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Effects of pH and titratable acidity on the growth and development of *Monilinia laxa* (Aderh. & Ruhl.) *in vitro* and *in vivo*.

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Abstract This investigation examines the effects of pH and titratable acidity on the growth and developments of a strain of *Monilinia laxa* (Aderhold & Ruhland) at seven different pH levels in Potato Dextrose Agar media and on peach fruit from formation to commercial maturity. The fungi growth was obtained by daily measurement of mycelia on the pH amended Potato Dextrose Agar. The sporulation performance was determined after 30 days of culture incubation. Fruits were inoculated with *M. laxa*, from fruit set to maturity, on weekly basis for brown rot susceptibility. The pathogen development, *in vitro,* was affected, by the pH (2.4–11.52) amended nutrient media. *M. laxa* exhibited variation in its growth and sporulation capacities on the seven pH amended PDA, preferring relatively moderate acidic conditions for optimum performance. In the in vitro analysis, there was mycelia growth at pH 2.40 to 8.84, while pH 11.52 did not support any mycelia growth. There was a continuous and stable increase in weight of fruit as it developed whereas the fruit size increased, then decreased and finally increased as the fruit develops. The acidity dynamics exhibited a non-sinusoidal waveform through the growth and development of the fruit. In all these characteristic variations, *M. laxa* did not develop infection or shown any brown rot incidence in the fruit until the period of commercial maturity.

Keywords Alkaline · Monilia · Physicochemical · *Prunus persica* · Tolerance

Abbreviations

BRI	Brown rot incidence
DAF	Days after floration
DAS	Days after fruit set
EpHV	Expected pH values
FS	Fruit size
FW	Fruit weight
JDs	Julian days
PDA	Potato dextrose agar
PP	Polypropylene
RPM	Revolutions per minute
RpHV	Real pH values
TA	Titratable acid.

Introduction

Brown rot in peaches (*Prunus persica* (L.) Batsch) is a disease primarily incited by *Monilinia* species which includes *M. laxa, M. fructigena, M. fructicola, and M. polystroma* (Gell et al. 2007; Jansch et al. 2012). The degree of susceptibility to infection by *Monilinia* spp. is

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variable throughout fruit development. Susceptibility is high during the early stages of fruit development, decreases during the green fruit stage and increases again during the ripening period (Gradziel 1994). Stages of peach development can generally be considered to occur in four phases which includes, fruit set, rapid cell division, cell expansion and ripening/maturation (Moing et al. 1998; Tutu and Ciornea 2011; Guidarelli et al. 2014).

In a pathogen-host interaction the growth and development of microorganism is influenced by different physicochemical conditions such as temperature and water activity (Pascual et al. 1997; Xu et al. 2001; Moral et al. 2012), light, aeration and pressure (Maharshi and Thaker 2012), pH and titratable acidity (Pascual et al. 1997; Dirlewanger et al. 1999; Holb 2004; Romero-Arenas et al. 2012). During this process the physicochemical conditions influence the microbial activity determining either the growth and reproduction or the inhibition of activity and the inactivation of the pathogen (Dirlewanger et al. 1999; Tutu and Ciornea 2011).

The pH and titratable acidity (TA) are interrelated concepts of organic acids (Tyl and Sadler 2017) controlling physicochemical factors that act in an additive and interactive mode to inhibit metabolic pathway (Dirlewanger et al. 1999). These two physicochemical components, though complementary in nature, are statutorily different. TA refers to the total concentration of free protons and undissociated acids in a fruit juice that can react with a strong base and be neutralized, hence it is any amount of base needed to neutralize such acidity and bring its pH to a neutral (pH 7), or slightly alkaline (pH 8.1) value, and pH represents the free hydrogen ion activity in the fruit juice (Lobit et al. 2002) or a means of expressing such H^+ ions concentration in a substrate (Tutu and Ciornea 2011). In peach fruit acidity is an important genetic quality (Dirlewanger et al. 1999) which influences both perception of sourness and sweetness found in varying proportions depending on the cultivar and the ripening stage (Lobit et al. 2002).

