Differential Fruit Maturity Plays an Important Role in Chili Anthracnose Infection

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Abstract

Correlation between physiological and biochemical changes that occurred during fruit ripening of Capsicum baccatum PBC80 and infection by Colletotrichum acutatum that caused anthracnose disease was studied. Fruit firmness, total soluble solids, pericarp color and % titratable acid (TA) increased during fruit development. Only the pericarp

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color (‘a’ value) and TA showed negative correlation ($R^2 = -0.48$ and -0.66 respectively) with lesion size after infection by *C. acutatum* MJ8 during fruit development. Lesion size on fruit inoculated at weekly intervals from 4 to 12 weeks after flowering (WAF) gradually decreased during the development from immature green to mature green fruit. A critical window where *C. acutatum* was unable to infect fruit occurred around 9 WAF depending on the growing season. Pericarp color (‘a’ value) showed a sudden change from negative to positive values during the critical window period. However when the ‘a’ value and anthracnose infection were reinvestigated at more frequent 3-day intervals, the ‘a’ values remained negative until 65 days after flowering, while *C. acutatum* was unable to infect chili fruit 6 days earlier. The decreasing trend in lesion size during fruit development suggested that fruit became fully resistant at the late mature green fruit stage, prior to fruit color change. Although the ‘a’ value could be used as a phenotypic marker to identify the fully ripe stage of chili it could not define the immature green from the mature green fruit stages. Hence making the ‘a’ value is an unreliable phenotypic marker to select for mature green fruit resistance.

**Keywords:** *Capsicum baccatum*, *Colletotrichum acutatum*, Differential infection, Fruit maturity

1. Introduction

Anthracnose caused by *Colletotrichum* spp. has been one of the most important diseases of fruits and vegetables in the tropical and subtropical areas worldwide. Over two decades, breeding for resistance to anthracnose in chili (*Capsicum annuum*) has been conducted in several Asian countries including Korea, Taiwan, Indonesia, India and Thailand [1]-[3]. However, no commercial resistant varieties of *C. annuum* have been developed, due to the lack of resistance in the *C. annuum* gene pool.

At least three species of *Colletotrichum* including *C. truncatum* (formerly *C. capsici*), *C. gloeosporioides* and *C. acutatum* have been reported to be associated with anthracnose disease in Thailand [2], [3] and south-east Asia [4]. Pathotypes of the *Colletotrichum* species involved in chili anthracnose have been identified based on qualitative differential reactions and infection of inoculated fruit at different maturity stages on a set of differential *Capsicum* genotypes [2], [5].

Different pathotypes are able to infect different chili genotypes at either mature green or ripe fruit stages. The mature green stage is where fruit is at full physiological development and still green in color (before color changes). The ripe stage is where fruit is fully ripe eg. completely red or yellow. For instance, pathotype 1 (PCc1-R) of *C. truncatum* was able to infect chili genotypes of *C. annuum*, *C. frutescens* and *C. chinense* but not genotypes of *C. baccatum* at the ripe fruit stage. Based on the recent study by Mongkolporn et al. [2], the total number of pathotypes of *C. truncatum* identified in Thailand was three on ripe and two on mature green fruit stages respectively; five and six from *C. gloeosporioides*; and none and three pathotypes from *C. acutatum*.

Breeding for anthracnose resistance has utilized *C. chinense* and *C. baccatum* as sources to introgress the resistance into *C. annuum*. Host resistance in these species has been shown to be differentially expressed at different fruit maturity stages [2]-[5]. Ko et al. [6] reported on differential resistance in green and ripe fruit of the same chili variety as revealed by high expression of *PepEST* gene
in the ripe fruit, which correlated to resistance to *Colletotrichum gloeosporioides* (Cg), while the green fruit was susceptible to Cg. Independent genetic studies have also identified resistance genes in *C. chinense* and *C. baccatum* that were differentially expressed at different fruit maturity stages [7]-[9]. Genetic studies of the resistance to anthracnose suggested that the inoculation bioassay for the resistance selection needed to be performed at different fruit maturity stages, so as to select for all resistance genes.

