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# Evaluation of screening methods and fruit composition in relation to anthracnose fruit rot resistance in blueberries

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Anthracnose fruit rot is an important disease of blueberries, and losses are common in humid growing regions. Most commercial cultivars are susceptible and the disease is usually managed with fungicides. However, a few cultivars are considered resistant. The objectives of this study were to: (i) compare different inoculation techniques for anthracnose fruit rot resistance screening, (ii) screen ripe fruit from a range of blueberry cultivars using selected techniques, and (iii) investigate the role of fruit characteristics in anthracnose fruit rot resistance. The following inoculation methods were evaluated on ripe fruit of a susceptible and resistant cultivar using a conidial suspension: spray, droplet, and injection inoculation of whole fruit; and droplet inoculation of the open surface of cut fruit. All whole-fruit inoculations yielded similar results. Despite the removal of the epidermis, resistance was also expressed in cut fruit but relatively fewer conidia were produced. The cut-fruit assay required substantially less time and half the amount of fruit to accomplish than whole-fruit assays. Detached ripe fruit from 24 cultivars in 2008 and 26 cultivars in 2009 were screened for resistance. Results from the cut-fruit assay correlated best with published resistance ratings. To determine the possible role of fruit characteristics in resistance, fruit pH, titratable acidity, sugar content and firmness were regressed against various fruit rot resistance measures. Fruit rot resistance was positively correlated with sugar content. On defined media, mycelial growth was restricted as sugar concentration increased and pH decreased, suggesting that fruit composition may play a role in the resistance phenotype.

*Keywords*: *Colletotrichum acutatum*, pH, ripe rot, sporulation, sugar content, *Vaccinium corymbosum*

## Introduction

Anthracnose fruit rot, caused by the fungus *Colletotrichum acutatum*, is a major disease of highbush blueberries (*Vaccinium corymbosum*) in humid growing regions. The disease is characterized by sunken areas on infected fruit that become covered by salmon-coloured conidial masses. Infections may occur as early as fruit set and remain latent until fruit ripening (Milholland, 1995). Most commercial cultivars are susceptible, and the disease is usually managed with fungicides (Miles & Schilder, 2008). The use of resistant cultivars with desirable horticultural characteristics would be a cost-effective and environmentally safe way of managing fruit rots. One of the difficulties in breeding for anthracnose fruit rot resistance is the labour and amount of fruit required for sufficient screening, which requires natural infection or artificial inoculation of fruit. Through a screening effort that took more than 10 years to complete, anthracnose fruit rot resistance profiles have been generated for the majority of older blueberry cultivars (Polashock *et al.*, 2005). In that study, the authors artificially inoculated immature fruit on potted plants with multiple strains of *C. acutatum* and rated disease incidence once the fruit ripened. Many of the newer cultivars were not included but were screened for resistance to post-harvest fruit rot in another study that relied on natural field infection rather than inoculation (Hancock *et al.*, 2008).

Fruit rot resistance may be manifested in two ways, namely reduced infection incidence (Polashock *et al.*, 2005) and reduced symptom severity or sporulation (Wharton & Schilder, 2008). Not much is known about the mechanism of anthracnose fruit rot resistance in blueberries. In previous studies, resistance to fruit infection by *C. acutatum* was not correlated with resistance to foliar infection (Ehlenfeldt *et al.*, 2006) or with the production of antimicrobial fruit volatiles (Polashock *et al.*, 2007). Recent studies have shown an active resistance response in ripe fruit of cv. Elliott leading to a restriction of fungal growth in fruit epidermal and subepidermal tissues (Wharton & Schilder, 2008). An accumulation of phenolic compounds (Wharton & Schilder, 2008), an oxidative burst following infection (Miles *et al.*, 2011), and the presence of putative antifungal compounds (Miles *et al.*, 2009) are thought to play a role in anthracnose fruit rot resistance in cv. Elliott.

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In previous studies performed here, cv. Elliott had a lower sugar content and higher pH than the susceptible cultivar Jersey, and it was hypothesized that fruit composition may play a role in resistance, either directly by affecting fungal growth or indirectly by modulating fungal or plant enzyme activity (Miles *et al.*, 2009). Fruit composition, i.e. sugar content, pH and titratable acidity, is an important determinant of flavour and sensory quality of blueberry fruit (Connor *et al.*, 2002; Hancock *et al.*, 2008). Ripe blueberries typically contain between 10% and 14% sugar (48% D-glucose, 49% D-fructose and 3% sucrose) by fresh weight and have a pH of 2·5 to 3·5, depending on the cultivar and study (Mathews *et al.*, 1987; Connor *et al.*, 2002; Hancock *et al.*, 2008). A better understanding of how these traits affect resistance to *C. acutatum* will provide further information about the nature of the resistance response and aid in the development of resistant cultivars.

Although disease resistance is an objective of many blueberry breeding programmes, no genotypic or phenotypic markers are currently available for rapid screening of blueberry breeding lines for anthracnose fruit rot resistance. Resistance screening of plants can only be accomplished when they bear fruit in sufficient quantities for evaluation, usually at 2–3 years of age. Relying on natural field infection is less labour intensive than using artificial inoculation but would require observations over multiple locations and/or years due to variability between sites and growing seasons. Furthermore, it would be difficult to compare cultivars directly due to widely varying fruit maturation dates, chilling requirements, and cold hardiness levels which prevent some cultivars from being grown in certain locations. Unless anthracnose fruit rot resistance is a specific priority of a breeding programme, new cultivars may not be evaluated for anthracnose fruit rot resistance prior to their release.

The infection process of *Colletotrichum* spp. on fruits has been studied in a number of plant pathosystems (Wilson *et al.*, 1990; Prusky *et al.*, 2000; Chillet *et al.*, 2007; Moral *et al.*, 2008). In general, as fruits start to ripen they become increasingly susceptible to infection.

Many physiological changes occur during fruit ripening, such as a reduction in fruit firmness, changes in pH and cell wall composition, and an increase in soluble sugars and secondary metabolites, such as anthocyanins (Sacher, 1973; Brady, 1987). In avocado, several factors have been associated with increased fruit susceptibility to infection by *Colletotrichum gloeosporioides* as fruit ripens, including an increase in fruit pH (Prusky *et al.*, 2000) and a decrease in preformed antimicrobial compounds (Prusky *et al.*, 1983) and pathogenicity factor inhibitors, such as epicatechin (Guetsky *et al.*, 2005). Other studies have shown that ammonia secretion by the fungus increases the pH of host tissues and is important for pathogenicity on almond (Diéguez-Uribeondo *et al.*, 2008), apple (Prusky *et al.*, 2001), avocado (Prusky *et al.*, 2001) and tomato (Prusky *et al.*, 2001; Alkan *et al.*, 2008). Soluble sugars may also play a role in defence responses during ripening. Guava cultivars that contained high levels of soluble sugars and ascorbic acid were also the most resistant to *Glomerella cingulata* (Singh & Sharma, 1981). In grapes, the accumulation of antifungal proteins and sugars during fruit ripening is an important defence mechanism against the fungal pathogens *Botrytis cinerea* and *Guignardia bidwellii* (Tattersall *et al.*, 1997; Salzman *et al.*, 1998).

