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Evaluating the Resistance of Grapevines Against Anthracnose by Pathogen Inoculation, Vineyard Inspection, and Bioassay with Culture Filtrate from *Elsinoe ampelina*

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Abstract

Breeding of grape cultivars resistant to anthracnose is one of the most important breeding goals in Korea. In this study, evaluation of resistance using bioassay of grape leaves with culture filtrates from *E. ampelina* and their ethyl acetate extracts was compared with pathogen inoculation and field screening. To evaluate the resistance to anthracnose disease in grape germplasm, European grapes, American grapes, and *Vitis* hybrids were tested. Bioassay with culture filtrates produced by the pathogen showed that 'Black Eye', 'Mario', 'Niunai', 'Rizamat', and 'Rosario Bianco' were sensitive, while 'Campbell Early', 'Niagara', and 'Honey Red' were tolerant to anthracnose. In the evaluation by pathogen inoculation, some cultivars such as 'Black Swan', 'Rizamat', 'Rosario Bianco', and 'Kaiji' were susceptible, while others such as 'Campbell Early', 'Niagara', 'Sheridan', and 'Izumo Queen' were found to be resistant to anthracnose. Vineyard evaluation showed the same results. The results of bioassay with culture filtrates of the pathogen were consistent with those from pathogen inoculation and screening in the vineyard.

Introduction

Grape (*Vitis* spp.), one of the world's most important fruit crops, is subject to a number of bacterial, fungal, and viral diseases (6). Grape anthracnose, caused by *Elsinoe ampelina* Shear, specially damages European grape (*V. vinifera*) and its hybrids grown in warm and humid climates (6). The pathogenic fungus attacks all aerial parts of the vine and overwinters in dead canes and fruits, making its control very difficult (3, 5). Developing resistant grape cultivars against anthracnose is needed to reduce the labor and cost of chemical spraying and to produce marketable grapes. In the development of grape cultivars resistant to the disease, the selection of resistant genetic resources is initially required in the grape breeding programs.

Screening of disease resistance in plants has been conducted through a survey of natural infection in the vineyards and pathogen inoculation in greenhouses. However, this process takes time, is very costly and inefficient in screening of perennial crops like grapevine especially for large scale management. Resistance to grape anthracnose has been evaluated in native grapes as well as other grape cultivars (3, 7, 9, 13, 14). In previous studies, there were reports on the development of an efficient screening system for resistance against anthracnose among grape cultivars by pathogen inoculation (17) and by the use of culture filtrates from *E. ampelina* (18). Hence, it is necessary to examine the degree of resistance in grape cultivars growing in the country using an efficient screening system.

Screening of grapes' resistance or tolerance to anthracnose is one of the most important steps in developing disease resistant plants. It is usually done by visual inspection

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of natural infection in the vineyards, and by pathogen inoculation in the greenhouse. Diagnosis of the disease or determination of disease resistance by visual inspection of symptom expression in vines is very difficult and confusing, as symptoms may be influenced by many environmental factors. A more detailed, quantitative and systematic screening to determine anthracnose resistance in grapevines is needed to select the resistant cultivars efficiently.

This study aims to evaluate resistance against anthracnose by an efficient screening system such as pathogen inoculation in the greenhouse, bioassay using culture filtrates containing phytotoxic agents, as well as vineyard inspection.

Materials and Methods

Pathogen. The pathogen used in this study was a virulent strain EA-1 (*E. ampelina*), which was isolated from the infected leaves by Dr. W.K. Kim in the National Institute of Agricultural Science and Technology, RDA, Suwon, Korea.

Pathogen inoculation. To screen anthracnose resistance by vineyard evaluation and pathogen inoculation, 61 grape cultivars were tested: 'Benifuji', 'Black Olympia', 'Campbell Early', 'Cheongsoo', 'Fujiminori', 'Honey Black', 'Hongdan', 'Kaiji', 'Kyoho', 'Niagara', 'Red Queen', 'Rizamat', 'Rosario Bianco', 'Ruby Okuyama', 'Sheridan', etc.