The behavior of microorganisms-host interaction shows variation in their growth and sporulation on different levels (Maharshi and Thaker 2012). Most microorganisms, especially non fungi, grow best around neutrality (pH 7), while fungi in general prefer slightly acidic conditions for their growth (Alexopoulos 1952; Yamanaka 2003; Agrios 2011; Maharshi and Thaker 2012). Some fungi species, however, favour neutral to slightly alkaline conditions (Maharshi and Thaker 2012). *Monilinia* spp. can acidify the host tissue in peaches and nectarines from pH 4.50 and 4.45, to pH 3.75 and 3.90, respectively (De Cal et al. 2013). For *M. laxa*, information related with the effect of pH on growth and development *in-vitro* and *in-vivo* is hardly available. *Monilinia laxa* can infect flowers, resulting in blossom blight, as well as both healthy and wounded fruit, resulting in brown rot (Rungjindamai et al. 2014); a disease able to produce millions of spores on a single fruit that can spread quickly within and between orchards, locations, and hosts (Fazekas et al. 2014).

The general objective of this experiment was to determine the modulating effect of pH and titarable acidity (TA) to pathogenic activities of *M. laxa.* This included (a) determining the effect of pH on the mycelia growth, sporulation and development of *M. laxa* on solid PDA, and (b) to determine the effect of pH and TA evolution on the brown rot incidence (BRI) depending on the growth and development of peach fruit.

Materials and methods

Culture pH media preparation, inoculation and incubation

The cultivation medium, potato dextrose agar (PDA), was prepared in line with the manufacturer's instruction following which 7.8 g was measured into seven Erlenmeyer flasks (500 mL) and 200 mL sterile water added to the different flasks and contents slightly heated in a microwave oven for proper dissolution of mixture. They were sterilized at 121 °C for 15 min and, in a laminar flow chamber (aseptic conditions), known quantities of H_2SO_4 and KOH chemicals, as previously determined by titration, were added with a pipetman into the flasks (pH 5.30) marked with the expected pH values (EpHV) at a warm (45 °C-60 °C) temperature. The solution was agitated with a magnetic stirrer at 110 rpm for about 30s for homogeneity. Known quantity of 20 mL was separately and aseptically decanted into 50 mL transparent polypropylene (PP) jars and a pH stripe indicator cut and dipped into the jars to observe the associated pH readings. The molten PDA was later measured with the pH meter to obtain the real pH value (RpHV) before pouring out into Petri dishes of 90 mm diameter at 20 mL /plate for inoculation with *M. laxa*. The desired pH was adjusted with 1 M Hydrogen sulphate (H_2SO_4) and 5 M Potassium hydroxide (KOH) for acidic, neutral or alkaline values using the pH meter. Known quantities

of H_2SO_4 or KOH were, aseptically, added to the sterilized PDA (in 500 mL Erlenmeyer flasks at a warm (45 °C-60 °C) temperature.

The pH amended PDA were poured out at 20 mL/Petri dishes of 90 mm diameter (five Petri dishes/treatment). Active growing (7 days old) *M. laxa* mycelia (6.5 mm) from a PDA culture was centrally plated in the Petri dish containing the real pH values (RpHV) and incubated at 23 °C. And later the mycelia growth and extent of sporulation were determined.

Measurements of mycelia growth rate and sporulation density

Measurements of mycelia growth were taken daily using a digital Venier meter (Mitutoyo CD-15 DCX, Tokyo Japan) at a cross section. This was done until a Petri dish was fully covered with the mycelia extension. Rate of extension and influence of pH on pathogen activity were determined from the mycelia growth. There were five replications for each treatment.

After the data collection on mycelial growth, the culture was subsequently evaluated for sporulation activity at the 30th day of incubation. Distilled water (5 mL) was added to each of the Petri dishes containing 25 μL Tween® 80 and the mycelia colony rasped with a sterile laboratory metal spatula. The rasped colony together with the Petri dishes was placed on a mechanical shaker at 175 rpm for 30 min. Each treatment was filtered through a 4-fold of cheese cloth into premarked test tubes. From this suspension, 25 μL was pippeted onto a hemacytometer (Neubauer Cell Counting Chamber) and examined under a microscope. Two different spore loads (40 counts/load) were made in each replication of the seven treatments.

