultures were grown on the above described agar media in 9 cm diam, plastic Petri dishes at 23 °C in darkness. The dishes in five replicates were inoculated with 4 mm plugs of each 20-day old *M. fructigena* and *M. polystroma* culture. Mean colony diameter, in mm, was determined after 4, 7, 10 and 20 days of incubation for each isolate. Moreover, acidity changes were assessed after 4, 7, 10, 20 and 30 days of incubation for each isolate. For this, a 4 cm^2 area of agar media including fungus bodies was cut from the dishes and put into 20 ml distilled water. The portion of culture media was crushed in the water and the pH was measured with a pH meter. At each assessment date, 2 replicates of each isolate were chosen for acidity determination. Experiments were conducted twice.

Assessment of stroma formation. For this experiment, isolates were grown on cherry decoction agar (CHA) prepared according to *Gams* et al. (1998) and *Van Leeuwen* et al. (2002). After a 30-day incubation, the area and the weight (wet and dry) of stroma formation per Petri dish was quantified using an image analyser (Quantimet 570, Cambridge Instrument, UK) and a weighing-machine (Mettler PM 600, Cowan Alexander LLC, Woodland Hills, CA, USA), respectively. Stroma was considered mature when it developed dark brown or black pigmentation (*Willetts*, 1968b).

For image analysis, the underneaths of plates were measured, because here the dark brown or black stroma formation contrasted well with the yellowish colour of the mycelial colony. Therefore, stroma could be separated precisely from other parts of the plate.

The procedure for measuring the wet and dry weight of stroma fromation was as follows. Five ml distilled water were poured on the surface of each culture and the mycelium was washed out with a paintbrush. Then, the stroma was taken with a sharp knife from the cleaned surface. The collected stroma with 10 ml water was heated in a heat-proof glass at 100 °C for 30 min., then filtered on cheesecloth in order to fully separate the stroma from the media. After separating the stroma, its wet weight was measured. Then, the stroma was dried at 73 °C for 18 hours by a Memmert universal oven (Memmert Model U, Memmert GmbH, Schwabach, Germany) and the dry weight of the stroma was measured.

Experiments were conducted twice.

Fruit study

Selected cultivars and isolates. Two apple cultivars 'James Grieve' and 'Cox's Orange Pippin' were used. Ripe fruits were inoculated with one isolate of *M. fructigena* (PD 4.96) and one of *M. polystroma* (JAP 2316).

Fruit inoculation and incubation. 85 ripe fruits were collected for each cultivar and brought into the laboratory. Then, 5 fruits were squashed separately in a mixer and the pH of the juice was determined. 40 fruits for each cultivar were artificially inoculated with 4 mm plugs of PD 4.96 isolate grown on PDA. The same inoculation was made with isolate JAP 2316. The infected fruits were placed separately in a box and incubated at 17 °C in darkness.

Assessment of acidity changes. The acidity of healthy and infected fruits was measured separately after 7, 14, 28 and 35 days of incubation for all cultivars and isolates. At each assessment date, 5 healthy and 5 infected fruits were disrupted in a mixer with 50 ml distilled water. Then, the H_3O^+ concentration was measured with a pH meter calibrated at 20 °C. The experiment was conducted twice.

Data analyses

Mean mycelial growth in mm, growth rate in mm d^{-1} and area of stroma formation were calculated for each isolate and for each acidity range, and subsequently the overall means for the two species were compared with a *t*-test. Mean growth rates were calculated as the value of colony diameter (mm) divided by the age of the culture (day). Mean fruit acidity changes were calculated for each inoculated cultivar and subsequently the overall means for the two species were compared with a *t*-test.

Results

Mycelial growth and growth rate on agar plates

Mycelial growth of both *Monilia* species was continuous on PDA agar plates. *M. polystroma* isolates grew faster than *M. fructigena* isolates (*Table 2*). The fastest mycelial growth was at pH 4.5 for *M. polystroma* and at pH 3.5 for *M. fructigena*. *M. polystroma* isolates covered the Petri dishes after a 7-day incubation at an initial pH 3.5 and pH 4.5, while it took 10 days for *M. fructigena* at the same initial pH ranges. It can be seen clearly that the best mycelial growth was between the initial pH 3.5 and 4.5 for both *M. fructigena* and *M. polystroma* isolates.