Selection for anthracnose resistance in chili breeding programs has relied on a detached fruit method [2], [8], [10]-[12]. In the study by Mahasuk et al. [7],[8] the fruit inoculation was performed separately on mature green and ripe fruit originating from the same plants, to ensure that the different resistance genes were selected simultaneously. However, inconsistent anthracnose symptoms often occurred on inoculated mature green fruit, that was close to color change ie. fruit breaker stage, but tended to turn red after inoculation; while some mature green fruit remained green throughout the inoculation and incubation period. The problem of anthracnose symptom inconsistency on the green fruit has considerably affected the selection for the green stage resistance [2], [8], [10], [12]-[14].

Both genetic studies of anthracnose resistance in chili by Mahasuk et al. [7], [8], and the pathotype study of *Colletotrichum* by Montri et al. [5] and Mongkolporn et al. [2] suggested that differential fruit maturity played a role in chili anthracnose resistance mechanisms. The inconsistency of symptom expression in the mature green fruit stage could have been affected by variations in the functionality of the mature green resistance gene, which could possibly be related to physiological changes in fruit maturity. In the other words, whilst the appearance (phenotype) of the mature green fruit stage remains unchanged, some internal physiological or biochemical changes may have taken place and thus altered the function of the genes. For breeding purposes, the efficiency of the resistance selection at mature green stage needs to be enhanced.

Physiological and biochemical changes during fruit development and maturity have been reported to be related to fruit firmness [15], fruit acidity and soluble sugars [16]-[18]. Fruit pericarp color also indicated fruit maturity stages in fruits especially for chili fruit [19]. Chili fruit has been categorized as a non climacteric fruit [17], [20], thus the ripening process is not activated by external ethylene.

The inheritance of chili anthracnose resistance in *C. baccatum* PBC80 by Mahasuk et al. [8] indicated that the resistance genes in mature green and ripe fruit stages were different and controlled by single dominant genes. Therefore, to investigate the function of resistance genes during mature green stage an isolate (MJ8; pathotype PCa-1G) of *Colletotrichum acutatum* was identified that could infect the mature fruit but not the ripe stage of *C. baccatum* PBC80 fruit [2]. This phenomenon suggested that the gene that conferred resistance to PCa-1G only functioned in the ripe fruit stage. Therefore, this study investigated physiological and biochemical changes that occurred during the ripening process in relation to anthracnose symptom development on chili fruit after inoculation. Simultaneously, the study also investigated the physiological/biochemical parameters that could be used as markers to select the mature green fruit from the immature green and breaker stages for inoculation.

2. Materials and Methods
2.1 Plant Material, Fungal Isolate and Inoculation

Plants of *Capsicum baccatum* ‘PBC80’, each were grown in 50-cm diameter plastic pots in a shade
The isolate MJ8 of *Colletotrichum acutatum*, classified as PCa1-G pathotype by Mongkolporn et al. [2], originated from an infected chili fruit of *C. annuum* collected in the Maejo district of Chiang Mai, Thailand; was grown on potato dextrose agar (PDA; Difco Laboratories, France) at 24°C for 14 to 21 days with 12 h photoperiod. Based on the study by Mongkolporn et al. [2], the isolate MJ8 could infect only the green fruit of PBC80, but not the ripe. Conidial suspensions were prepared by adding sterile distilled water to the sporulating culture and gently rubbing with a sterile bent glass rod. The conidial suspensions were adjusted with a haemocytometer to a concentration of 10⁶ conidiospores/ml for immediate use in bioassay experiments. Spores of the isolate MJ8 were injected into chili fruit following the standard bioassay used to screen for resistance in previous studies [1], [2], [5], [7], [8]. Each fruit was injected with 1 μl (~1,000 spores) using a microinjector consisting of a Micro Syringe model 1705 TLL and a dispenser PB600-1 (Hamilton, Switzerland).

