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Vegetative Compatibility Grouping and Pathogenicity of *Colletotrichum gloeosporioides* Isolates from Different Host Plants

Il-Pyung Ahn, Soonok Kim, Kyung-Hwan Im[1] and Yong-Hwan Lee*

School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea
[1]Department of Biology, University of Incheon, Incheon 402-749, Korea

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A total of 57 isolates of *Colletotrichum gloeosporioides* were recovered from diseased tissues of Hall's crab apple (*Malus haliana*), 3 cultivars of edible apple (*M. pumila* var. *dulcissima*), red pepper (*Capsicum annum*), and grapevine (*Vitis vinifera*) fruits. All isolates showed strong virulence on their own host plants. Isolates from edible apple exhibited high level of cultivar specificity in pathogenicity tests. Ten isolates from apple cultivar 'Fuji' were virulent on 'Jonathan' and 'Rall's Genet'. However, 12 isolates from 'Jonathan' and 'Rall's Genet' were not virulent on 'Fuji'. Among the 24 isolates from red pepper, only seven and two isolates were infective on edible apple and grapevine fruits, respectively. All six isolates from grapevine were only virulent on their own host. These isolates were grouped into five vegetative compatibility groups (VCGs), A, B, C, D, and E, by demonstrating heterokaryosis through complementation using nitrate-nonutilizing (*nit*) mutants. Among them, isolates belong to VCG-A and VCG-D accounted for 24 and 17 isolates; those in VCG-A exhibited wide host range involving Hall's crab apple, all three edible apple cultivars, and red pepper. On the other hand, isolates of VCG-D and VCG-E showed limited host range specific to red pepper and grapevine, respectively. Taken together, the data suggest that among *C. gloeosporioides* isolates, the concepts of pathotype and/or forma specialis may exist, and that three is a relationship between host specificity and VCG grouping among *C. gloeosporioides* isolates.

Keywords **:** *Glomerella cingulata*, host range, mycelial compatibility group, host range.

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. (teleomorph; *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk), a homothallic Ascomycete, causes anthracnose disease in more than 197 species of plants including crops, weeds, and trees (Farr et al., 1989). This fungal pathogen causes serious diseases on apple (*Malus pumila* var. *ducissima*), red pepper (*Capsicum annuum*), and grapevine (*Vitis vinifera*) in Korea (Park et al., 1992; Park and Kim, 1992). Controlling anthracnose diseases has been dependent on multiple applications of fungicides during growing season. However, recent emergence of chemical-resistant isolates has reduced the disease control efficacy of fungicides, and environmental regulations have restricted their use (Delp, 1980; Park et al., 1990). Although developing and introducing disease resistant germplasms are under progress, these efforts have been impeded due to the lack of information about the host specificity of the fungal pathogen and the genetic structure of pathogen population.

The taxonomy of *C. gloeosporioides* is in flux state. von Arx (1957) subsumed 594 species into a large one, *C. gloeosporioides*, based on mycological characteristics. As a result, this species is composed of genetically diverse isolates or isolates exhibiting different phenotypes, e.g. host specificities (Alahakoon and Brown, 1994; Cisar et al., 1994; Daniel et al., 1973; Freeman and Shabi, 1996), cultural characteristics (Bernstein et al., 1995; Freeman and Shabi, 1996), and lower intraspecific similarity than those of other *Colletotrichum* spp. (Freeman et al., 1996; Freeman and Shabi, 1996; Kelemu et al., 1999). Classification of *C. gloeosporioides* into subspecies or forma specialis has not yet been clearly established due to inconsistent pathological interactions between *C. gloeosporioides* isolates and host plants (Davis et al., 1984; Freeman et al., 1996; Kelemu et al., 1999; Oh et al., 1998; Oh, 1995). Similar phenomena have been reported between other *Colletotrichum* species and host plants including *C. lagenarium*-cucumber (Goode, 1958), *C. orbiculare*-various hosts (Barnes, 1972), *C. gloeosporioides* f. sp. *aeschynomene*-various plants (TeBeest, 1988), and *C. gloeosporioides*-avocado and almond (Freeman et al., 1996). These suggest that more consistent and handy tools are necessary to assess pathogenic variation among *Colletotrichum* species and to develop efficient anthracnose management strategy in various crops.