In blueberries, resistance to anthracnose fruit rot is expressed in green as well as in ripe fruit (Wharton & Schilder, 2008; Miles *et al.*, 2011). It is not known whether the same mechanism governs resistance at both developmental stages. Because immature fruit of all blueberry cultivars is resistant to anthracnose fruit rot unless physically damaged, the focus here is on resistance expressed in ripe fruit. To improve techniques for resistance screening in blueberry breeding and evaluate fruit characteristics as possible phenotypic markers of resistance, a study was conducted with the following objectives: (i) to compare different inoculation techniques for anthracnose fruit rot resistance screening, (ii) to screen ripe fruit from a range of blueberry cultivars using selected techniques, and (iii) to investigate the role of fruit characteristics in anthracnose fruit rot resistance. The overall goal of this project is to facilitate the development of anthracnose fruit rot-resistant blueberry cultivars.

## Materials and methods

### Plant and fungal material

Ripe blueberry fruit was collected from the Michigan Blueberry Growers Association (MBG) variety trial field in Grand Junction, MI, USA on 27 July and 7 August in 2008 and 2009 and stored at 4°C for no more than 3 days prior to testing. Approximately 50% of the fruit of selected cultivars was ripe at the time of harvest. Germplasm types were categorized into five groups: northern highbush (*Vaccinium corymbosum*), southern highbush (adapted *V. corymbosum* with minimal chilling requirement, some crossed with *V. darrowi* and/or *V. ashei*), half high (*V. corymbosum* × *V. angustifolium*), and intermediate (between southern and northern highbush blueberries in chilling requirement with genetic contributions from southern highbush) (Table 1). The cultivars included in this study are commonly grown in Michigan and account for more than 95% of the cultivated blueberries in the state (Kleweno, 2007). A single-conidium isolate of *C. acutatum* isolated from blueberry fruit in Grand Junction, MI, USA in August 2006 was used for inoculations. This isolate (CA001) was the most virulent of 25 isolates in a preliminary test and was used for all experiments. Fungal cultures of *C. acutatum* were grown and stored in accordance with Miles *et al.* (2011). For inoculum production, sporulating cultures were flooded with 3 mL of sterile deionized water (SDW), and conidia were dislodged using a sterilized L-shaped glass rod. Conidia were counted using a haemocytometer and diluted to $1 \times 10^6$ conidia $mL^{-1}$ with SDW.

Table 1 Anthracnose fruit rot resistance profiles of different blueberry cultivars after artificial inoculation with *Colletotrichum acutatum* as measured by infection incidence (proportion of fruit infected), infection severity (the percentage of the fruit surface supporting sporulation), and sporulation capacity (number of conidia produced on the cut surface of a half berry). Ripe fruit was collected from a field planting in Grand Junction, MI, USA in July and August of 2008 and 2009 ($n$ = 10 unless otherwise noted and SE = standard error of the mean)

| Cultivar | Germplasm type | Infection incidence (proportion ± SE) (average of 2008 and 2009) | Infection severity (percentage ± SE) (2009 only)[a] | Sporulation capacity (no. of conidia × $10^6$ ± SE) (average of 2008 and 2009) |
|---|---|---|---|---|
| Aurora | NHB | 0·64 ± 0·05 | 3·9 ± 0·5 | 0·56 ± 0·02 |
| Berkeley | NHB | 0·92 ± 0·03 | 8·8 ± 0·8 | 4·56 ± 0·20 |
| Bluechip | NHB | 0·75 ± 0·13[b] | –[c] | 14·97 ± 3·25 |
| Bluecrop | NHB | 0·98 ± 0·02 | 21·6 ± 2·0 | 12·30 ± 0·90 |
| Bluehaven | NHB | 0·88 ± 0·06 | 7·7 ± 1·1 | 0·51 ± 0·07 |
| Bluejay | NHB | 0·63 ± 0·04 | 14·4 ± 3·4 | 3·12 ± 1·75 |
| Blueray | NHB | 1·00 ± 0·00 | 27·2 ± 3·6 | 10·81 ± 2·75 |
| Bounty | NHB | 0·98 ± 0·02 | 26·9 ± 2·8 | 7·60 ± 1·60 |
| Brigitta | NHB | 0·66 ± 0·03 | 7·5 ± 1·7 | 2·15 ± 0·67 |
| Caroline | NHB | 0·65 ± 0·13[b] | –[c] | 1·90 ± 1·15 |
| Collins | NHB | 0·98 ± 0·01 | 22·7 ± 2·1 | 1·68 ± 0·60 |
| Darrow | NHB | 0·98 ± 0·01 | 15·0 ± 2·4 | 7·62 ± 1·80 |
| Denise | NHB | 0·86 ± 0·06 | 9·6 ± 1·9 | 3·34 ± 0·65 |
| Draper | NHB × SHB | 0·68 ± 0·07[a] | 8·4 ± 2·3 | 1·05 ± 0·80[a] |
| Duke | NHB | 0·70 ± 0·04[a] | 14·9 ± 1·5 | 7·25 ± 0·60[a] |
| Earliblue | NHB | 0·70 ± 0·04 | 36·3 ± 6·4 | 2·87 ± 0·55 |
| Elliott | NHB | 0·47 ± 0·06 | 2·4 ± 0·2 | 0·29 ± 0·08 |
| Jersey | NHB | 0·98 ± 0·02 | 21·6 ± 1·4 | 3·35 ± 0·31 |
| Lateblue | NHB | 0·50 ± 0·06[b] | –[c] | 3·00 ± 0·55 |
| Liberty | NHB | 0·52 ± 0·04 | 4·3 ± 0·8 | 1·00 ± 0·33 |
| Nelson | NHB | 0·70 ± 0·06[b] | –[c] | 14·10 ± 5·00 |
| Northblue | HH | 0·70 ± 0·07 | 9·9 ± 0·9 | 8·04 ± 0·10 |
| Northland | NHB | 0·81 ± 0·05 | 8·9 ± 0·7 | 0·98 ± 0·48 |
| Rose | NHB | 0·57 ± 0·08 | 6·1 ± 1·3 | 4·13 ± 1·10 |
| Rubel | NHB | 0·98 ± 0·02 | 14·3 ± 1·3 | 2·07 ± 0·80 |
| Toro | NHB | 0·81 ± 0·04 | 5·4 ± 0·9 | 5·62 ± 2·15 |

Abbreviations for germplasm type as previously defined in Materials and methods. NHB, northern highbush; SHB, southern highbush; HH, half high (*V. corymbosum* × *V. angustifolium*); NHB × SHB, intermediate.
[a]Data were collected only in 2009 and therefore $n$ = 5.
[b]Data were collected only in 2008 and therefore $n$ = 5.
[c]No data collected.

## Inoculation methods

Ripe fruit of cvs. Elliott (resistant) and Jersey (susceptible) was inoculated with a conidial suspension ($10^6$ conidia $mL^{-1}$) using four different methods: (i) applying a 10 $\mu$L droplet into the calyx cup, (ii) spraying the berries until runoff, (iii) injecting 50 $\mu$L with a syringe into the interior of the fruit, and (iv) applying a 50 $\mu$L droplet to the open surface of a cut fruit. For the cut-fruit assay, berries were cut in half longitudinally with a sterile scalpel and placed cut side up. There were five replicates per cultivar with five fruits per replicate. Fruit in all treatments was incubated on wire mesh screens at 22–24°C and 100% humidity for 10 days post-inoculation (dpi), except cut fruit which was incubated for 3 dpi. After incubation, conidium production was quantified by placing five inoculated fruit in 5 mL of sterile water and gently inverting the tube for 5 min. Conidium concentration per mL was determined using a haemocytometer as the average of three separate counts (Table 1).