Plant material, fruit size and weight determination

Two peaches (*Prunus persica* (L.) Batsch) were the source of plant materials. The Plant materials were 'Babygold 9' and 'Crown Princess' from the collection of the Aula Dei-CSIC, Zaragoza. There were three trees per cultivar. Number two (middle tree) was marked and labeled after fruit setting for use in the evaluation. The marked tree was not thinned to enable enough fruits for sample harvest. Numbers 1 and 3 trees were however given the normal orchard treatments (e.g. fruit thinning). Fruits selected for analysis were all of similar maturity and size at each developmental stage. Inoculation with *M. laxa* was completed on 'Babygold 9' and not on 'Crown Princess' after observing that there was no infection at the early stage of inoculation. And the aim was to preserve enough samples for other essential analysis due to fewer available fruits on the 'Crown Princess'.

The range of complete floration and fruit settings in the two cultivars (Table 1) occurred in the early to mid-season of spring between 10/03/2014 and 11/04/2014; and the range of harvest date occurred in the early to mid-season of summer. 'Babygold 9' reached 100% floration and fruit setting on the 14/03/2014 (72 JDs) and the 11/04/2014 (100 JDs) respectively and harvested on the 21/08/2014 (232 JDs). 'Crown Princess' reached 100% floration and fruit setting on the 10/03/2014 (68 JDs) and fruit setting on the 11/04/2014 (100 JDs) and harvested on the 18/06/2014 (168 JDs).

Five fruits per cultivar were used for evaluations on a weekly basis. Fruits of visually uniform size were harvested for each period. Fruit size (mm) was determined by measuring the two diagonal sections with a digimatic venier caliper (Mitutoyo CD-15 DCX, Tokyo Japan). The fruit weight (g) was determined on a precision weighing machine.

Development of pH and titratable acidity

TA and pH were determined as explained in previous studies (Cantín et al. 2009; Abidi et al. 2015). In brief, three fruits were used per cultivar for this purpose. On a weekly basis after setting, fruits were harvested, cleaned with tap water, peeled and cut into thin slices. Five grams was weighed out into an automatic titrator tube and mashed dry with a polytron machine (Ika T-18 Ultra Turrax Digital High Speed Homogenizer, Germany) and later 45 mL of distilled water measured into the titrator tube. The pH automatic valuator machine was first calibrated using a buffer (for pH 4 and pH 8). This is after cleaning and flushing the electrodes with distilled water then filled with electrolyte (KCl) for compensation of any possible loss due to evaporation. The known quantity of the solution (50 g) in the automatic valuator was used to determine the pH with TA values accordingly. This was repeated every week until the cultivar reached commercial maturity date (Larena et al. 2005).

Evaluation for susceptibility to Monilinia

Five fruits per cultivar were used for this determination. Fruits were disinfected according to Obi et al. (2017).

Table 1 Complete floration, fruit settings and harvest in two peach cultivars (gestation period)

Activity	'Babygold 9'	'Crown Princess'	BBCH-scale
Floration (100%)	14/03/2014 (72 JDs)	10/03/2014 (68 JDs)	65
Fruit setting (100%)	11/04/2014 (100 JDs)	11/04/2014 (100 JDs)	73
Harvesting date	22/08/2014 (233 JDs)	12/06/2014 (162 JDs)	87

Using a Pipetman P100, fruits were inoculated with the isolate of *M. laxa* in the method of Obi et al. (2017). In brief each fruit, unwounded, was inoculated with 25 μL spore load of the 25×10^3 cfu mL^{-1} conidia suspension. Inoculated samples were incubated at 23 °C and 40–60% RH and duly observed for brown rot incidence for seven days.

Statistical analysis

The size, weight, pH and TA of fruit, including their standard errors were analyzed using the SPSS-23 statistical software (Statistical Product and Service Solutions Inc., Chicago USA). An earlier normality test was realized on parameters with the Kolmogorov-Smirnov test ($P \geq 0.05$), enabling the presentation of frequency of histograms. The same statistical software was used for mean standard errors (SE) and Pearson's correlations. An ANOVA test was used to compare differences between means and a post hoc test of the Duncan (DMRT) was used to measure for separation ($P \leq 0.05$) between pairs of means.

Result

Mycelial growth

Mycelial growth or extent of colonization was evaluated at seven levels of pH. There was mycelia growth in all the pH levels except at pH 11.52. The highest mycelia growth was at pH 6.40 (80.61 mm) at the rate of 8.96 mm/day. The lowest mycelial growth was at pH 2.40 (11.60 mm) at the rate of 1.29 mm/day (Fig. 1). Mean values varied according to the extent of colonization.