Pathogen-free dormant cuttings were randomly collected from each cultivar and used for the study. Five rooted cuttings with three-nodes of each cultivar were individually used for pathogen inoculation. Several colonies of the pathogenic fungus were transferred to Fries liquid medium and incubated in a shaking incubator (140 rpm) at 28°C for 10 days. Pellets of cultures harvested by centrifugation were ground with a homogenizer in sterile distilled water, poured on a V-8 juice agar medium and incubated at 28°C under a near ultraviolet lamp to produce spores of pathogen for two days. Spores of pathogen were collected by scraping off the plates with sterilized distilled water, adjusted to 10^5 spores/m\ and sprayed onto rooted cuttings with eight to ten leaves. Cuttings inoculated with spore suspension were incubated in a humid chamber (28°C) for 48 hrs, and moved to the greenhouse. Two weeks after the inoculation, the lesions on the 7 leaves per shoot and 3 shoots per plant were counted in a greenhouse. Inocula adjusted to 10^5 spores/m\ of *E. ampelina* were sprayed on the grapevines with eight to ten true leaves, and resistance against anthracnose among the grape cultivars was evaluated by counting the lesions formed in the $3cm^2$ area of the leaves and in the 10 cm long shoots from the apex. Three cuttings from each cultivar were inoculated with sterile distilled water as a control.

Vineyard inspection. For the field test, naturally infected anthracnose lesions were counted in the vineyard from July to September each year for three years.

Bioassay with culture filtrates. After incubating the pathogen in Fries medium at 28°C for 21 days, fungal cell-free culture filtrates (CFCF) of *E. ampelina* were collected from supernatant by centrifugation at 11,000 rpm for five minutes and sterilized through ultra-filtration (pore diameter, 0.2μm). The upper 4th fully expanded leaf from the shoot apex of 42 grape cultivars were collected and injured slightly with a pencil tip or a scalpel. Thirty microliters of culture filtrates were dropped on the wounded portion of grapevine leaves using several dilution ratios. The fresh Fries medium used for the production of CFCF from *E. ampelina* were applied on the wounded portion of grapevine leaves as a control. The leaves treated with culture filtrates or media were incubated in a dark moist chamber for three days at 28°C. The size of the necrosis around the wounded spot was scored into 5 grades by visual inspection for the resistance evaluation.

Table 1. Susceptibility of grape leaves and shoots to *E. ampelina* inoculation and in field test.