Nitrate-nonutilizing (*nit*) mutants have been used to

*Corresponding author.
Phone) +82-2-880-4674, FAX) +82-2-873-2317
E-mail) yonglee@snu.ac.kr
Present address of the first author: Plant Biotechnology Division, National Institute of Agricultural Biotechnology, RDA, Suwon 441-100, Korea

investigate vegetative compatibility in numerous plant pathogenic fungi (Anagnostakis, 1987; Puhalla, 1985) including *Colletotrichum* spp. (Brooker et al., 1991; Vaillancourt and Hanau, 1994). Several researches suggested the close linkage between loci controlling vegetative incompatibility (*vic* or *het* loci) and genes involved in virulence or pathogenicity (Puhalla, 1985; Wasilwa et al., 1993). Vegetative compatibility group (VCG) analysis has been used as a powerful method to classify races (Larkin et al., 1990; Wasilwa et al., 1993) to assess genetic relationship among fungal populations (Chacko et al., 1994; Jacobson and Gordon, 1991), and to estimate the virulence level or host specificity of the isolates (Ahn et al., 1998). In addition, virulent population of *Colletotrichum* spp. exhibits simpler VCG structure compared with those of saprophytic ones (Cisar et al., 1994).

This study investigated the host specificity of *C. gloeosporioides* isolates from various plants, their genetic structures with VCG analysis, and the relationship between host specificity and VCG of *C. gloeosporioides*.

Materials and Methods

Fungal isolates. A total of 57 isolates of *C. gloeosporioides* was recovered from Hall's crab apple (*Malus haliana*), three cultivars of edible apple (*M. pumila* var. *dulcissima*), red pepper (*Capsicum annum*), and grapevine (*Vitis vinifera*) fruits showing typical anthracnose symptoms. All diseased tissues were collected between August and October 1993, from Kyonggi, Chonnam and Kyungbuk provinces in Korea. Symptomatic tissues were surface-sterilized with 0.1% sodium hypochlorite for 2 min and were placed on water agar. After single conidium isolation, fungal isolates were identified as *C. gloeosporioides* based on the mycological characteristics described by Sutton (1980), and subsequently were cultured on potato dextrose agar (PDA) plates. Fungal stock was maintained as conidial suspension amended with 20% glycerol at -70°C until use.

Pathogenicity tests. Pathogenicity tests were performed as described by Bernstein et al. (1995) with some modification. Each isolate was grown on PDA plate in 16 h photoperiod at 26°C for 10 days. Conidia were recovered by scraping them off with 250 ppm Tween 80. Mycelia were removed by passing through four layers of cheesecloth, and the concentration of conidial suspension was adjusted to 10^6 conidia/ml. Twenty (20) microliters of inoculum was dropped on each fruit surface wounded slightly with sterilized pins. Fully-ripened fruits (*Malus haliana*, *M. pumila* var. *dulcissima* cv. 'Fuji', 'Jonathan', and 'Ralls Genet', *Capsicum annuum* cv. 'Kumtap', and *Vitis vinifera* cv. 'Tano Red') were washed with detergent and water to remove fungicide residues, then surface-sterilized for 2 min with 0.5% sodium hypochlorite and rinsed with sterile distilled water. Inoculated fruits were kept in a humid plastic chamber at 26°C with 12 h photoperiod regime. All pathogenicity tests were conducted more than three times for each isolate with ten replicates. Fruits of apple cultivar 'Jonathan' inoculated with isolate MhSW-4 and treated with 250 ppm Tween 80 were included in each virulence test as a positive and a negative control, respectively. Disease incidence was estimated at 14 days after fungal inoculation.