Sporulation capacity

Sporulation capacity was determined using the *M. laxa* colonies of the seven different levels of pH after 30 days of incubation. Sporulation was found to be highest at pH 5.30 with mean conidia concentration of over 1×10^5 cfu mL^{-1} and lowest at pH 8.84 with mean conidia of less than 5×10^4 cfu mL^{-1} (Fig. 2). There was no sporulation of *M. laxa* at pH 2.40, 3.01 and 11.52. There were, however, significant differences ($P \leq 0.05$, Duncan's test) in the sporulation capacity among pH 4.21, 5.30, 6.40 and 8.84.

Development of pH and TA in 'Babygold 9' and 'Crown Princess'

The evolution of pH and TA in fruits of 'Babygold 9'and 'Crown Princess' cultivars were non-sinusoidal waveforms. This waveform is best demonstrated in the 'Babygold 9′ (Figs. 3 and 5) which has a longer period of gestation. In this particular cultivar the pH peak (highest) was at 208 JDs (77 BBCH scale) which corresponds to 136 and 108 days of complete floration and fruit setting respectively. The lowest dip was at 215 JDs (78 BBCH scale) which was equivalent to 143 and 115 days after floration (DAF) and days after setting (DAS) respectively. From this position the pH increased along with the remaining growth and commercial maturity of the fruit.

In all this non-sinusoidal waveform dynamics of pH in 'Babygold 9', the pattern in TA evolution was always in the contrary (Fig. 3), hence the lowest dip was at 208 JDs (77 BBCH scale) which corresponds to 136 and 108 DAF and DAS respectively. The highest peak was at 215 JDs (78 BBCH scale) which was equivalent to 143 and 115 DAF and DAS respectively. From this position the TA decreased along with the remaining growth of the fruit.

Growth in fruit size and weight

The growth in fruit size (FS) and weight (FW) is presented in Fig. 4. FS was at steady increase for well over 194 JDs (75 BBCH scale). This pattern slightly changed at 201 JDs (76 BBCH scale) where there was a slight

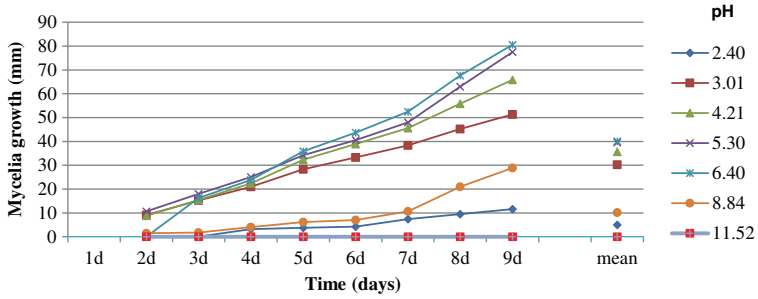

Fig. 1 Mycelia growth rate of *Monilinia laxa* (mm), *in vitro*, on the 7 different pH. *M. laxa* performed best at pH 6.4 and lowest at pH 2.4. No mycelium at pH 11.52. Each treatment was replicated five times and experiment repeated twice

slope that subsequently ascended at the 208 JDs (77 BBCH scale) and then a gentle smooth increase in size until the fruits' period of commercial maturity and subsequent harvest on the 232 JDs (79 BBCH scale).

In the same vein, FW was at a steady increase for over a period of 201 JDs (76 BBCH scale). This pattern changed at the 208 JDs (77 BBCH scale) when a noticeable rapid increase was observed. This increase appeared to have continued until the fruits' period of commercial maturity and subsequent harvest on the 232 JDs (79 BBCH scale).

Effect of pH and TA on brown rot incidence

Figure 5 represents the effect of pH and TA on brown rot incidence along with the growth and development of the fruit. After the highest increase in pH 4.39 ± 0.10 at 208 JDs (77 BBCH scale) and the lowest dip in pH 3.63 ± 0.10 at 215 JDs (78 BBCH scale), the pH re-initiated and continued to increase until fruit maturity. The reverse was the case with the TA which started to decrease until maturity (pH and TA linked with broken lines at 208 JDS (77 BBCH scale). Hence at the full commercial maturity, the fruit was associated with pH 4.19 ± 0.10 and TA 0.41 ± 0.01 (Mg 100 g^{-1} FW). From the beginning of fruit inoculation (145 JDs) (65 BBCH scale) with *M. laxa* to 222 JDs (78 BBCH scale) there was no incidence of brown rot until when the fruit was inoculated at the 232 JDs (79 BBCH scale) of commercial maturity.