The growth rate was the fastest after a 4-day incubation at all ranges of initial pH for all isolates (data not shown). Mean growth rate after a 7-day incubation was 7.9, 6.9, 7.8, 11.0, 11.8, and 6.1 mm d^{-1} at pH 5.4 (control), 6.5, 5.5, 4.5, 3.5 and 2.5, respectively, for *M. fructigena*. The corresponding growth rate for *M. polystroma* was 10.6, 10.4, 11.5, 12.2, 12.2 and 8.1 mm d^{-1} at pH 5.4 (control), 6.5, 5.5, 4.5, 3.5 and 2.5, respectively.

The pH of agar plates changed during the incubation period (*Table 3*). During the incubation period, the pH hardly changed in agar plates of either *Monilia* species with an initial pH of 2.5 and 3.5. However, considerable changes were detected at the initial pH values between 4.5 and 6.5. After 10 and 20 days of incubation, plates attained a pH among 4.2–4.9 and 5.3–5.8 for *M. fructigena* and *M. polystroma* isolates, respectively. These results showed that *M. fructigena* was likely to grow mycelium and form stroma under a lower acidity content compared to *M. polystroma*.

Area and weight of stroma formation on agar plates

First stroma formation was detected after 10 and 15 days of incubation in plates of *M. polystroma* and *M. fructigena* isolates, respectively. After a 30-day incubation, *M. polystroma* isolates produced twice or three times more stroma generally compared to *M. fructigena* isolates (*Table 4*; *Figure 1*). For both brown rot fungi, the amount of the mature stroma increased from pH 3.5 to 5.5 and it slightly decreased at pH 6.5. The largest amount of stroma formation developed at pH 4.5 and 5.5 for *M. fructigena* and *M. polystroma* isolates, respectively. No stroma developed at pH 2.5 for either fungi.

Table 2 Mycelial growth of *Monilia fructigena* and *M. polystroma* isolates on PDA at 23 °C in darknes

Initial pH	Assessment dates in days after inoculation				
	1.5	4	7	10	20
M. fructigena					
Control (5.4)	11.7^{aab}	34.8^{b}	55.3^{b}	65.8^{b}	79.2^{bc}
6.5	10.2^{a}	32.2^{b}	48.7^{bc}	62.4^{b}	77.4^{bc}
5.5	10.4^{a}	34.5^{b}	54.7^{b}	66.2^{b}	81.1^{ab}
4.5	9.8^{a}	53.8^{a}	77.2^{a}	85.0^{a}	85.0^{a}
3.5	***10.2**^{a}*	***58.7**^{a}*	***82.4**^{a}*	***85.0**^{a}*	***85.0**^{a}*
2.5	7.2^{b}	24.2^{c}	42.7^{c}	58.7^{b}	75.5^{c}
$SED_{0.05}$	2.48	7.22	10.44	8.55	4.11
M. polystroma					
Control (5.4)	19.2^{ab}	52.4^{c}	74.2^{b}	85.0^{a}	85.0^{a}
6.5	18.1^{b}	48.2^{c}	72.8^{b}	85.0^{a}	85.0^{a}
5.5	18.4^{b}	57.3^{bc}	80.4^{a}	85.0^{a}	85.0^{a}
4.5	***22.2**^{a}*	***70.3**^{a}*	***85.0**^{a}*	***85.0**^{a}*	***85.0**^{a}*
3.5	20.1^{ab}	64.7^{ab}	85.0^{a}	85.0^{a}	85.0^{a}
2.5	7.8^{c}	32.2^{d}	56.8^{c}	76.4^{b}	82.3^{b}
$SED_{0.05}$	3.14	10.27	5.31	4.54	2.12

[a] Values are means of 5 replicates of four *M. fructigena* or *M. polystroma* isolates.

[b] Values within columns followed by different letters are significantly different at P = 0.05.