To further evaluate whole- and cut-fruit inoculation methods, a range of blueberry cultivars (24 in 2008 and 26 in 2009) was screened for anthracnose fruit rot resistance. Ripe whole fruit was spray inoculated as described above followed by evaluation of infection incidence (proportion of fruit infected) and severity (percentage of the fruit surface supporting sporulation) after 10 days. Cut fruit was drop inoculated and sporulation was measured with a haemocytometer as the number of conidia produced per fruit half.

To determine the utility of spectrophotometry for measuring sporulation, conidia were harvested from a 4-day-old microconidiating culture of *C. acutatum*. A dilution series from $1{\cdot}0 \times 10^5$ to $1{\cdot}0 \times 10^7$ conidia $mL^{-1}$ was used to develop a standard curve for conidium concentration based on optical density of the conidial suspension. The absorbance was read with an EL 800 Universal Microplate Reader (BioTek Instruments) at a wavelength of 590 nm. The actual conidium concentration of each sample was determined microscopically with a haemocytometer. All measurements were made on triplicate subsamples. The same procedures were used to quantify the number of conidia produced on cut fruit surfaces of seven

blueberry cultivars (Bluecrop, Blueray, Elliott, Jersey, Liberty, Nelson and Rubel) in 2008. In addition, fruit from an $F_1$ population resulting from a cross of cvs. Draper (resistant) and Jewel (susceptible) was used to validate the spectrophotometric procedure. Plants were grown in Interlachen, FL, USA and fruit was collected in April and May 2011 when approximately 50% of the fruit on a bush was ripe. Fruit was cooled immediately, shipped within 3 days after harvest, and inoculated and evaluated as described above.

### Assessment of fruit characteristics

Ripe fruit of different blueberry cultivars was harvested at appropriate times from early July to late August over 4 years (2005 to 2008) from the MBG variety trial in Grand Junction, MI, USA. Sugar content, pH and titratable acidity (TA) were measured in juice extracted from 25 berries per cultivar (five replicates of five berries) that were blended at high speed in a tissue homogenizer (Ultra Turrax T25; Janke and Kunkel Co.). Sugar content was determined using a handheld refractometer (Westover model RHB-32; Southwest United Industries). Results are reported as percentage sugar content (wt/wt) on a fresh-weight basis. TA was determined using 10 mL of juice diluted to 100 mL with SDW, titrated with 0·1 M sodium hydroxide to pH 8·2, and expressed as percentage citric acid (wt/wt) on a fresh-weight basis. Fruit firmness was determined on a sample of 50 fruit per cultivar per year using a portable firmness measuring instrument (Timm *et al.*, 1996). Data were reported as force (newtons $mm^{-2}$) required to deform the surface of the fruit. These results represent an expansive data set including more cultivars and years of data collection than previously reported by Hancock *et al.* (2008).

### Effect of sugar concentration on mycelial growth *in vitro*

To determine the effect of sugar concentration on fungal growth, a minimal medium was used as described by Allen & Kuc (1968), consisting of 0·7% $KH_2PO_4$, 0·4% $KNO_3$, 0·3% $Na_2HPO_4$, 0·1% $MgSO_4$, 0·03% $CaCl_2$, 1·5% BACTO-agar and varying concentrations of sugar (ranging from 4 to 20%). The sugar used in the media was 100% D-glucose, 100% D-fructose, or a 50/50 mixture of both sugars. The medium was adjusted to a final pH of 6·5 and autoclaved at 121°C and 100 kPA for 30 min. Hyphal plugs (~5·5 mm in diameter) were transferred from a 7-day-old potato dextrose agar (PDA) culture of *C. acutatum* to Petri plates containing the defined minimal medium. There were three replicate plates per treatment. Plates were incubated without Parafilm for 7 days in the dark at 25°C. After incubation, the diameter of each colony was measured in two perpendicular directions, and the average diameter was calculated after subtracting the diameter of the initial hyphal plug. The experiment was conducted three times.

### Effects of pH and sugar concentration on mycelial growth in liquid media

To determine the effects of pH and the interaction of pH and sugar concentration on mycelial growth, liquid media were used, namely potato dextrose broth containing 0·4% potato starch and 2% dextrose, and the minimal medium described above containing 4% D-glucose and 4% D-fructose without the addition of agar. Both media were adjusted to a final pH of 2·0, 2·5, 3·0, 3·5, 4·0, 5·0 and 6·5 using dilute hydrochloric acid as measured with an Accumet AB15 pH meter (Thermo Fisher Scientific). The media were sterilized with a 0·45 $\mu$ syringe filter (Millipore) and placed in 2·95 mL aliquots in 14 mL polypropylene Falcon tubes (Becton Dickinson Labware). A 50 $\mu$L conidial suspension ($10^6$ conidia $mL^{-1}$) from a 3- to 7-day-old culture was added to each tube and cultures were shaken at 300 rpm at 22–24°C. After 7 days, the mycelial suspensions were filtered through pre-weighed, 9 cm diameter Whatman filter paper disks in a Büchner funnel by applying a vacuum. Filter disks were allowed to air dry for 4 days and the dried mycelium was weighed. To study the interaction of pH and sugar concentration, the minimal medium as described above was used with sugar concentrations of 8, 10, 12, 14% and 16% (50/50 D-glucose/D-fructose) at three pH levels (3·0, 4·0 and 5·5). Each treatment was replicated three times and the experiments were conducted twice.

### Statistical analyses

All statistical analyses were performed with the StatGraphics statistical computer program (StatPoint Inc.) and SigmaPlot version 11 (SYSTAT Software). For the different inoculation techniques, the data were statistically analysed using a paired Student's *t*-test ($\alpha$ = 0·05) after checking for normality and equality of variance. For the cultivar resistance screening experiments, statistical differences were determined by one-way ANOVA and Fisher's Protected LSD test ($P$ = 0·05). As the factor 'year' had no significant effect, the data from 2008 and 2009 were pooled to calculate means and standard errors. For fruit composition analysis, all experiments were analysed by ANOVA according to a completely randomized design. Previously reported resistance ratings using proportion decayed fruit from Polashock *et al.* (2005) were used for regression analyses. Linear and power (log-log) regressions were run using SigmaPlot for obtaining $P$ and $r$ values. For investigating the combined effect of pH and sugar, a two-factor ANOVA was used to analyse main and interaction effects of sugar content and pH ($\alpha$ = 0·05).

## Results

### Effect of inoculation method on resistance phenotype

The three whole-fruit inoculation methods (droplet, spray, and injection) resulted in similar infection phenotypes with abundant sporulation on the fruit surface

10 days after inoculation (Fig. 1). In each method, cv. Jersey fruit yielded similar numbers of conidia ($23{\cdot}2 \times 10^6$ to $26{\cdot}5 \times 10^6$ conidia $mL^{-1}$), whereas conidium production was much lower in cv. Elliott ($2{\cdot}8 \times 10^6$ to $4{\cdot}0 \times 10^6$ conidia $mL^{-1}$) (Fig. 2). The variance of the data was highest in the injection and lowest in the spray inoculation procedure.

In the cut-fruit method, fungal sporulation was evident on the cut fruit surface of both cultivars after 3 days. However, cv. Jersey showed more darkening and maceration of the internal fruit tissues than cv. Elliott (Fig. 1d,i). No significant necrosis or sporulation was observed in the water control of either cultivar (Fig. 1e,j). Overall, the cut-fruit method yielded about 10-fold fewer conidia per berry than the whole-fruit inoculation methods, but the difference between the cultivars was proportionally the same. However, if the cut fruit was incubated longer than 3 days, significant background infection became apparent, because these were field-collected berries (data not shown).