Discussion

In general, according to the obtained results in this investigation, there was an ample influence of pH and titratable acidity (TA) in the solid PDA and the peach cultivars inhibiting the growth of the *M. laxa*. According to Tutu and Ciornea (2011), the pathogen-host interaction involves the process of nutrition, which leads to either growth/reproduction or the inhibition of activity and the inactivation of the pathogen as a result of available acidity. In the pH-amended PDA, there was no effective pathogen growth/development under a high acidic condition. Similarly, *M. laxa* developed no infection in the fruit until commercial maturity at pH 4.19 ± 0.10. This is relatively a moderate state of non-acidity in the fruit. Previously, peach fruits with a non-acid character have been characterized at maturity by a pH higher than 4.0 (Dirlewanger et al. 1999).

Mycelia growth (colonization) and sporulation are the most accurate variables to effectively compute the degree of disease development (Douds 1994; Gigot et al. 2009; Miles et al. 2009; Burnett et al. 2010; Obi et al. 2017). Consequently, determining the influence

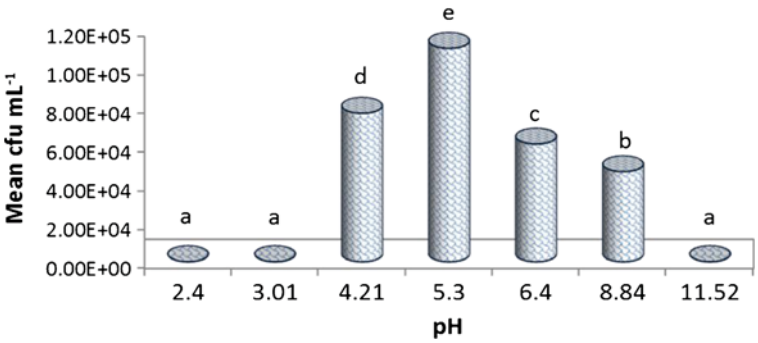

Fig. 2 Sporulation capacity of *Monilinia laxa* on 7 different pH at 30 days of incubation. Different mean letters indicate significant differences ($P \leq 0.05$, Duncan's test) among pH. *M. laxa* sporulated highest at pH 5.30. No sporulation at pH 2.40, 3.01 and 11.2. Each treatment was replicated five times and experiment repeated twice

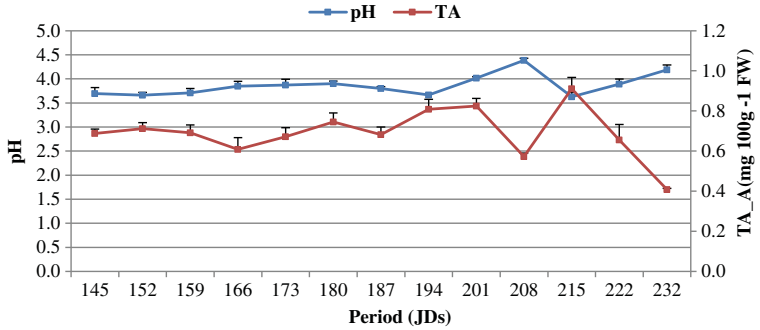

Fig. 3 The evolution of pH and TA plus SE in 'Babygold 9' fruit. Fruits were harvested once a week and each treatment replicated 3 times. There was significant difference ($P \leq .0.05$, Duncan test) in pH of fruits among weeks of harvest. However, no significant difference in the pH of fruit among 145, 152 and 194 JDs; and among 166, 173,180, 187, and 222 JDs. There was significant difference ($P \leq .0.05$, Duncan test) in TA of fruits among weeks of fruit harvest. However, no significant difference in TA of fruits among 145, 152, 159 and 187 JDs; and among 166 and 222 JDs. (See supplementary Table 1 for details)

of pH and TA on the development of *M. laxa,* at both *in vitro* and *in vivo* levels is an avenue to understanding the epidemiology of brown rot and subsequent development of disease management strategies to effectively combat the problem (Tian and Bertolini 1999; Hong et al. 2000).