Table 3 Changes of acidity of agar plates inoculated with *Monilia fructigena* and *M. polystroma* isolates after 4, 7, 10, 20 and 30 days of incubation

Assessment dates in days after inoculation	Initial pH of cultures					
	Control (5.4)	6.5	5.5	4.5	3.5	2.5
M. fructigena						
4	4.2^{a}	4.4	4.2	4	3.6	2.7
7	4.1	4.1	4.2	3.9	3.5	2.8
10	4.0	4.1	4.0	3.9	3.5	2.8
20	*4.2*	*4.5*	*4.4*	*4.3*	3.6	2.8
30	*4.2*	*4.9*	*4.6*	*4.3*	3.5	2.8
M. polystroma						
4	4.3	4.5	4.3	4.1	3.6	2.8
7	4.2	4.6	4.2	4.1	3.6	2.8
10	*5.7*	*5.7*	*5.4*	*5.2*	3.6	2.8
20	*5.7*	*5.8*	*5.8*	*5.3*	3.6	2.8
30	*5.7*	*5.8*	*5.8*	*5.3*	3.6	2.8

[a] Values are means of 5 replicates of four *M. fructigena* or *M. polystroma* isolates.

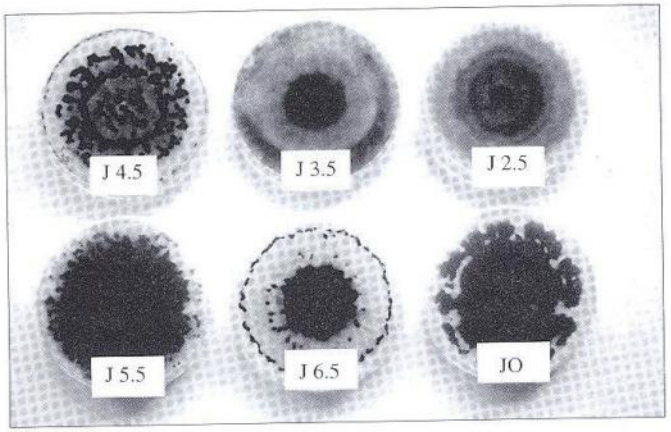

Figure 1 Stroma formation of *Monilia polystroma* under different pH ranges grown on PDA at 23 °C after a 30-day inoculation (initial pH 2.5, 3.5, 4.5, 5.5, 6.5 and control corresponding to J2.5, J3.5, J4.5, J5.5, J6.5 and JO, respectively)

Fruit acidity

Results of the fruit experiment showed that pH rapidly increased in the inoculated fruits for both cultivars (*Table 5*). Healthy fruits were quite acidic (pH ≤ 3.5) during incubation periods. The first rapid increase of pH in inoculated fruits was assessed after a 21-day incubation. By this time, the fruits were rotten and stroma formation had started to develop. During the stroma formation, the pH of the infected fruits increased and reached pH 4.6–5.4, depending on cultivar and fungus isolate. In both cultivars, stroma developed at a significantly higher pH for *M. polystroma* than for *M. fructigena*.

Discussion

Our study indicated that the most favourable initial hydrogen-ion concentration for mycelial growth was between pH 3.5 and 5.5 for both *M. fructigena* and *M. polystroma* (*Table 2*). This result is in agreement with a study of Willetts (1968a) who demonstrated that the mycelial growth of *M. fructicola* after a 4-day incubation was the highest at pH 4.0–4.8. These results support the fact that both fungi develop more rapidly on ripe than unripened fruits in the nature. This is due to the fact that the acid content is high (pH < 3) during fruit development and lower acidity (i.e. higher pH) occurs during ripening and harvest (*Soltész & Szabó*, 1997), because most fruits transform acids into sugars during ripening, which probably increases pH inside the fruits.

Our results demonstrated that both fungi increased the pH of agar plates or inoculated fruits during mycelial growth or stroma development (*Tables 3* and *5*). During the rotting process, the fungus disrupts the host cells (or the artificial media) by its enzymes (*Najim & Turian*, 1979), and it decomposes e.g. the sugars, acids and other nutrients of the host. This fungus-host interaction probably resulted in an increase of pH, which additionally stimulated mycelial growth and stroma development.