Vegetative compatibility. Nitrate-nonutilizing (*nit*) mutants were induced and their phenotypes were determined as previously described by Brooker et al. (1991). Fresh mycelial blocks grown on PDA were transferred on the minimal medium (MM) containing 3.0% potassium chlorate ($KClO_3$) and incubated at 26°C in the dark. Thin, fast-growing, chlorate-resistant sectors were recovered at 14 days after inoculation. It was determined in the phenotype of each mutant whether aerial mycelia were formed or not on MM containing nitrate, nitrite (0.5 g/L), ammonium titrate (1.0 g/L), hypoxanthine (0.2 g/L), and uric acid (0.2 g/L) as the sole nitrogen source. *nit1* and NitM were recovered from all isolates because complementation responses were most evident between these two phenotypes. Pairing of *nit1* and NitM mutants from each isolate was conducted to identify whether the isolated were vegetatively self-compatible or self-incompatible. Mycelial plugs of two *nit* mutants were paired on minimal medium (MM) containing sodium nitrate as the sole nitrogen source and incubated at 26°C for 2 weeks with two replicates. Development of aerial mycelia was observed at the contact region of mycelia if two isolates belonged to the same VCG.

Results and Discussion

Generation of *nit* mutants. Three (3) to 31 *nit* mutants were obtained from each of the 57 isolates on MM containing 3.0% chlorate (Table 1). Generation of *nit* mutant was repeated until stable *nit1* and NitM phenotypes were recovered from each isolate. Spontaneous chlorate-resistant sectors were readily recovered. However, there was a significant variation according to fungal isolates with respect to mutant recovery rate as well as phenotype ratios. The *nit* mutants could be divided into three classes; *nit1* (a mutation of nitrate reductase structural locus), *nit3* (a mutation of nitrate-assimilation pathway specific locus), and NitM (mutations that affect the assembly of a molybdenum-containing cofactor necessary for nitrate

Table 1. Frequency and phenotypes of *nit* mutants recovered from *C. gloeosporioides* isolates

Phenotypes of *nit* mutants[a]	No. of mutants[b]	No. of isolates[c]
nit1	449	57
nit3	23	11
NitM	121	57
CRUN[d]	329	31

[a] Identification of nitrate non-utilizing (*nit*) mutants were determined as described by Brooker et al. (1991).
[b] Numbers indicate the total numbers of the *nit* mutants generated.
[c] Numbers indicate the number of isolates generating each phenotype among isolates tested.
[d] Chlorate-resistant and utilizing nitrate mutants.

Table 2. Origin of hosts, vegetative compatibility groups, and virulence of *C. gloeosporioides* isolates

Origin of host	No. of isolates	No. of isolates exhibiting virulence[a]						VCG[b]
		M. haliana	*M. pumila* var. *dulcissima* cv.			*C. annuum*	*V. vinifera*	
			Fuji	Jonathan	Rall's Genet			
M. haliana	5	5	5	5	5	1	0	A
M. pumila var. *dulcissima* cv.								
Fuji	10	0	10	10	10	2	0	A, B
Jonathan	7	2	0	7	7	0	0	A, C
Rall's Genet	5	0	0	5	5	0	0	A
C. annuum	24	0	6	1	0	24	2	A, B, D
V. vinifera	6	0	0	0	0	0	6	E

[a] Virulence tests were conducted more than 3 trials with 10 replicates and disease incidence was recored at 14 days after inoculation.
[b] Vegetative compatibility grouping was conducted on the basis of heterokaryon formation (formation of aerial mycelia) between *nit1* and NitM mutants. Complementation tests were performed more than twice with two replicates.

reductase activity). The most common mutant phenotypes were *nit1* (46.5%) and followed by NitM (33.8%) and *nit3* (19.7%). These ratios of mutant phenotypes are, in general, consistent with those of Brooker et al. (1991) for *C. gloeosporioides* and other *Colletotrichum* spp. (Beynon et al., 1995). In addition, large numbers of chlorate-resistant and utilizing nitrate (CRUN) mutants were induced. A similar phenomenon was also observed in another fungal species, *F. poae* (Liu and Sundheim, 1996).