Sporulation itself is a function of colonization (Douds 1994). Colonization concerns dimension (size or area) occupied by infection while sporulation deals with population (conidia or spores) involved in an occupied or diseased area. This implies that sporulation is the subsequent effect of colonization due to infection (Xu et al. 2001). However, we have found according to our results that a high rate of colonization did not equate to high rate of sporulation. Hence the extent of lesion or colonization does not always equate to the degree of sporulation in a host-pathogen interaction but depends on available level of acidity as shown by this experiment.

For example, in our assay the mycelia growth or extent of colonization at pH 6.40 and 5.30 were 80.61 mm and 77.50 mm producing mean conidia concentrations of 60,800 and 110,800 spores mL^{-1} respectively (Figs. 3 and 4). Though the extent of colonization was higher at pH 6.40 than at pH 5.30, sporulation was found to be higher at pH 5.30 than at pH 6.40 with about 45.13%. This appears to suggest that sporulation of *M. laxa* increases as the pH increases, reaches its maximum in the acidic region, and then descends as the pH approaches neutral and then alkaline.

We are of the view, therefore, that *M. laxa* could sporulate at a wide range of pH between 3.5 and 9.5 with the optimum between pH 4.5 and 5.5. This is similar to, and in agreement with, the results of Agarwal and Sarboy (1978) that acidic pH favours fungi growth with best performance within the range of pH 3.5–6.5. Pascual et al. (1997) observed a pH 4–6 range for optimum

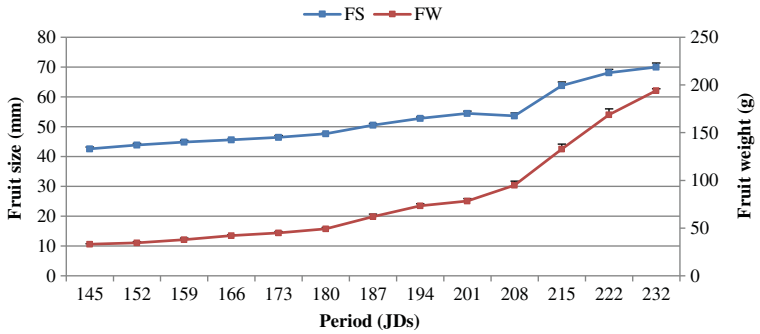

Fig. 4 The evolution of size (mm) and weight (g) plus SE in 'Babygold 9' from setting to fruit maturity. Fruits were harvested at weekly basis and each treatment replicated five times. There was significant difference ($P \leq 0.05$, Duncan test) in fruit size between weeks. However, no significant differences found in the size of fruits harvested at 194, 201 and 208 JDs; 222 and 232 JDs. There was significant difference ($P \leq 0.05$, Duncan test) in fruit weight between weeks. No significant difference found in weight of fruit harvested at 145 and 152 JDs; 194 and 201 JDs. (See supplementary Table 1 for details)

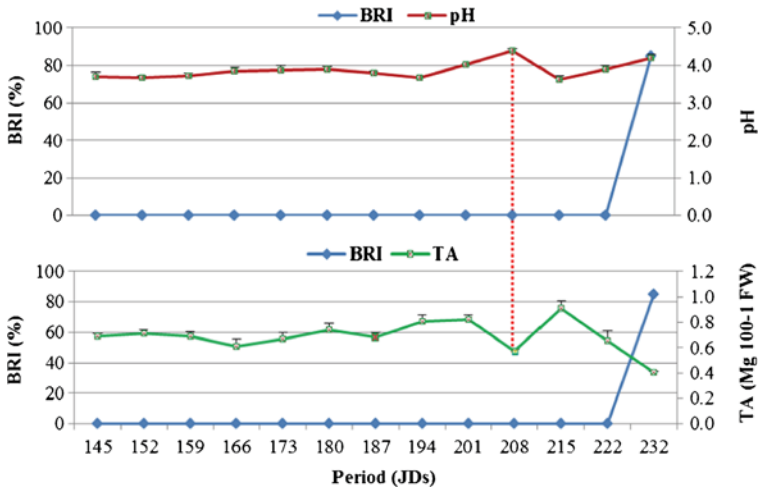

Fig. 5 The effect of pH and titratable acidity (TA) plus standard error (SE) on the susceptibility to brown rot incidence (BRI) of 'Babygold 9' from immature to fruit maturity. Five fruits were inoculated and three fruits evaluated for acidity at weekly basis. The broken line between pH and TA at 208 JDs indicates a clear and unique non-sinusoidal characteristics of acidity in the evolution of peach fruit

