 ROLE OF TWO INOCULATION METHODS IN EXPRESSION OF ANTHRACNOSE RESISTANCE GENES IN CHILI (CAPSICUM ANNUUM L.)

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Pre-and postharvest anthracnose fruit rot is a major disease of chili (Capsicum spp.) in tropical production systems. Anthracnose disease of chili (Capsicum annuum L.) can cause substantial postharvest losses in fruit quantity and quality by creating an entry point for aflatoxins. Colletotrichum acutatum is the most prevalent in North East Thailand causing chili anthracnose. Identification of germplasm resistant to anthracnose requires reliable techniques of C. acutatum inoculation. The two inoculation methods (microinjection and spray inoculation) on anthracnose resistance were investigated with the cross between resistant (AVPP0207; P_R) and susceptible (KKU-P31118; P_S) Capsicum annuum L. parents. Fifteen green mature fruits of six populations (P_R, P_S, F_1, F_2, BC_1P_S and BC_1P_R) were inoculated by spray and microinjection methods using C. acutatum-Ca153 at concentrations of 1x10^7 and 5x10^5 conidia/ml, respectively. Lesion diameters were measured and recorded five days after inoculation (DAI) for the microinjection method and the percent disease severity (PDS) was recorded seven DAI for the spray method. Frequency distribution of the disease score in F_2 and backcross plant suggested that a single dominant gene was responsible for resistance when plants were challenged with the spray method. However, a single recessive gene provided resistance when plants were challenged with the microinjection method. Linkage analysis between these two monogenic resistance genes identified through the two inoculation methods showed both genes inherited independently at 47.45 cM distance. A possible reason for different modes of inheritance will be discussed, as well as the
appropriate inoculation method from this study in our anthracnose resistance breeding program to reduce postharvest losses of chili.

Keyword: Dominant gene, recessive gene, inheritance, linkage, microinjection, spray

INTRODUCTION

Pre- and postharvest anthracnose fruit rot is a major disease of chili (Capsicum spp.), which can cause substantial postharvest losses and create entry point for aflatoxins. In chili samples from Thailand, very high and unacceptable levels of aflatoxin contamination (up to 60 parts per billion (ppb)) have been reported, which is significantly beyond the accepted level for safe consumption (≤ 20 ppb). Safe foods secure people’s health, increase market access, and foster development. There has always been awareness on production of safer food worldwide and concern among stakeholders. Quality of fresh production is one of the key factors having significant relationship with the consumer acceptability and marketability; and has always been a major concern of stakeholders from production level to marketing and consumption (Shewfelt, 1999). Postharvest disease development is a major constraint to the quality and shelf life of product, especially, for anthracnose fruit rot is a major disease of chili (Capsicum spp.) in tropical production systems. The infected fruit was decay due to rapid disease development during storage ripening. Anthracnose (Colletotrichum SP.) is main disease problems of chili production with many countries. During the rapid increase in the severity and incidence of this infecting in chili growing areas, its management became a worldwide concern to ensure the postharvest fruit quality (Than et al., 2008). Breeding for anthracnose resistance is effective techniques for disease control. In recently year, anthracnose caused C. acutatum has become aggressive species, but the resistant cultivars have limited. Due to the resistant source were reported within chili wild species, which are interspecific barrier with normal species (Komworn et al., 2014; Yoon et al., 2006). However, the resistance genes were introgressed into the common species and the introgressive lines were available used (Lee et al., 2010). In addition, the information on inheritance of anthracnose is general required for predictable result. The present of resistance gene actions the methods for screening are considered importance for understanding response mechanism of the genes. Microinjection method is widely used
technique for identified resistance gene action (Kanchana-udomkan et al., 2004, Mahasuk et al., 2009a, Temiyakul et al., 2012, Lin et al., 2007), but this technique is considered harsh method (Mahasuk et al., 2013). Thus AVRDC center developed the lesser harsh ones called high pressure spray method, which is most nearly natural infection. However, the two inoculation methods are different part infection of chili fruit surface, MI method has deliver fungal spores into epidermal cell (wounding), while HP method has airbrush to deliver fungal spore into the surface of chili fruit (non-wounding). The two methods are lack comparison and correlation information for inheritant study (Mahasuk et al., 2013). This study aimed to investigate inheritance of the resistance derived from PBC932 in response to two different inoculation methods (MI & HP).