A linear regression yielded the best fit for a standard curve of conidium concentration against optical density of an aqueous suspension of *C. acutatum* conidia from culture ($R^2 > 0{\cdot}99$) (Fig. 3a). Conidia harvested from inoculated cut fruit surfaces from the Draper × Jewel population in 2011 also showed a strong positive linear correlation with optical density ($R^2 = 0{\cdot}90$), although there was an occasional outlier (Fig. 3b). A linear function also described the standard curve for conidia from cut fruit surfaces of selected blueberry cultivars in 2008. However the relationship was not as strong as for the previous curves ($R^2 = 0{\cdot}81$) (Fig. 3c).

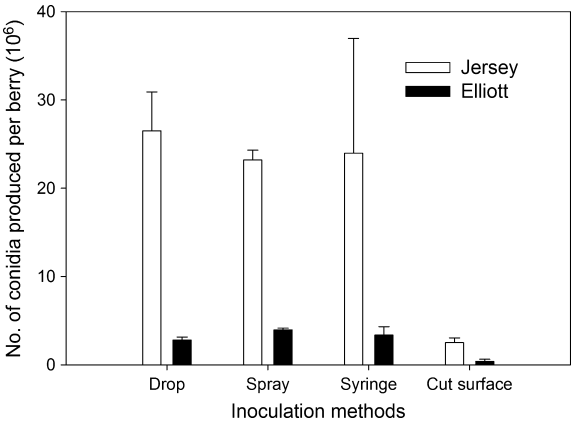


Figure 2 Conidium production on fruit of blueberry cultivars Jersey and Elliott after inoculation with a *Colletotrichum acutatum* conidial suspension using different inoculation methods: applying a 10 μL droplet into the calyx cup, spraying the berries until runoff, injecting 50 μL into the interior of the fruit with a syringe, and applying a 50 μL droplet to the open surface of a cut fruit. All treatments were incubated for 10 days after inoculation, except the cut-fruit treatment (3 days). Bars denote the standard error of the mean ($n$ = 5 with 10 fruits per replicate).

## Correlation of fruit resistance results with previously published resistance ratings

The results of screening different blueberry cultivars for anthracnose fruit rot resistance in the whole- and cut-fruit assays were consistent from 2008 to 2009, as there were no significant effects of year or cultivar × year. *P*-values were 0·32 (whole fruit) and 0·51 (cut fruit) for the effect of year, and 0·13 (whole fruit) and 0·21 (cut fruit) for cultivar × year. Data were therefore reported as an average of the 2 years (Table 1), except for infection severity which was only rated in 2009. Water controls in the whole-fruit inoculations showed evidence of background infection, primarily in the susceptible cultivars.

Of the resistance measures evaluated, sporulation capacity in the cut-fruit assay was most strongly

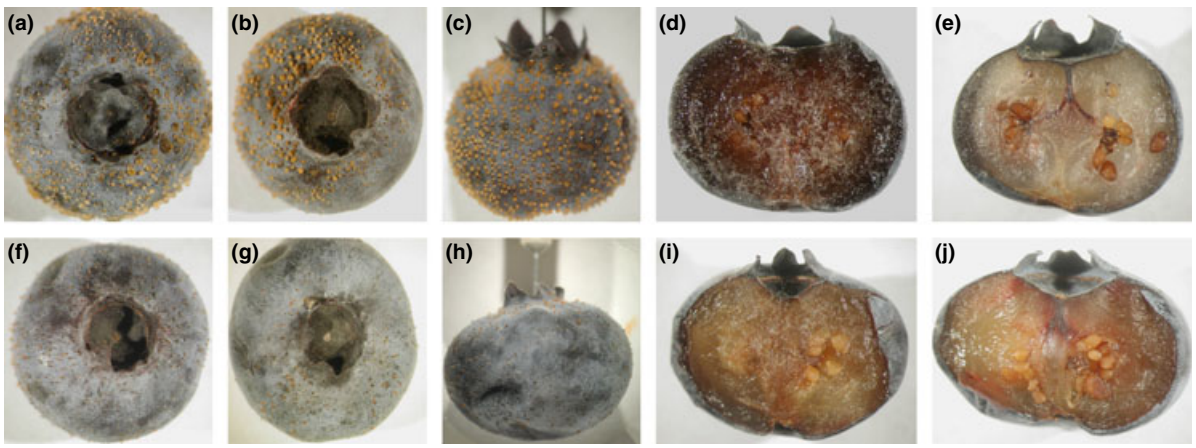


Figure 1 Signs and symptoms of fruit infection by *Colletotrichum acutatum* on two different blueberry cultivars, Jersey (a–e), and Elliott (f–j) after inoculation with a conidial suspension using different techniques: applying a 10 μL droplet into the calyx cup (a,f), spraying the berries until runoff (b,g), injecting 50 μL into the interior of the fruit with a syringe (needle still visible) (c,h), and applying a 50 μL droplet to the open surface of a cut fruit (d,i). Fifty microlitres of sterile deionized water served as a control in the cut fruit experiments (e,j). All treatments were incubated for 10 days after inoculation, except the cut-fruit treatments (3 days).

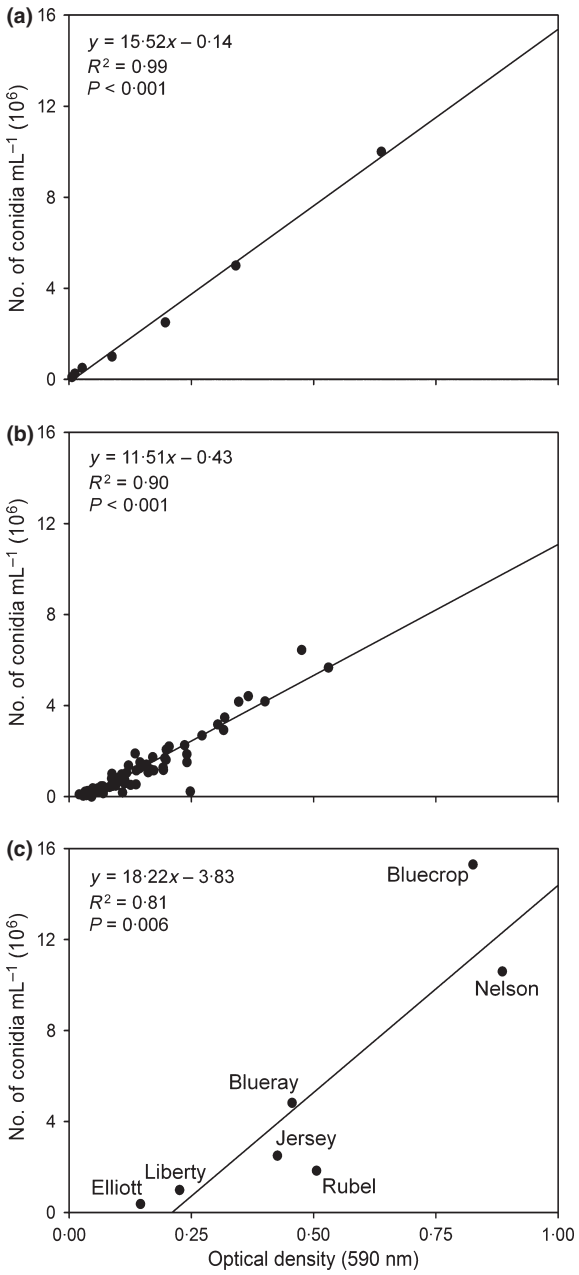


Figure 3 Relationship between the concentration of an aqueous suspension of *Colletotrichum acutatum* conidia and optical density at 590 nm. (a) Conidia produced on PDA, (b) conidia from cut fruit surfaces from a Draper × Jewel F1 population, (c) conidia produced on cut fruit surfaces of selected blueberry cultivars.