Cultivar (species)	No. of lesions			
	Pathogen inoculation		Field screening	
	Leaves	Shoots	Leaves	Shoots
Beniyamabico (*Vitis* hybrid)	5.3 a[a]	7.8 abc	6.1 fgh	8.1 c
Hokkou (*V. vinifera*)	5.2 a	6.8 bcdefg	9.2 ab	7.4 c
Black Eye (*Vitis* species)	5.2 a	9.7 a	9.8 a	9.8 a
Black Sanjaku (*V. vinifera*)	5.1 a	4.9 fghi	7.1 def	5.7 d
Rizamat (*V. vinifera*)	4.9 ab	8.7 ab	9.1 abc	7.8 c
Black Swan (*V. vinifera*)	4.9 ab	9.8 a	8.5 bcd	8.6 bc
Zhana (*V. vinifera*)	4.8 abc	7.8 abc	5.6 fghi	7.5 c
Thompson Sds (*V. vinifera*)	4.5 abcd	4.6 fghi	9.1 abc	7.9 c
Guroryu (*Vitis* species)	4.5 abcd	3.9 hijkl	6.5 ef	5.2 de
Benizuiho (*Vitis* hybrid)	4.5 abcd	6.3 cdef	2.1 klmno	6.8 cd
Rosario Bianco (*V. vinifera*)	4.4 abcd	7.7 bcd	6.1 fgh	2.4 jklm
Manicure Finger (*V. vinifera*)	4.4 abcde	7.3 bcde	5.2 fghi	6.5 cd
Kaiji (*V. vinifera*)	4.4 abcde	7.5 bcde	8.0 cde	7.2 c
Ruby Okuyama (*V. vinifera*)	4.2 abcde	7.1 bcdef	7.1 def	3.8 ghi
Mario (*V. vinifera*)	4.2 abcde	3.8 hijkl	9.3 ab	9.1 b
71068 (*Vitis* hybrid)	4.2 abcde	3.6 hijkl	8.1 cde	3.7 fgh
Niunai (*V. vinifera*)	3.9 abcde	3.8 hijkl	9.2 ab	7.6 c
Seto Giant (*Vitis* species)	3.6 bcdef	6.7 bcdefg	3.8 jklm	5.3 de
Ryuho (*Vitis* hybrid)	3.6 bcdef	3.5 hijkl	6.2 fg	3.7 ghi
Pione (*Vitis* hybrid)	3.6 bcdef	6.0 cdefgh	2.1 klmno	2.9 ijkl
Kyoho (*Vitis* hybrid)	3.6 bcdef	6.0 cdefgh	3.5 jklmn	2.7 ijkl
Centennial Seedless	3.6 bcdef	6.0 cdefgh	4.2 hij	4.5 efgh
Beniizu (*Vitis* hybrid)	3.6 bcdef	5.7 defgh	1.7 opq	3.4 ghij
Takao (*Vitis* hybrid)	3.5 cdef	5.2 efghi	7.5 def	7.4 c
Koho (*Vitis* hybrid)	3.5 cdef	3.3 ijklm	3.9 jklm	2.6 jklm
Benifuji (*Vitis* hybrid)	3.5 cdef	5.5 defghi	2.3 jklmno	2.7 jklm
Rhodo Berry (*Vitis* hybrid)	3.2 defg	4.1 hijk	4.6 ghi	4.3 efgh
Kitasaki Red (*Vitis* hybrid)	3.2 defg	3.4 ijklm	6.1 fgh	4.8 def
Sekirei (*V. vinifera*)	3.1 defg	2.9 jklm	6.1 fgh	4.4 efg
Neo Muscat (*V. vinifera*)	3.1 defg	2.9 jklm	3.5 jklmn	2.5 ijkl
Fujiminori (*Vitis* hybrid)	3.1 defg	4.9 fghi	3.1 jklmn	2.5 jklm
Black Olympia (*Vitis* hybrid)	3.1 defg	5.3 efghi	7.2 def	4.2 efgh
Red Queen (*Vitis* hybrid)	3.0 efg	4.5 ghij	2.1 klmno	2.7 ijkl
Ryogyoku (*Vitis* hybrid)	2.9 efg	2.5 jklmn	3.5 jklmn	2.7 ijkl
Kokuou (*Vitis* species)	2.5 fgh	2.9 jklm	3.4 jklmn	3.4 ghij
Red Globe (*V. vinifera*)	2.4 fgh	4.3 hijk	3.2 jklmn	3.9 ghi
Ichikimar (*V. vinifera*)	2.4 fgh	4.0 hijkl	2.3 jklmno	2.5 jklm
Hongdan (*Vitis* hybrid)	2.3 fghi	3.9 hijkl	1.9 mnop	1.9 klmn
Venus (*V. vinifera*)	2.1 ghij	2.2 klmn	1.4 opq	1.6 klmn
Honey Black (*Vitis* hybrid)	2.1 ghij	3.8 hijkl	0.4 pq	0.5 mno
Unibala Seven (*V. vinifera*)	1.0 ghijk	3.5 ijklm	2.5 jklmno	3.6 ghi