MATERIALS AND METHODS

Plant genetic

The anthracnose resistant parent ‘AVPP0207’ (BC3F4 of IR x PBC932) (‘Pₛ’) was crossed with to the susceptible line parent ‘KKU-P31118’ (C. annuum) (‘Pᵣ’), which is otherwise is a high yielding, and highly pungency and resistant to CMV resistant genotype. Seed from five F₁ plants were grown in greenhouse during dry season 2012 and were used to produced the seeds of F₂ generation seed. Pollen from these F₁ plants was bulked and used to generate backcrosses to the resistant parent ‘Pₛ’ (F₁BC₁Pₛ) and the susceptible parent ‘Pᵣ’(F₁BC₁Pᵣ). In the dry fall season of 2012, all plants of both the parents and all the generations [Pₛ (10 plants), Pᵣ (10 plants), F₁ (10plants), F₂ (120 plants), F₁BC₁Pₛ (60 plants), F₁BC₁Pᵣ (60 plants)] were established in an open field nursery at AVRDC–The World vegetable Center (Shanhua, Taiwan). From each individual plant, 30 maturegreen fruits were harvested for inoculation during February 2013.

Inoculum Pathogen preparing and inoculation

—Microinjection method: Fruits were inoculated at two places (proximal and distal ends) individually with 1μl conidia suspension (5x10⁵ conidia/ml) of C. acutatum (AVRDC isolate ‘Coll-153’), using a syringe fitted with a 22-gauge needle 0.6-0.75 mm needle in length, to inject 1μl conidia suspension (5x10⁵ conidia/ml) (AVRDC, 1999) at two (proximal and distal) locations on each fruit. Spray method: using flockyto spray conidia suspension (1x10⁷) whole chili fruit till the fruit are soaked. Inoculated Fruits inoculated with both the of each methods were arrayed.
kept in an incubation chamber in a randomized complete block design (RCBD), with 3 replications. There were a total of 15 fruits per plant per method in each replication. The inoculated fruits were then incubated for 5 and 7 days of microinjection and spray methods, respectively, at 25°C and 95-98% RH.

**Evaluation** Disease scoring and Statistical analysis

Evaluation of Microinjection: Disease lesion diameter (mm) was measured individually, and averaged over all 24 lesions for each plant. Plants displaying mean lesion diameter less than 4 mm were classified as resistant, while those with mean lesions larger than 4 mm were classified as susceptible. Evaluation of spray method was assessed by using percent severity of disease per fruit. Each population was analyzed using Chi-square goodness-of-fit test.

**RESULTS AND DISCUSSION**

Inheritance of Resistance at microinjection method

Inheritance of resistance to *C. acutatum* was examined at microinjection and spray method at green mature fruit stage. At the microinjection method, fruit of the cross ‘AVPP0207 x KKU-P31118’ and its genetic populations F₁, F₂, F₁BC₁Pₛ, F₁BC₁Pᵣ were also inoculated with *C. acutatum* (Coll-153), again using average lesion diameter of ≤ 4 mm as the criterion for the resistant class. Segregation data for resistant and susceptible plants are presented in Table 1. As expected, all the 10 plants of the both parents, the parent line ‘AVPP0207’ was all resistant and ‘KKU-P31118’ were resistant and was all susceptible, respectively. All the 10 F₁ plants displayed all were susceptible plants, suggesting resistant trait control by a recessive gene effect in nature. The F₂ generation plants segregated into 47 resistant (R) and 73 susceptible (S) plants, fitting a 1R:3S Mendelian ratio (χ² (1:3) = 0.01, P = 0.09) of monogenic single recessive genes displaying control of resistance. The In F₁BC₁Pₛ generation, all 60 plants were susceptible reaction. However, segregation of susceptible in the F₁BC₁Pᵣ generation of 30 resistant plants and 30 susceptible plants, which was provides a good fit to 1R:1S (χ² (1:3) = 2.05, P = 1.00). Hence confirming control of resistant trait under the microinjection method, this cross gives a generally good fit to a segregation model of single recessive genes.
Inheritance of Resistance at under spray method

At spray method of all genetic populations also inoculated and investigated as green fruit stage. Segregation data are presented in Table 4. The parentline ‘AVPP0207’ was all resistant, and ‘KKU-P31118’ was all susceptible. F1 plants all 10 plants showed resistant, while the F2 generation segregated 96 resistant to 22 susceptible plants in the F2 generation. This approximates a 3R:1S ratio ($\chi^2 (3:1) = 2.54, P= 0.11$), which is characteristic of a model of single genes interacting in dominant. Almost all the F1 BC1P1 progeny were susceptible (1R:1S plants) as would be expected, but the F1 BC1P2 generation all resistant 60 plants as fit the expected 1R:0S ratio.

Goodness-of-fit test of independent control of resistance with microinjection and spray methods
Based on the inherent mode outlined above (resistance controlled independently in microinjection method by a single recessive gene, and in the spray method by a single dominant gene, four compound phenotypes are expected to segregate in to segregate in predictable ratios in each parental, hybrid, and segregating generation. The independent segregation of resistance at the two methods is assumed.