correlated with cultivar resistance ratings published by Polashock *et al.* (2005) ($r = 0{\cdot}86$; $P < 0{\cdot}01$). Infection incidence in the whole-fruit assay was positively correlated with infection severity on whole fruit ($r = 0{\cdot}69$, $P < 0{\cdot}01$) and sporulation capacity on cut fruit ($r = 0{\cdot}40$, $P = 0{\cdot}04$) but only weakly correlated with resistance ratings by Polashock *et al.* (2005) ($r = 0{\cdot}33$, $P = 0{\cdot}21$). Infection severity on whole fruit was also correlated with sporulation capacity on cut fruit ($r = 0{\cdot}63$, $P = 0{\cdot}002$) and resistance ratings by Polashock *et al.* (2005) ($r = 0{\cdot}64$, $P = 0{\cdot}01$) (Table 2).

### Correlation of anthracnose resistance with certain fruit characteristics

Average weight per berry ranged from 1·10 to 3·07 g, sugar content from 10·3 to 13·9%, pH from 2·53 to 3·28, titratable acidity from 0·4 to 1·2%, and fruit firmness from 262 to 519 newtons $mm^{-2}$ (Table 3). Anthracnose fruit rot resistance as measured by sporulation capacity on cut fruit, infection severity on whole fruit, and proportion decayed from Polashock *et al.* (2005) was negatively correlated in a linear fashion with percentage soluble sugar in the fruit ($r = -0{\cdot}53$, $P = 0{\cdot}02$; $r = -0{\cdot}44$, $P = 0{\cdot}09$; $r = -0{\cdot}62$, $P < 0{\cdot}01$, respectively), i.e. the higher the sugar content, the more resistant the fruit. The correlation between infection incidence on whole fruit and sugar content showed the same trend but was not significant ($r = -0{\cdot}24$, $P = 0{\cdot}34$). Additionally, infection severity on whole fruit showed marginally significant negative correlations with berry weight ($r = -0{\cdot}44$, $P = 0{\cdot}09$) and titratable acidity ($r = -0{\cdot}45$, $P = 0{\cdot}08$) (Table 4). Correlations with fruit firmness were not significant (Table 4).

### Mycelial growth at high sugar content and low pH

Colonies of *C. acutatum* grew faster on solid media with D-glucose than with D-fructose (Fig. 4d). Growth on the medium with a 50/50 mixture of D-glucose and D-fructose was more similar to growth with D-glucose than with D-fructose. However, mycelial growth was reduced to a similar extent as concentrations of both sugars increased. Average colony diameter was reduced by 37% and 35%, respectively, as D-glucose and D-fructose concentrations increased from 4% to 20%. On the medium with a 50/50 mixture of D-glucose and D-fructose, the largest reduction in growth occurred between 12% (Fig. 4b) and 16% (Fig. 4c) sugar content, reducing average colony diameter by about 1 cm (Fig. 4d). In a more detailed experiment using a 50/50 mixture of D-glucose and D-fructose within the physiological range of blueberries, the biggest reduction in mycelial growth was seen between 11% and 13% sugar content (Fig. 4e).

When investigating the effect of initial medium pH on mycelial growth of *C. acutatum* in liquid media, a similar pattern was observed on both media with slightly more growth in potato dextrose broth than in the minimal medium. In the latter, mycelial growth was significantly reduced below an initial pH of 4·0 and no growth had occurred at an initial pH of 2·5 after 7 days of incubation. In potato dextrose broth, a similar reduction was observed but in this case, no growth occurred at a pH of 2·0. An initial pH of 4·0 appeared optimal for mycelial growth (Fig. 5a). If the fungus grew, the pH was modified in the culture to 5·6 to 6·5 by day 7 depending on the initial pH (data not shown).

Table 2 Pearson correlation coefficients (*r*) and statistical significance (*P*) for regressions between different measures of anthracnose fruit rot resistance after artificial inoculation of a range of blueberry cultivars with *Colletotrichum acutatum*. Infection incidence (proportion of fruit infected), infection severity (the percentage of the fruit surface supporting sporulation), sporulation capacity (number of conidia produced on the cut surface of a half berry) and previously published resistance ratings (Polashock *et al*., 2005) were subjected to linear regression. All values were log transformed prior to regression

| Dependent variable (Values from Table 1) | Independent variable | Number of cultivars in common | *r* Value | *P* value |
|---|---|---|---|---|
| Infection incidence | Infection severity | 22 | **0·69** | <0·001 |
| | Sporulation capacity | 26 | **0·40** | 0·044 |
| | Proportion decayed (Polashock *et al*., 2005) | 18 | 0·31 | 0·208 |
| Infection severity | Sporulation capacity | 22 | **0·63** | 0·002 |
| | Proportion decayed (Polashock *et al*., 2005) | 15 | **0·64** | 0·010 |
| Sporulation capacity | Proportion decayed (Polashock *et al*., 2005) | 18 | **0·86** | <0·001 |

*r*-values in bold indicate statistically significant correlations at $\alpha \leq 0{\cdot}05$.

The combined effect of initial pH and sugar content in the physiological range of blueberries showed that the fungus was able to grow under all conditions, but mycelial growth was most curtailed by high sugar content combined with a low pH (Fig. 5b). Unfortunately, pH levels lower than three could not be tested due to the fungus' inability to grow at low pH in the minimal medium, but by extrapolation, mycelial growth would have been even more reduced and in some cases virtually absent at the more acidic pH levels typical of blueberries, i.e. 2·5 to 3·2

Table 3 Characteristics of fruit of different blueberry cultivars collected from a field planting in Grand Junction, MI, USA from 2005 to 2008. Values shown are averages and standard errors over 4 years