2.2 Physiological Changes during Fruit Development

For both experimental repeats 10 fruit were harvested weekly from the one plant (this ensured genetic uniformity for all fruit) for studying the fruit development from 4 to 12 weeks after flowering. Every week, five fruit were inoculated with *C. acutatum* MJ8 to study the severity of *C. acutatum* infection and the other five were used to study physiological and biochemical changes. Each of the five fruit harvested at different ages were nondestructively assessed for fruit firmness and pericarp color. Fruit firmness was measured using a Fruit Hardness Tester (5 kg; N.O.W., Japan) that measures the force in kg, required to penetrate the pericarp tissue, the reading were converted to Newton (N) by multiplying with 9.80665 ms⁻² [21].

Pericarp color was measured at three positions ie. top, center and end of the fruit using a Color Reader CR10 (Minolta, Japan), and was recorded as ‘L’, ‘a’ and ‘b’ values. ‘L’ represented color whiteness or brightness, ‘a’ redness/greenness, and ‘b’ yellowness/blueness.

2.3 Biochemical Changes during Fruit Development

After being measured for color and firmness, the fruit were assessed for total soluble solid (TSS) and titratable acidity (TA). Fruit pedicels and calyx were removed before the fruit was cut into small pieces (~0.5 x 1 cm). Approximately one gram of the fruit pericarp (without seed) was ground with liquid nitrogen and then the pericarp extract was divided into two portions for TSS and TA analyses. The fruit TSS was measured using a hand refractometer (PAL-1, ATAGO, Japan). Three readings (°Brix) per fruit were taken and then averaged for further statistical analysis. The fruit TA was measured by titratability of 100 µL of the fruit extract which had been homogenized with 1 mL distilled water. 0.1 N NaOH was used as a standardized titration solution. TA was recorded as mean volume (µL) of 0.1 N NaOH required, which was converted to % TA and three measurements per fruit were performed and averaged.

2.4 Anthracnose Evaluation during Fruit Development

Anthracnose lesions at the inoculation sites were evaluated at 9 days after inoculation (DAI) on the basis of percent lesion size in proportion to fruit size [5] for five replications and then averaged.
2.5 Investigation of Anthracnose Infection in Relation to ‘a’ Value during the Critical Period from Weeks 8 to 10

Another set of fruit was harvested from one plant of the second repeat at 3-day intervals during the critical window of fruit ripening from weeks 8 to 10 after flowering. The ‘a’ values and anthracnose lesion sizes were measured every 3 days from 56 to 71 days after flowering following the same methods as previously described.

2.6 Statistical Analysis

Analysis of variance was performed using MINITAB statistical software Release 13. Least Significant Difference was used for mean separations (\( P = 0.05 \)). Correlation analysis was performed to investigate relationship between physiological and biochemical attributes of fruit and anthracnose development using correlation analysis (Minitab Release 13).

3. Results

3.1 Fruit Firmness and Pericarp Color

Fruit firmness ranged from 0.600 to 0.633 N during fruit development from weeks 4 to 12 (Figure 1). The fruit firmness reached the peak (0.633 N) at week 10 and then appeared to remain constant. Visual color of chili fruit pericarp was green from week 4 until 8, started to change color at week 9, then gradually turned to red after week 10 and was fully red in week 12. Pericarp color as measured with a color meter also correlated with the visual color. The greenness of the

Figure 1 Fruit firmness, pericarp color (L, a, b), total soluble solid (TSS), and titratable acid (TA) of Capsicum baccatum PBC80 during fruit development from weeks 4 to 12 after flowering (grown in May-Nov 2009); mean values with the same letter are not significantly different at \( P = 0.05 \), based on least significant difference; Error bars show the standard errors of the mean value.
pericarp had ‘a’ values below zero (-6.57 and -5.33) during fruit ages of 4 to 8 weeks (Figure 1). A sudden increase in ‘a’ value occurred during weeks 8 to 9 from -5.33 to +5.70, then reached the highest value of +29.80 at week 12. ‘L’ and ‘b’ values showed similar trends to each other with only a small increase from weeks 4 to 12 (Figure 1).

3.2 Total Soluble Solid (TSS) and Titratable Acidity (TA)

TSS during fruit development increased significantly from weeks 4 to 7 with a sharp increase during weeks 6 to 7 (6.83 to 10.10 °Brix), followed by change after week 7 (Figure 1). Fruit from weeks 4 to 9 had low acid (TA; 0.51 to 0.73%). A sharp increase of TA was detected during weeks 9 to 10 (0.73 to 1.64%), with a small increase after week 10 (Figure 1).