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Table 1. (*continued)*

Cultivar	No. of lesions			
	Pathogen inoculation		Field screening	
	Leaves	Shoots	Leaves	Shoots
Tensyu (*Vitis* hybrid)	1.9 ghijk[a]	2.3 jklmn	2.1 klmno	2.4 ijkl
Neomat (*Vitis* species)	1.9 ghijk	2.6 jklmn	1.8 mnopq	2.2 jklm
Morgen Schon (*V. vinifera*)	1.8 ghijk	2.4 jklmn	2.1 klmno	1.9 klmn
Fuen (*Vitis* hybrid)	1.8 ghijk	1.9 klmn	2.7 jklmno	2.5 jklm
Takasumi (*Vitis* hybrid)	1.6 ghijk	2.5 klmn	2.8 jklmno	3.9 ghi
Shigyoku (*Vitis* hybrid)	1.5 ghijk	2.2 klmn	2.1 klmno	1.2 lmn
Kokuyou (*Vitis* species)	1.5 ghijk	1.9 lmno	1.7 mnopq	0.8 mno
Campbell Early (*Vitis* hybrid)	1.3 hijkl	2.2 klmn	2.2 klmno	1.8 klmn
Choryu (*Vitis* hybrid)	1.2 hijkl	2.1 klmn	1.7 klmnopq	1.2 lmn
Tamasizuku (*Vitis* species)	1.1 ijkl	1.2 hijkl	1.9 mnop	1.7 klmn
Black Rose (*V. vinifera*)	1.0 ijkl	1.9 lmno	2.0 lmno	2.9 ijkl
Christmas Rose (*Vitis* species)	0.9 jkl	1.5 mno	1.2 opq	1.5 klmn
Niagara (*V. labrusca*)	0.8 jkl	1.7 mno	0.2 pq	0.3 mnop
Tamnara (*Vitis* hybrid)	0.7 jkl	1.2 hijkl	0.2 pq	0.1 mnop
Sheridan (*Vitis* hybrid)	0.7 kl	0.9 no	0.1 pq	0.9 mno
Cheongsoo (*Vitis* hybrid)	0.7 fg	1.9 hijk	0.3 pq	0.7 mno
Beni Pizzutello (*V. vinifera*)	0.6 kl	0.4 no	0.1 pq	0.2 mnop
Kourgan Rose (*V. vinifera*)	0.1 l	0.4 no	0.9 pq	1.2 lmn
Izumo Queen (*V. vinifera*)	0.0 l	0.0 o	0.0 q	0.0 o
Benianyo (*Vitis* species)	0.0 l	0.0 o	0.9 pq	1.0 lmn

[a]Mean separation within columns by Duncan's multiple range test, $P=0.05$.

Bioassay with ethyl acetate extracts. Culture filtrates were extracted with ethyl acetate and extracts were dissolved in acetone, diluted into 1/2, 1/4, and 1/8 with distilled water, and applied to the wounded leaves with the same method as bioassay of culture filtrates.

Results and Discussion

Pathogen inoculation. Sixty one grape cultivars were tested by scoring the numbers of lesions per 3cm^2 area in the leaves after pathogen inoculation. Cultivars such as 'Black Swan', 'Rosario Bianco', and 'Kaiji' with more than 4 lesions per 3cm^2 area in the leaves were rated as susceptible, while 'Kyoho' and 'Benifuji' were moderately susceptible. 'Campbell Early', 'Niagara', 'Sheridan', and 'Izumo Queen' were resistant to anthracnose (Table 1). More than 7.0 lesions appeared in the 10 cm shoots from the top in grape cultivars such as 'Black Swan', 'Rizamat', Rosario Bianco', 'Kaiji', 'Ruby Okuyama' making them susceptible to anthracnose. There were less than 2.0 lesions in the shoots from 'Campbell Early', 'Niagara', 'Sheridan', and 'Izumo Queen', thus, they were rated as resistant to anthracnose.