| Cultivar | Germplasm type | Years tested[a] | Berry weight (g) | Sugar content (%) | pH | Titratable acidity (%) | Firmness (N mm$^{-2}$)[b] |
|---|---|---|---|---|---|---|---|
| Aurora | NHB | 05, 06, 07, 08 | 2·0 ± 0·2 | 13·4 ± 0·5 | 2·8 ± 0·1 | 1·1 ± 0·0[c] | 415·6 ± 85·9 |
| Berkeley | NHB | 07 only | 2·0 | 12·3 | 2·8 | 0·6 | 227·7 |
| Bluecrop | NHB | 05, 06, 07, 08 | 2·0 ± 0·1 | 11·2 ± 0·3 | 2·8 ± 0·1 | 0·9 ± 0·1 | 354·6 ± 67·9 |
| Bluegold | NHB | 05, 06, 07, 08 | 1·9 ± 0·2 | 12·6 ± 0·3 | 2·8 ± 0·1 | 1·0 ± 0·1 | 342·7 ± 66·1 |
| Bluehaven | NHB | 06, 07 | 1·9 ± 0·3 | 11·8 ± 0·2 | 2·7 ± 0·0[c] | 0·8 ± 0·0[c] | 146·2 ± 95·5 |
| Bluejay | NHB | 05, 06, 07, 08 | 1·8 ± 0·1 | 12·5 ± 0·3 | 3·0 ± 0·1 | 0·7 ± 0·0[c] | 299·6 ± 54·5 |
| Blueray | NHB | 05, 06, 08 | 2·3 ± 0·1 | 10·5 ± 0·8 | 3·0 ± 0·1 | 0·7 ± 0·1 | 365·8 ± 89·3 |
| Bluetta | NHB | 05, 06, 08 | 1·4 ± 0·1 | 10·4 ± 0·2 | 2·8 ± 0·1 | 0·8 ± 0·2 | 396·8 ± 99·4 |
| Brigitta | NHB | 05, 06, 07, 08 | 2·6 ± 0·3 | 12·1 ± 0·6 | 2·8 ± 0·1 | 0·9 ± 0·0[c] | 463·4 ± 90·1 |
| Chanticleer | NHB | 06, 07, 08 | 3·1 ± 0·2 | 10·5 ± 0·5 | 2·8 ± 0·1 | 1·1 ± 0·1 | 276·4 ± 79·9 |
| Darrow | NHB | 05, 06 | 2·6 ± 0·1 | 12·2 ± 0·9 | 2·6 ± 0·0[c] | 1·2 ± 0·0[c] | 519·2 ± 21·.7 |
| Draper | NHB × SHB | 05, 06, 07, 08 | 2·2 ± 0·2 | 12·2 ± 0·9 | 2·8 ± 0·1 | 1·1 ± 0·1 | 519·2 ± 124·6 |
| Duke | NHB | 05, 06, 07, 08 | 1·9 ± 0·0[c] | 10·3 ± 0·2 | 3·0 ± 0·1 | 0·7 ± 0·1 | 572·5 ± 148·6 |
| Earliblue | NHB | 05, 06, 07 | 1·2 ± 0·0[c] | 11·1 ± 0·7 | 2·9 ± 0·0[c] | 0·7 ± 0·1 | 361·7 ± 80·9 |
| Elliott | NHB | 05, 06, 07, 08 | 1·8 ± 0·2 | 12·9 ± 0·9 | 2·8 ± 0·1 | 1·1 ± 0·1 | 341·8 ± 69·7 |
| Jersey | NHB | 05, 06, 07, 08 | 1·6 ± 0·2 | 13·4 ± 0·4 | 3·2 ± 0·2 | 0·5 ± 0·0[c] | 319·9 ± 64·3 |
| Jewel | SHB | 08 only | 2·5 | 11·5 | 2·6 | 0·7 | 211·1 |
| Lateblue | NHB | 06, 07, 08 | 2·1 ± 0·1 | 12·9 ± 0·5 | 2·8 ± 0·1 | 0·9 ± 0·1 | 167·1 ± 54·6 |
| Legacy | NHB × SHB | 05, 06, 07, 08 | 2·2 ± 0·1 | 13·2 ± 0·6 | 3·1 ± 0·1 | 0·6 ± 0·1 | 373·2 ± 75·1 |
| Liberty | NHB | 05, 06, 07, 08 | 2·4 ± 0·3 | 13·3 ± 0·7 | 2·9 ± 0·1 | 0·9 ± 0·0[c] | 518·8 ± 114·6 |
| Nelson | NHB | 05, 06, 07, 08 | 2·1 ± 0·3 | 12·2 ± 0·2 | 2·8 ± 0·1 | 1·0 ± 0·0[c] | 318·8 ± 53·4 |
| O'Neal | SHB | 06, 08 | 1·7 ± 0·1 | 12·5 ± 0·2 | 3·28 ± 0·4 | 0·5 ± 0·0[c] | 121·2 ± 89·8 |
| Ozark Blue | NHB × SHB | 05, 06, 07, 08 | 2·2 ± 0·1 | 11·3 ± 0·3 | 2·9 ± 0·1 | 0·8 ± 0·1 | 354·0 ± 66·5 |
| Patriot | NHB | 07 only | 2·2 | 11·9 | 2·5 | 1·0 | 310·3 |
| Rubel | NHB | 05, 06, 07 | 1·1 ± 0·1 | 13·9 ± 0·3 | 2·9 ± 0·1 | 0·9 ± 0·1 | 424·9 ± 110·2 |
| Sapphire | SHB | 08 only | 1·8 | 13·1 | 3·0 | 0·4 | 262·0 |
| Spartan | NHB | 05, 06, 07, 08 | 2·3 ± 0·1 | 11·4 ± 0·1 | 3·0 ± 0·1 | 0·6 ± 0·0[c] | 396·2 ± 75·4 |
| Star | SHB | 06, 08 | 1·5 ± 0·2 | 10·8 ± 0·4 | 3·1 ± 0·3 | 0·6 ± 0·1 | 161·2 ± 125·5 |
| Toro | NHB | 05, 06, 07, 08 | 2·6 ± 0·0[c] | 11·1 ± 0·3 | 2·8 ± 0·1 | 0·9 ± 0·0[c] | 374·0 ± 73·2 |

Abbreviations for germplasm type as previously defined. NHB, northern highbush; SHB, southern highbush; HH, half high (*V. corymbosum* × *V. angustifolium*); NHB × SHB, intermediate.

[a]To calculate means, five replicates of five berries (25 in total) were used in each year of study. The values displayed in the table are the averages of the yearly means.

[b]Force required to indent blueberry fruit.

[c]Indicates standard error values <0·05.

Table 4 Pearson correlation coefficients (*r*) for regressions of various measures of anthracnose fruit rot resistance after artificial inoculation of blueberry fruit with *Colletotrichum acutatum* (Table 1) against fruit characteristics (Table 3). Infection incidence (proportion of fruit infected), infection severity (percentage of the fruit surface supporting sporulation), sporulation capacity (number of conidia produced on the cut surface of a half berry) and previously published resistance ratings (Polashock *et al.*, 2005) were subjected to linear regression against fruit variables

| | Independent variable | | | | |
|---|---|---|---|---|---|
| Dependent variable | Berry weight (g) | Sugar content (%) | pH | Titratable acidity (%) | Firmness (N mm$^{-2}$)[a] |
| Infection incidence | −0·11 | −0·24 | 0·16 | 0·28 | 0·06 |
| Infection severity | **−0·44**[b] | **−0·44**[b] | 0·42 | **−0·45**[b] | −0·07 |
| Sporulation capacity | 0·17 | **−0·53**[c] | 0·10 | −0·03 | 0·03 |
| Proportion decayed (Polashock *et al.*, 2005) | 0·29 | **−0·62**[c] | −0·30 | 0·26 | 0·18 |

Statistically significant *r*-values are indicated in bold.
[a]Force required to indent fruit surface.
[b]Indicates statistical significance at $P \leq 0{\cdot}10$.
[c]Indicates statistical significance at $P \leq 0{\cdot}05$.

(Table 3). At the typical sugar content of ripe blueberries (10·4% to 13·9%; Table 3) moderate growth suppression would be expected.