In agar plates and inoculated fruits, the rapid increase of pH started when the fungus started to produce stroma (*Table 3* and *5*). The pH increase was significantly higher for *M. polystroma* than for *M. fructigena*. The most favourable initial pH for stroma formation was between 4.5 and 5.5 for both fungi (*Tables 2, 4* and *5*). *Willetts* (1968a) indicated also that low acidity and high level of nutrient content are necessary for stroma development of *M. fructicola*. In the same study, it was also found that the greatest area of stromata were formed in cultures of initial pH 4.8. However, he also noted that when stroma formed, there was a competition for nutrients between macroconidia and stroma (*Willetts*, 1968b, 1969).

Above results reviewed together with previous research by others can be interpreted in the life cycle of *M. fructigena*.

Table 4 Stroma formation of *Monilia fructigena* and *M. polystroma* isolates on PDA at 23 °C in darkness after a 30-day incubation

Stroma formation	Initial pH of cultures					
	2.5	3.5	4.5	5.5	6.5	Control (5.4)
M. fructigena						
area (cm^2/Petri dish)	0	0	18.9	16.8[a]	4.67	20.3
wet weight (g/Petri dish)	0	0	1.12	1.05	0.67	1.16
dry weight (g/Petri dish)	0	0	0.11	0.10	0.06	0.12
M. polystroma						
area (cm^2/Petri dish)	0	11.6**[b]	39.3**	51.3**	29.8**	49.8**
wet weight (g/Petri dish)	0	0.86**	2.06*	2.87**	1.67**	2.82**
dry weight (g/Petri dish)	0	0.07**	0.19*	0.33**	0.20**	0.34**

[a] Values are means of 5 replicates of four *M. fructigena* or *M. polystroma* isolates.

[b] Significantly different values between *M. fructigena* and *M. polystroma* by *t*-test at P = 0.01 (*) and P = 0.001 (**) are indicated in superscript.

Table 5 Changes of acidity in fruits (cv. 'James Grieve' and cv. 'Cox's Orange Pippin') inoculated with *Monilia fructigena* and *M. polystroma* isolates after 7, 14, 28 and 35 days of incubation

Assessment days	James Grieve			Cox's Orange Pippin		
	Healthy	Inoculated		Healthy	Inoculated	
		M. fructigena PD 4.96	*M. polystroma* JAP 2316		*M. fructigena* PD 4.96	*M. polystroma* JAP 2316
7	2.9	3.1	3.2ns[a]	3.1	3.1	3.1ns
14	3.1	3.5	3.5ns	3.2	3.6	3.7ns
21	3.3	4.5	4.7ns	3.4	3.9	4.2*
28	3.3	4.6	5.2*	3.4	4.1	4.5*
35	3.4	5.1	5.4*	3.5	4.6	4.9*

[a] Significantly different values between *M. fructigena* and *M. polystroma* by *t*-test at P = 0.01 (*) are indicated in superscript. ns = not significant at P = 0.05.

During infection, the pathogen penetrates into the wounds of injured fruits and the initial coloniser hyphae start to grow. These hyphae can even colonize under a high acidic content, however, they grow slowly under these circumstances. This feature can be observed in the field when an unripe fruit becomes infected and the rotting process is slow or sometimes stops. If the first hyphae can colonise the host tissue successfully, then the fungus begins to decompose the host's sugars and acids with its enzymes, which results in higher pH and increasing mycelial colonisation of the host. This process can also be observed in nature when a fruit starts to rot slowly and soon after the rot increases exponentially. The more mature the fruit is, the faster the rotting is. One of the key factors is probably the increase of pH during fruit maturation which provides a better condition for the mycelial and stroma development of the fungus.

The above described process is probably valid for *M. polystroma*, however, the fungus enzymes may decompose more acid compounds in the fruit than *M. fructigena*, therefore, pH is higher during hyphae colonisation and stroma formation (*Table 5*). Moreover, the growth rate is faster and stroma formation is more abundant for *M. polystroma* than for *M. fructigena* (*Batra & Harada*, 1986; *Van Leeuwen*, 2002; *Holb*, 2003), which contributes to the fact that *M. polystroma* is a more aggressive and a more dangerous pathogen than *M. fructigena* in fruit orchards. Therefore, its introduction to European countries should be delayed as much as possible.

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