growth of *Penicillium oxalicum*. Furthermore, Amiri et al. (2009) found, as most suitable, pH 3.6 to enable selective isolation and enumeration of three *Monilinia* spp. of stone fruits. However n our work, *M. laxa* sporulated best under acidic conditions at pH 5.3, while in the work of Pascual et al. (1997) the fungi sporulated best under a neutral/alkaline range of pH 7–8. Gupta et al. (2010) observed maximum growth and sporulation of *F. solani* at pH 5.5; hence *M. laxa* could be associated with the greater percentage of fungi that grow well in marginally acidic condition. It is equally significant to note here that *M. laxa* has had less attention, notwithstanding the fact that it is as important as *M. fructicola* and *M. fructigena* in the study of epidemiology and management of brown rot (Rungjindamai et al. 2014).

The evolution of pH and TA in the growth and development of peach fruit was a non-sinusoidal waveform (Fig. 3). This is found to corroborate the report of Moing et al. (1998). High pH value gives a corresponding lower content in the TA and vice versa. This was obvious, for example, at 208 JDs (77 BBCH scale) of 'Babygold 9' (Fig. 3) where the highest pH peak corresponded to the lowest dip in TA value. Also this tends to validate the work of Dirlewanger et al. (1999) that TA and pH in peach are negatively correlated, and Lobit et al. (2002) that pH and TA, the most common measure of acidity with perceived sourness or sugariness in peach fruit, correlate inversely.

The non-sinusoidal waveform peak of pH reached by fruits at 208 JDs (77 BBCH scale), which corresponds to 136 and 108 days of complete floration and fruit setting respectively, must have occurred during the cell expansion phase. In peach fruit there is usually a reduction in organic accumulation, which results in fruit with lower acidity and higher pH as was determined in our work at 208 JDs (77 BBCH scale). This tends to support Moing et al. (1998) that such physicochemical activity occurs well before ripening. Stages of peach development are considered to occur in four phases which includes: fruit set, rapid cell division, cell expansion, and ripening/maturation (Tutu and Ciornea 2011).

Hence the most resistant period to pathogen infection in peach fruit is during the stages covering pit hardening to pre-harvest (Keske et al. 2011). It could, therefore, be inferred that immature peaches are very resistant to brown rot because of high levels of acidic pH found in the epidermal cells. This high level of pH could have inhibited brown rot incidence at the immature stages in'Babygold 9'due to acidification activities. Gluconic acid has been reported as the main organic acid associated with the enhancement of peach acidification in host-pathogen interactions (De Cal et al. 2013). *M. laxa* colonized the 'Babygold 9′ at the commercial stage of maturity (232 JDs) (79 BBCH scale) when the acidic pH had reduced, probably aided by local acidification of the host tissue (De Cal et al. 2013).

It is noteworthy, however, that in the inoculation of uninjured 'Babygold 9' with the normal conidia from immature to mature fruit state, the effects of inoculating with mycelia on both injured and uninjured immature fruits were also determined simultaneously. The observations indicate that: at 145 JDs (65 BBCH scale) (pH 3.69 ± 0.13) there was no BRI when fruit was inoculated with 25 μL of 25×10^3 cfu mL^{-1} spores on artificial injury. There was also no BRI in fruits without artificial injury inoculated with 25 μL of 25×10^3 cfu

mL^{-1} spores. There was, however, BRI in the immature fruit with artificial injury inoculated with a 6.5 mm mycelia PDA. In addition, at 222 JDs (78 BBCH scale) (pH 3.89 ± 0.11), within the fruit colour break, there was no BRI in fruits with intact skin (no artificial injury) when inoculated with 25 μL of 25×10^3 cfu mL^{-1} spores. To the contrary, artificially injured fruits showed BRI when inoculated with 25 μL of 25×10^3 cfu mL^{-1} spores.