## Discussion

This study represents a broad approach to screening blueberry cultivars for anthracnose fruit rot resistance, comparing and improving screening methods, and correlating resistance ratings to a previous study. When the susceptible cultivar Jersey and the resistant cultivar Elliott were inoculated, the quantity of conidia produced was similar in fruit inoculated by syringe injection compared to droplet and spray inoculation. There was also a difference between the cultivars of similar proportion by drop-inoculating the open surface of cut fruit. This suggests that anthracnose fruit rot resistance in cv. Elliott fruit is expressed not only in the skin but also in the flesh of the blueberry. While *Colletotrichum* studies generally do not involve inoculation of wounded or cut fruit because wounding is not required for infection, similar resistance levels were found in five olive cultivars whether the fruit was wounded or not prior to inoculation (Moral *et al.*, 2008). As the majority of soluble phenolic compounds in blueberries are found in the epidermal and subepidermal layers of the fruit peel, e.g. anthocyanins and flavonol glycosides (Robards *et al.*, 1999), mechanisms other than the presence of soluble phenolic substances may play a role in the resistance response.

Under natural conditions, infection usually takes place on green fruit followed by a latent period until the fruit ripens, at which time the infection progresses until most of the fruit is covered with acervuli (Miles & Schilder, 2008). Infection incidence tends to increase rapidly during fruit ripening, with later harvests having poorer fruit quality (Miles & Schilder, 2008). Due to the labour and space involved in inoculating green fruit on plants in the field or greenhouse, this study chose to inoculate detached ripe fruit collected from field-grown plants. Three different disease evaluations were compared, including disease incidence and severity on whole fruit and sporulation capacity on cut fruit. The whole-fruit inoculations did not seem to correlate well with the other data sets, which is likely due to background infections as indicated by the water controls. It is difficult to avoid background infection in the field unless plants are grown in dry regions or protected from precipitation. Therefore, using whole ripe fruit from field-grown plants may not provide accurate resistance ratings. Furthermore, due to the variation in flowering and ripening times of different cultivars, it is possible that some escape infection or, conversely, are exposed to higher than average doses of inoculum. While an escape of infection due to timing of flowering or fruit ripening may lead to 'field resistance', this would not be a reliable indicator of cultivar performance across regions.

The cut-fruit inoculation assay for blueberries showed the strongest correlation with previously reported anthracnose fruit rot resistance ratings by Polashock *et al.* (2005) in New Jersey and appears to be an efficient method for fruit rot resistance screening. Furthermore, due to the relatively short incubation period, interference from background infections is mostly eliminated as acervuli from natural infections on the fruit epidermis are not able develop to any significant extent during the 3-day incubation period. As local pathogen strains were used in both studies, the strong correlation suggests that strain variation is not a big issue, and that a cultivar that is resistant in one region can also be expected to be resistant in another region. It should be pointed out that Polashock *et al.* (2005) rated disease incidence (proportion of fruit decayed) whereas this cut-fruit assay measured the quantity of spores produced per half berry, which could be interpreted as a measure of disease severity. Disease incidence and severity are often correlated but not necessarily congruent. In addition to a direct relationship between inoculum abundance and measures of disease incidence and severity, sporulation and fruit softening must be sufficiently severe to be visible to the naked eye against the dark background of a blueberry fruit, thereby affecting visual incidence ratings.

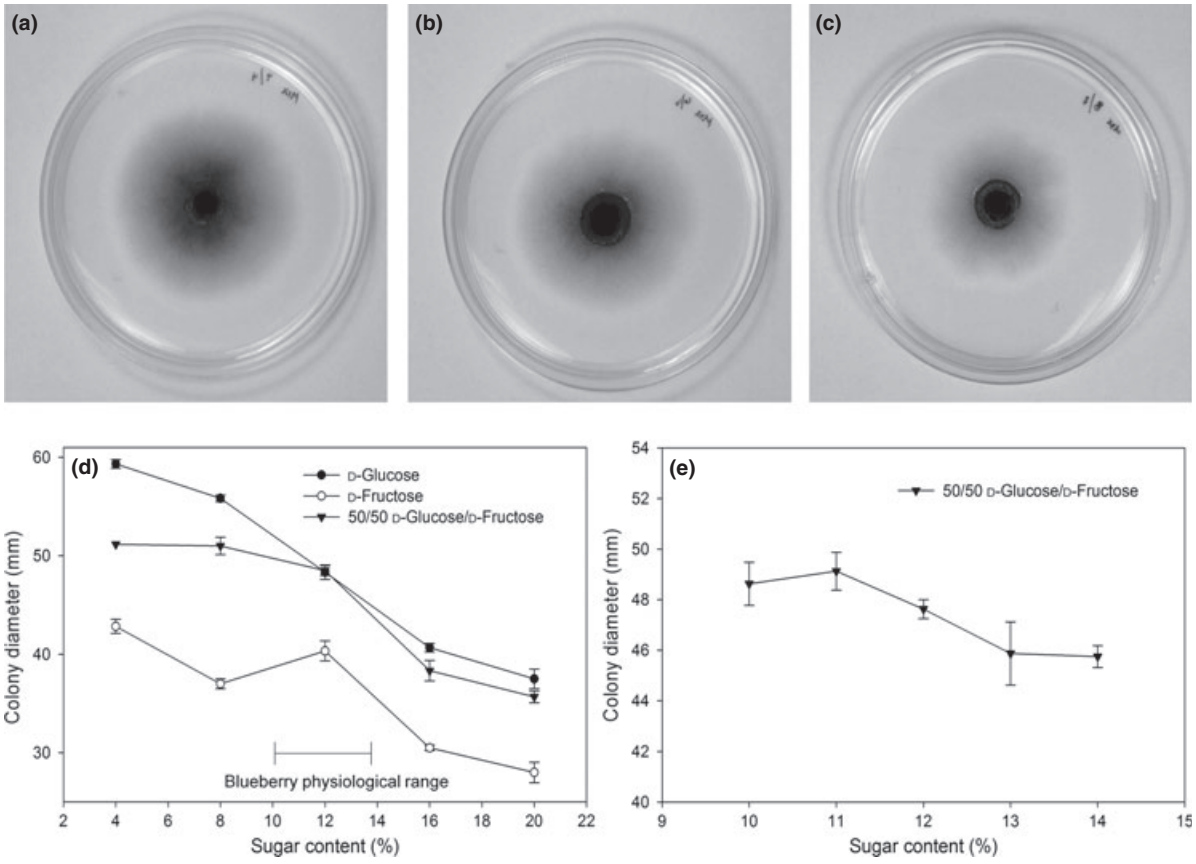


Figure 4 Mycelial growth of *Colletotrichum acutatum* on minimal medium with different sugar (50/50 D-glucose/D-fructose) concentrations: 8% (w/v) (a), 12% (w/v) (b), and 16% (w/v) (c) after 7 days. (d) The effect of sugar content in minimal medium on mycelial growth of *C. acutatum* after 7 days using D-glucose, D-fructose and a 50/50 D-glucose/D-fructose mixture. (e) The effect of sugar content in minimal medium on mycelial growth of *C. acutatum* after 7 days using a 50/50 D-glucose/D-fructose mixture in the physiological range for blueberries. In (d) and (e), error bars denote standard error of the mean.

The cut-fruit assay is an improvement over conventional anthracnose fruit rot resistance screening assays, in that testing can be done on younger bushes that produce small amounts of fruit, facilitating screening earlier in the breeding process. This way, moderately to highly susceptible genotypes can be excluded before much effort has been expended in their selection and maintenance. It does not preclude conducting other screening assays at a later stage. Because microscopic quantification of conidia requires training and is somewhat tedious, a possible improvement is the use of spectrophotometric methods in quantification of sporulation. A linear correlation was found between optical density and conidium concentration in several experiments, with the strongest correlation observed for conidia harvested from culture. Because pigments and debris released from injured blueberry fruit may affect optical density readings, it is advisable to develop a standard curve for cut fruit from representative cultivars prior to conducting a large-scale screening assay. While use of optical density for quantifying conidia from cut fruit may be slightly less accurate, it is more time efficient and requires less training than microscopic observation.