3.3 Anthracnose Infection and its Relationship to Fruit Ripening

Anthracnose symptoms developed on young chili fruit inoculated at 4 weeks after flowering with 11.7% lesion size, then at each subsequent fruit inoculation during fruit development, lesion size was progressively smaller until there was no visible infection after inoculation at 9 weeks after flowering (Figure 2). Correlations of each parameter ie. fruit firmness, pericarp color (a, b, L), TSS, and TA, to anthracnose lesion size showed that only the ‘a’ value and TA had significant negative relationship with the anthracnose lesion size (R² = -0.485 and -0.667 respectively) (Table 1), while the other physiological characters did not correlate with anthracnose lesion size.

![Figure 2](image)

**Figure 2** Percent anthracnose lesion during fruit development (weeks 4 to 12) on *Capsicum baccatum* PBC80 fruit inoculated with *Colletotrichum acutatum* MJ8, at weekly intervals grown in May-Nov 2009. Mean values with the same letter are not significantly different at \( P = 0.05 \), based on least significant difference.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fruit firmness</th>
<th>a value</th>
<th>b value</th>
<th>L value</th>
<th>Total soluble solid</th>
<th>Titratable acidity</th>
<th>Anthracnose lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruit firmness</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a value</td>
<td>0.273(0.19)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b value</td>
<td>0.245(0.13)</td>
<td>0.552**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L value</td>
<td>0.116 (0.19)</td>
<td>0.349*</td>
<td>0.892**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total soluble solid</td>
<td>0.314 (0.06)</td>
<td>0.645**</td>
<td>0.666**</td>
<td>0.480**</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Titratable acidity</td>
<td>0.371*</td>
<td>0.48*</td>
<td>0.248 (0.14)</td>
<td>0.078 (0.64)</td>
<td>0.275 (0.10)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Anthracnose lesion</td>
<td>-0.315 (0.06)</td>
<td>-0.485**</td>
<td>-0.196 (0.25)</td>
<td>0.013 (0.93)</td>
<td>-0.276 (0.10)</td>
<td>-0.667**</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Significantly different at \( P = 0.05 \), ** significantly different at \( P < 0.01 \), Numbers in brackets are probability.
A critical window in fruit ripening occurred between 8 to 10 weeks after flowering when sudden changes in fruit ripening occurred, and infection (lesion development) ceased. During this time the ‘a’ values became positive (week 9) and TA sharply increased (week 10).

Although fruit maturity was delayed by one week for fruit development in December 2009 to May 2010, all the measurements showed similar trends to those observed in May-Nov 2009 (Figure 3). Fruit development from immature green to ripe stage took 12 and 13 weeks after flowering. The critical windows for fruit ripening and in two timings anthracnose resistance occurred during weeks 8-10 i.e. 8-9 (May-Nov 2009) and 9-10 (Dec 2009-May 2010).

### 3.4 Anthracnose Infection in Relation to ‘a’ Value during the Critical Period from Weeks 8 to 10

The change in the ‘a’ value and anthracnose infection of fruit from the plants grown from Dec 2009-May 2010 was investigated during weeks 8 to 10. At 3-daily intervals from 56 to 71 days after flowering...
the level of infection by *C. acutatum* decreased from 4% at 56 days to zero at 59 days, and then remained zero from 59 days onwards (Figure 4). The ‘a’ value remained negative (green) until 65 days after flowering then became positive (red) at ripe maturity. The point when the ‘a’ value suddenly changed from negative to positive could be an indicator to differentiate between the immature and mature green fruit. However, timing for the changes in infection (lesion size became zero – host resistance) and the fruit color (‘a’ became positive) were not correlated (Figure 4).

### 4. Discussion

#### 4.1 Phenotypic and Physiological Changes during Fruit Development

Fruit development in *C. baccatum* PBC80 could be defined in four stages based on the changes of pericarp color and fruit size as follows: 1) immature green from weeks 4 to 7, when the fruit was enlarging and green in color; 2) mature green from weeks 7 to 8, when the fruit had reached maximum size and was still green; 3) breaker period from weeks 8 to 9, when the fruit developed more red but was not yet fully red; and 4) ripe after week 9, when the fruit was completely red. The number of weeks to each stage varied slightly depending on the fruit production season.