The significance of these observations is that the degree of susceptibility to infection by *Monilinia* spp. is variable throughout fruit development (Gradziel 1994; Guidarelli et al. 2014). Our findings support this assertion because in our experiment at the early stage of growth and development, immature fruit could not exhibit brown rot symptoms when inoculated with conidia, neither through injury site nor on intact epidermis, but could only develop infection when inoculated with mycelium when injured. Furthermore, at colour-break 222 JDs (78 BBCH scale), peach fruit at pH 3.89 ± 0.11 could develop infection with conidia inoculation only through an injury and on the fruit epidermis. Hence skin injury could be responsible for the incident of brown rot on immature peach fruits observed in orchards (Northover and Biggs 1990; Holb 2004).

Further, FW at maturity (194.04 ± 1.99) was significantly different ($P \leq 0.05$, Duncan test) from the rest of the FW at development. Also the pH (4.19 ± 0.10) and TA (0.41 ± 0.01) values at maturity were all significantly different from those of the development values (Supplementary Table 1). Pearson's correlation shows inverse correlations between pH and all the pathological activities on peach cultivars at harvest. Harvest date (HD) significantly correlated with fruit size (FS) ($r = 0.912$, $P < 0.01$); fruit weight (FW) ($r = 0.889$, $P < 0.01$); and pH ($r = 0.440$, $P < 0.01$). Also FS significantly correlated with FW ($r = 0.980$, $P < 0.01$). There was a significant correlation between pH and FW ($r = 0.356$, $P < 0.05$). As expected, however, pH inversely correlated significantly with TA ($r = -0.604$, $p < 0.01$).

Finally while there was a continuous and stable increase in weight of fruit as it develops, the reverse was the case for fruit size. There was fluctuation in the size as the fruit developed. Fruit size increased, later decreased and finally increased as the fruit developed. The dynamics in pH and TA values also occurred when there were clear changes in fruit size and weight evolution. In addition, the pH 4.19 ± 0.1 at which *M. laxa* could infect the peach in this work relatively correlate with the range of pH (3.5–9.5) in the solid PDA considered to support sporulation *in vivo*. Hence brown rot infection and expression in peach fruit is dependent on the influence of pH and TA and in extension upon the stage of the fruit growth (Emery et al. 2000; Holb 2004; Gell et al. 2009). This study encompasses the necessity to know the development of fruit maturity in new and old varieties in relation to potential *Monilinia* infection in immature fruits.

This study has shown that *M. laxa* exhibited variation in its growth and sporulation capacities on the seven pH amended PDA levels, preferring relatively moderate acidic conditions for optimum performance. We found, in the *in vitro* analysis, that there was mycelia growth at pH 2.40 to 8.84, while pH 11.52 did not support mycelia growth. The pH 5.30 supported the highest sporulation while pH 6.40 encouraged the highest colonization extent or mycelia growth. This supports the findings of Holb (2004) that the most favorable initial hydrogen-ion concentration for mycelial growth occurs between pH 3.5 and 5.5. We found that there was a continuous and stable increase in weight of fruit as it develops, the reverse being the case in fruit size. The fruit size increased, decreased and finally increased as the fruit develops. The pH dynamics exhibited non-sinusoidal waveforms through the growth and development of the fruit. In all these physicochemical variations, *M. laxa* could not develop infection or show any brown rot incidence in the fruit until the period of commercial maturity. On the basis of this study it can be concluded that pH and titratable acidity have great impacts on the growth activity of *M. laxa* in a host-pathogen association both in solid PDA substrate and in peach fruit growth and development and could be important determining elements to be given attention in peach breeding programs.

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Compliance with ethical Standard

Conflict of interest The authors have declared that there is no conflict of interest exists.

Research involving Human Participants and/or Animals This article /manuscript does not contain any studies with human and /or animal subjects performed by any of the authors.

Informed consent This article is as a result of a general study in the evaluation of peach germplasm for tolerance to brown rot by *Monilinia laxa* at the Aula Dei-CSIC peach collection, Zaragoza. I, Vitus Ikechukwu Obi, testify on behalf of all co-authors (Yolanda Gogorcena and Juan Jose Barriuso) that our article.
Title: "Effects of pH and titratable acidity on the growth and development of *Monilinia laxa* (Aderh. & Ruhl.) *in vitro* and *in vivo*",
a) has not been published in whole or in part elsewhere;
b) is not currently being considered for publication in another journal;
c) and that all authors have been personally and actively involved in substantive work leading to the manuscript, and will hold ourselves jointly and individually responsible for its content.

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