A positive linear correlation between fruit sugar content and anthracnose fruit rot resistance suggests that soluble sugars may play a role in the resistance response. However, it is most likely an additive effect as indicated by the relatively low *r* values and the fact that some moderately susceptible cultivars had fairly high soluble sugar concentrations.

Diseases have been classified as high- and low-sugar diseases (Horsfall & Dimond, 1957; Vanderplank, 1984). Low-sugar diseases are characterized by heightened resistance to pathogens when tissues contain more sugar, a concept termed 'high-sugar resistance' (Horsfall & Dimond, 1957; Vanderplank, 1984). Soluble sugars have been found to repress photosynthetic genes as well as induce a number of defence-related genes and this shift in expression could contribute to the overall resistance response (Herbers *et al.*, 1996). It was found that high sugar concentrations in artificial media had a negative impact on hyphal growth of *C. acutatum,* presumably by increasing osmotic stress. This reduction was more pronounced with D-glucose than with D-fructose. The results suggest that internal sugar content in fruit may play a role in slowing the growth of *C. acutatum* during the

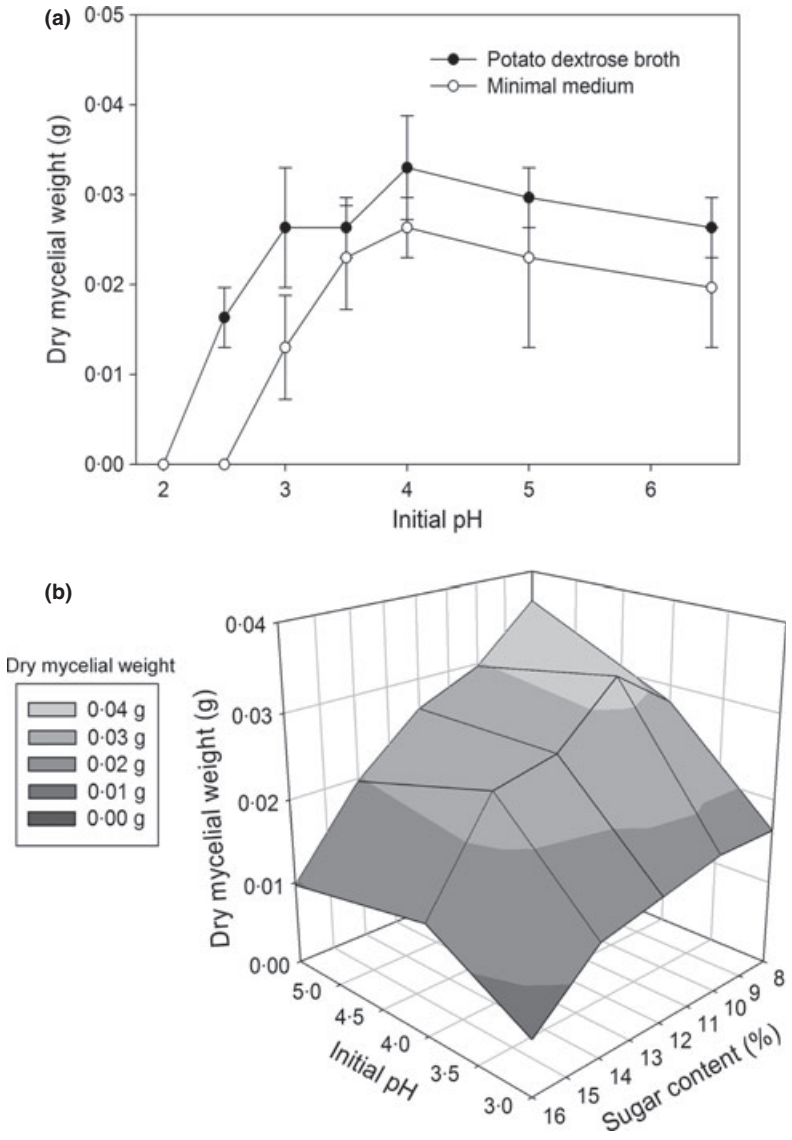


Figure 5 (a) The effect of initial pH in potato dextrose broth and minimal medium on the amount of mycelial growth (dry weight) of *Colletotrichum acutatum* after 7 days in 3 mL cultures. Initial pH is indicated here because the pH changed as cultures grew. Error bars denote standard error of the mean. (b) The effect of initial pH and sugar content (using a 50/50 D-glucose/D-fructose mixture) on mycelial growth (dry weight) of *C. acutatum* after 7 days. Different degrees of shading indicate different mycelial weights, with darker shades indicating less growth.

colonization of the fruit and should be investigated further. As sugar has been shown to induce the expression of pathogenesis-related proteins in *Arabidopsis thaliana*, a similar response may occur in blueberries (Thibaud *et al.*, 2004).

It is interesting that a correlation was not seen between pH and anthracnose fruit rot resistance ratings despite the fact that hyphal growth of *C. acutatum* was significantly inhibited in artificial media at pH values between 2·5 and 3·0, which are similar to natural pH levels in ripe blueberries. However, the method of measuring the pH of blueberry fruit was fairly crude, in that it involved the maceration of the entire fruit. By doing so, potential pH variation in different fruit tissues, such as the exocarp and mesocarp, might have been missed (Prusky *et al.*, 2000). Because the epidermis is the first physical barrier encountered by the pathogen, the specific pH of this tissue may be critical for the establishment of *C. acutatum*. Based on other pathosystems, it is likely that the fungus is able to modify the pH in its immediate surroundings, thereby reducing its effect (Diéguez-Uribeondo *et al.*, 2008). Modulation of pH by *Colletotrichum* spp. has been shown to play an important role in the colonization of other plant hosts (Prusky *et al.*, 2001). The observation of a pH change following a period of growth by *C. acutatum* in artificial media supports this assumption.

The role of berry weight and titratable acidity in fruit rot resistance appears to be limited as indicated by relatively low correlation coefficients and marginal statistical significance. However, if the rate of colonization is the same, the fungus would be expected to take longer to colonize large than small berries, which may be manifested in proportionally less surface area colonized in heavier (larger) berries. The percentage of titratable acidity may be related to berry size and therefore not be directly correlated with infection severity. As no significant correlations were found between fruit firmness and any of the resistance ratings, it is presumed not to play a role in fruit rot resistance.

This study has identified a relatively rapid and simple screening assay for anthracnose fruit rot resistance that can be used early in the selection process of blueberry germplasm. This would not preclude later screening under greenhouse or field conditions. Relying on natural infection only may introduce confounding effects of weather and inoculum availability as flowering and ripening times differ among blueberry cultivars. Artificial inoculation of fruit with no background infection is most desirable to identify an accurate resistance phenotype. Despite evidence for the role of sugar content in anthracnose fruit rot resistance, this characteristic is not suitable as a single marker due to the moderate strength of the correlation. However, selection for high sugar content may inadvertently yield anthracnose fruit rot-resistant cultivars as an added benefit. More investigation is required to elucidate the molecular and biochemical mechanism(s) of anthracnose fruit rot resistance in blueberry in order to identify specific markers for resistance screening and novel strategies for disease management.

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