Virulence tests. Cross-virulence among four different host species and 57 isolates of *C. gloeosporioides* were tested in all possible combinations. All *C. gloeosporioides* isolates were highly virulent on their own original host species and cultivars. Cultivar specificity was observed among *C. gloeosporioides* isolates from three edible apple cultivars. All ten isolates from cultivar. 'Fuji' were virulent on the other two apple cultivars, 'Jonathan' and 'Rall's Genet'. Isolates from cvs. 'Jonathan' and 'Rall's Genet' were all cross-infective with each other's original host cultivars. On the other hand, all of them were not virulent on cv. 'Fuji' (Table 2). These virulence tests strongly suggest that races (pathotypes) may exist among isolates from edible apples. Similar results were described in the *C. gloeosporioides* f. sp. *aeschynomene* infecting Northern jointvetch (TeBeest, 1988) and *C. orbiculare* infecting cucurbit (Wasilwa et al., 1993).

On the other hand, only two isolates among 22 apple pathogens were virulent on red pepper and none of these were virulent on grapevine (Table 2). These results strongly indicate that the virulence spectrum of *C. gloeosporioides* isolates from apple was restricted within certain hosts. Limited host spectrum was more evident in *C. gloeosporioides* isolates from grapevine. All isolates were virulent only on grapevine, but not on other plants such as apples and red pepper. Taken together, this strongly implies that forma specialis (f. sp.) may exist among *C. gloeosporioides* isolates tested in this study. However, isolates from red pepper were virulent not only on pepper but also on apples and grapevine, though a limited number of isolates exhibited cross infectivity. This cross infectivity would be problematic in the establishment of f. sp. concept in *C. gloeosporioides* isolates. Further experiments with more number of isolates are required to propose subspecies levels in this fungus.

Vegetative compatibility. All pairings were conducted more than twice in the dark due to the occasional occurrence of inconsistencies and confusion of heterokaryosis between the same pair isolates.

A total 57 isolates of *C. gloeosporioides* were grouped into five VCGs, A, B, C, D, and E, by demonstrating heterokaryosis through complementation using *nit* mutants (Table 3).

Twenty-four isolates belonged to VCG-A, the largest group. This VCG included isolates from apples and red

Table 3. Origin of hosts and vegetative compatibility grouping of *C. gloeosporioides* isolates

Origin of hosts	No. of isolates	Vegetative compatibility groups (VCGs)[a]				
		A	B	C	D	E
M. haliana	5	5	0	0	0	0
M. pumila var. *dulcissima* cv.						
Fuji	10	8	2	0	0	0
Jonathan	7	2	0	5	0	0
Rall's Genet	5	5	0	0	0	0
Subtotal	22	15	2	5	0	0
C. annuum	24	4	3	0	17	0
V. vinifera	6	0	0	0	0	6
Total	57	24	5	5	17	6

[a] Vegetative compatibility grouping was performed as described in Table 2.

pepper. All five isolates from Hall's crab apple were in VCG-A. Fifteen of the edible apple isolates also belonged to VCG-A. These data indicate that VCG-A is a major group in apples and is composed of more than one group of isolates aggressive on different host cultivars and species. Seventeen isolates from red pepper belonged to VCG-D, indicating that this is the major group among red pepper isolates. Furthermore, none of the isolates from other hosts belonged to the unique VCG-D group. More interestingly all six isolates from grapevine form another group, the VCG-E. These data suggest that there is a correlation between source of isolates and VCGs, although a few exceptions were observed. This is in accordance with host specificity of the isolates in pathogenicity as described in Table 2. However, the fact that apple isolates showing different host/cultivar specificity belonged to the same VCG indicates that a VCG does not correspond to a pathotype. Similar complex relationship between VC grouping and pathotypes (races) were also described in *Fusarium oxysporum* f. sp. *melonis* (Jacobson and Gordon, 1991).

In conclusion, the data suggest that there exist host specificity of isolates obtained from different hosts of *C. gloeosporioides*. Furthermore, a strong correlation was evident between host specificity and VCG of isolates. These could be an indication of genetic differences among *C. gloeosporioides* isolates infecting different host plants.

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