Vineyard inspection. For the field test, anthracnose symptoms were counted in the vineyard from July to September each year for 3 years. Based on lesions on the leaves naturally infected with anthracnose in the vineyard, cultivars such as 'Black Eye', 'Mario', 'Niunai', 'Rizamat', and 'Rosario Bianco' with many lesions were rated as

Table 2. Reaction of grape cultivars to *E. ampelina* culture filtrates and ethyl acetate extracts.

Cultivar	Reaction by dilution ratio							
	Culture filtrates				Ethyl acetate extract			
	1	2	4	8	1	2	4	8
71068 (*Vitis* hybrid)	+++	+	+	-	+++	+	±	-
Benianyo (*Vitis* species)	-	-	-	-	-	-	-	-
Benifuji (*Vitis* hybrid)	++	±	-	-	++	±	-	-
Beniizu (*Vitis* hybrid)	++	±	-	-	++	±	-	-
Benizuiho (*Vitis* hybrid)	++	+	±	-	++	±	-	-
Black Eye (*Vitis* species)	+++	++	+	-	+++	++	+	-
Black Olympia (*Vitis* hybrid)	++	++	±	-	++	+	±	-
Black Sanjaku (*V. vinifera*)	++	++	±	-	++	++	-	-
Campbell Early (*Vitis* hybrid)	-	-	-	-	-	-	-	-
Choryu (*Vitis* hybrid)	++	+	±	-	++	+	+	±
Fuen (*Vitis* hybrid)	±	-	-	-	±	±		-
Fujiminori (*Vitis* hybrid)	++	+	+	±	+	+	-+	-
Guroryu (*Vitis* species)	+++	+	±	-	+++	+	±	-
Hokkou (*V. vinifera*)	+++	+	+	-	+++	+	±	-
Honey Black (*Vitis* hybrid)	++	+	-	-	++	±	-	-
Honey Red (*Vitis* hybrid)	+	+	±	-	+	±	-	-
Kaiji (*V. vinifera*)	+++	++	±	-	+++	++	±	-
Kitasaki Red (*Vitis* hybrid)	++	±	-	-	++	±	-	-
Koho (*Vitis* hybrid)	+	±	-	-	±	±	-	-
Kokuou (*Vitis* species)	+	-	-	-	+	-	-	-
Kokuyou (*Vitis* species)	+	±	-	-	+	±	-	-
Kyoho (*Vitis* hybrid)	++	+	+	-	++	+	±	-
Mario (*V. vinifera*)	+++	++	+	±	+++	+	±	-
Neo Muscat (*V. vinifera*)	+	+	±	±	+	+	+	+
Niagara (*V. labrusca*)	-	-	-	-	-	-	-	-
Niunai (*V. vinifera*)	+++	+	+	-	++	±	±	-
Pione (*Vitis* hybrid)	+	+	-	-	+	±	-	-
Red Queen (*Vitis* hybrid)	+++	+	-	-	+++	+	±	±
Rhodo Berry (*Vitis* hybrid)	++	+	-	-	+	+	-	-
Rizamat (*V. vinifera*)	+++	++	+	±	+++	++	+	±
Rosario Bianco (*V. vinifera*)	+++	++	+	±	+++	++	+	±
Ruby Okuyama (*V. vinifera*)	+++	+	-	-	+++	±	-	-
Ryogyoku (*Vitis* hybrid)	±	±	-	-	±	-	-	-
Ryuho (*Vitis* hybrid)	++	±	-	-	++	±	-	-
Sekirei (*V. vinifera*)	++	+	±	-	++	+	±	-
Shigyoku (*Vitis* hybrid)	++	+	±	-	++	+	±	-
Takao (*Vitis* hybrid)	+++	++	+	-	+++	++	-	-
Takasumi (*Vitis* hybrid)	+	+	-	-	+	+	-	-
Tamasizuku (*Vitis* species)	±	-	-	-	±	-	-	-
Tensyu (*Vitis* hybrid)	+	+	±	-	+	±	±	-
Thompson Sds (*V. vinifera*)	+++	++	+	±	+++	++	±	-
Venus (*V. vinifera*)	±	±	-	-	±	±	-	-