Based on both inoculation methods (microinjection vs. spray) there could be four different resistant categories of plants: RR (resistant by both microinjection resistance and spray resistant), RS (resistant by microinjection resistance and susceptible by spray susceptible), SR (susceptible by microinjection susceptible and resistant spray resistant), SS (susceptible by both microinjection and spray susceptible). Both parents displayed expected ratio, F2 generation fit the model ($\chi^2 (29RR:54RS:2SR:33SS) = 20.98$, $P = 0.03$) and ($\chi^2 (9:3:3:1) = 6.46$ $P = 0.1066$). Poor linear correlations between average lesion diameters of green vs. red fruit in the several generations (Table 2) suggested that genetic control of resistance may be independent in the for two methods. Independent assortment of resistance at the MI and SP methods is supported by comparison of predictions of frequencies of recombinant phenotypes under contrasting models. For the independence analysis of the resistance genes by MI and by SP, 118 F2 plants were available. Goodness-of-fit test for a 9:3:3:1 combined phenotype segregation was performed and suggested that the two R genes were independent and linked with genetic distance of 47.45 cM (Table 2). Hypersensitive reaction in both spray and microinjection in PBC932 or derived resistance???

Anthracnose inheritance studies in chili pepper cultivars have shown that resistance may be controlled by one, two dominant gene (Lin et al., 2007) or recessive gene (Pakdewaraporn et al., 2005). Monogenic recessive resistance was found in the cultivar PBC932 to C. capsici (Pakdewaraporn et al., 2005), AR line to C. acutatum at green fruit stage (Kim et al., 2008b) and PBC80 at green fruit stage (Mahasuk et al., 2009b). Monogenic and dominant inheritance was also observed in the genotype PBC80 at ripe fruit stage (Mahasuk et al., 2009b), PI594137 (Kim et al., 2008a) to C.acutatum.

In addition, two complementary dominant genes showed within resistant line derived from PBC932 ‘0038-9155’ at green fruit stages, but showed two recessive gene in red fruit stage to C. acutatum-Coll 153 by used microinjection method. (Lin et al., 2007). While previous studies, the similar pattern of resistance to difference inoculation method (microinjection and high pressure spray method) has been found single dominant gene action (Mahasuk et al., 2013). The close fit of compound results to a model in which the resistances at MI and HP method are considered to segregate
independently. For supports this hypothesis. The two infection mechanisms have
different response, based on their were inoculated in different part of fruit surface
maybe indicated that the genetic control were difference between two parts of chili fruit
(surface and epidermal cell) and is considered gene for gene concept. On the other
hand, some studies implied cuticular waxes has barrier to infection (Oh et al., 1999),
thus HP methods was less aggressive than MI method.

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Table 1 Segregation for resistance (R) and susceptibility (S) in a crossbetween KKU-P31118 (P<sub>R</sub>) and AVPP0207 (P<sub>s</sub>)

<table>
<thead>
<tr>
<th>Population</th>
<th>Phenotypic data (number of plants)</th>
<th>Spray method</th>
<th>Microinjection method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>P&lt;sub&gt;R&lt;/sub&gt;</td>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>P&lt;sub&gt;s&lt;/sub&gt;</td>
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<td>10</td>
<td>0</td>
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<td>F&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td>10</td>
<td>0</td>
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<td>F&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>96</td>
<td>22</td>
</tr>
<tr>
<td>BC&lt;sub&gt;1&lt;/sub&gt;P&lt;sub&gt;s&lt;/sub&gt;</td>
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<td>30</td>
<td>30</td>
</tr>
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<td>BC&lt;sub&gt;1&lt;/sub&gt;P&lt;sub&gt;R&lt;/sub&gt;</td>
<td></td>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>

R = resistance; S = susceptibility; χ<sup>2</sup> = chi-square test; P = probability.
Table 2 Test of independent assortment in 118 F$_2$ plants (9 : 3 : 3 : 1) for monogenic control of anthracnose resistance based on spray (SP) and microinjection (MI) inoculations

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>E</th>
<th>O</th>
<th>$\chi^2$</th>
<th>$P$</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>R by SP and S by MI</td>
<td>67</td>
<td>54</td>
<td>20.98</td>
<td>0.003159</td>
<td>47.45%</td>
</tr>
<tr>
<td>R by SP and R by MI</td>
<td>22</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S by SP and S by MI</td>
<td>22</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S by SP and R by MI</td>
<td>7</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E = expected frequency of plants; O = observed frequency of plants; $\chi^2$ = chi-square test; $P$ = probability; RF = recombination frequency.