Among three types of fruit pericarp color, ‘L’, ‘a’ and ‘b’, the ‘a’ (greenness/redness) appeared to be the best for differentiating maturity stages of chili fruit into the three stages ie. green (immature and mature), breaker and ripe red. The green stage was indicated with minus ‘a’ values, while the breaker and ripe stages were indicated with positive ‘a’ values, which related to more redness in the fruit. However, ‘a’ value could not differentiate between immature and mature green fruit stages.

Fruit firmness slightly increased throughout fruit maturity development period due to the thickness of pericarp and increased levels of pectin resulting in more flexibility of pericarp when aged [20], [21]. The more mature fruit, therefore, required more force to penetrate the pericarp.

The sudden increase in TSS at week 7 may have been due to a sharp increase in concentration of soluble sugar in the chili extract, which was reported to be composed of hexose [23] and sucrose [24], at the mature green stage. A high rate of accumulation of TSS during the mature stage has been reported in other studies on pepper [18], [21]. The sudden increase in TA in PBC80 chili fruit at week 9 after flowering (ripening stage) was most likely due to higher ascorbic acid content than in green fruit stage [16], [18], [24], [25].

#### 4.2 Differential Fruit Maturity and Anthracnose Infection

Isolate MJ8 (pathotype PCa1-G) infected only the green fruit but was not able to infect the ripe fruit as previously found by Mongkolporn et al. [2]. The severity of infection in the green fruit as measured by lesion size decreased at each weekly interval until no lesions were observed at weeks 9 and 10 in experiments 1 and 2 respectively. Similar results were reported by Biles et al. [17] for *Phytophthora capsici* infection in non inoculated fruit where susceptibility decreased with increased ripening.

The mechanism of host resistance during fruit maturity was interesting in that there was a gradual reduction in lesion size at each weekly interval which may have indicated that host resistance was being less expressed at each weekly interval during fruit ripening. Tadessa et al. [21] and Pretel et al. [27] showed that the reduced greenness (increased redness) in chili
corresponded to chlorophyll degradation and carotenoid synthesis, which has been suggested to be linked to disease resistance expression in chili fruit [28], [29]. To prove this link between maturity and reduction in resistance, further studies are required to measure transcriptional expression of the resistance genes during these fruit maturity stages. There may have been other physiological or genetic mechanisms either suppressing transcription of the resistance gene(s) in green fruit resulting in a susceptible phenotype; or transcription of the resistance gene(s) gradually increased as the fruit ripened (turned red) until the expression of the resistance gene was strong enough to prevent infection. Mongkolporn and Taylor [30] reported that most of the resistance genes expressed in mature green and ripe fruit stages of PBC80 were different, with only a few genes in common. However, these common genes showed differential levels of expressions in the two fruit stages. Therefore, differential fruit maturity seems to play an important role in chili anthracnose resistance.

Although TA and ‘a’ values correlated with resistance to anthracnose infection, only critical changes in ‘a’ appeared to correlate with the onset of resistance (weeks 8 to 9 after flowering in the rainy season experiment), while the critical change in TA values occurred during weeks 9 to 10 after flowering. Therefore, weeks 8 to 9 were considered as a critical window of change when fruit color was turning from green to red, and the anthracnose infection was unsuccessful. Measuring the ‘a’ value may be a more convenient procedure compared to measuring TA for predicting fruit maturity and the expression of resistance. Nevertheless, the expression of host resistance occurred six days before redness color was detected by the color reader, which may limit the usefulness of using ‘a’ values to predict fruit maturity and resistance. This event suggested that host resistance was fully functional in the late mature green fruit, which may have been the cause of inconsistent symptoms of the anthracnose on inoculated green fruit. Therefore, the recommended procedure to identify fruit at the immature green and mature green fruit stages is to tag each newly opened flower and record the number of days to fruit maturity.

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