+++: necrotic area over 3mm from wounded spot, ++; necrosis of 2-3 mm over wounded spot, +; necrosis spreading to form area on wounded spot, susceptible, ±; slight necrosis -; no necrosis.

susceptible; 'Campbell Early', 'Niagara', and 'Sheridan' with less than 2.0 lesions were rated as be resistant. In the shoots, 'Black Eye', 'Mario', 'Niunai', 'Rizamat', and 'Rosario Bianco' were also found to be susceptible, and 'Campbell Early', 'Niagara', and 'Honey Red' were found to be resistant.

Culture filtrate and extract bioassay. Forty two cultivars among the grapevines evaluated for the resistance by pathogen inoculation and vineyard inspection were tested as to their reaction for *E. ampelina* culture filtrates and ethyl acetate extracts from culture filtrates. Bioassay results showed that some cultivars were tolerant to the culture filtrates, some were susceptible, while others were moderately resistant (Table 2). Among 42 grape cultivars, 'Rosario Bianco', 'Kaiji', and 'Rizamat' were found to be susceptible, 'Kyoho' and 'Benifuji' were moderately susceptible, and 'Sheridan', and 'Niagara' cultivars were resistant to culture filtrates of *E. ampelina* . The spectrum of sensitivity of grapes to culture filtrates was consistent with that of ethyl acetate extracts from culture filtrates in a number of grape cultivars. The spectrum of sensitivity to both culture filtrates and ethyl acetate extracts was also consistent with anthracnose susceptibility in a number of grape cultivars by pathogen inoculation test and vineyard test. Culture filtrates of pathogen at highly diluted concentrations as low one-eighth induced necrosis on leaves of the susceptible cultivar, 'Rosario Bianco'. This was also observed when undiluted droplets of the culture filtrates were placed on the leaves. In contrast, culture filtrates did not induce necrosis even with undiluted concentration on the leaves of resistant cultivar 'Niagara'.

It has been reported that grape cultivars showed varied tolerance to anthracnose. For instance, *V. vinifera* was highly susceptible, *V. labrusca* and hybrids were resistant or moderately resistant, and *V. rotundifolia* was immune to *E. ampelina* (3, 7, 9, 13, 14).

At present, evaluating resistance to anthracnose is done by planting the progeny or cuttings in the vineyard and symptoms are rated from natural infection (8, 14). Mortensen (8) tested a method of artificial inoculation in young grape seedlings with *E. ampelina* and found that inoculation method was less reliable than several years of vineyard observations during the warm, humid summer months. He reported that because of failure of sporulation of the pathogen in PDA (potato dextrose agar) culture, he was not able to develop any reliable sources of spores for artificial screening of young seedlings. Sporulation of *Elsinoe* species was very difficult to induce in artificial media (12, 15). In this study, however, a system for sporulation of *E. ampelina* in the medium by using liquid culture, grinding, and incubation of the pathogen in a V-8 juice agar under the near ultraviolet has been proposed. Jayasankar et al. (2) selected disease resistant plants in vitro selection of *V. vinifera* 'Chardonay' with *E. ampelina* culture filtrate. Since the culture filtrates contained some toxic compounds related to virulence of pathogenic fungi and induction of resistance in grapevines, techniques using toxic compounds from culture filtrates can be developed as an alternative approach to screen efficiently for resistance to anthracnose in the future.

Results of the this study showed that resistant cultivars identified through greenhouse screening by artificial inoculation and by bioassay with culture filtrates from the pathogen were also found to be resistant to anthracnose infection under vineyard conditions. The present study also showed that resistant cultivars identified from greenhouse screening by artificial inoculation and vineyard test were also found to be resistant to culture filtrates of the pathogen. Furthermore, results of the three screening methods showed the same pattern in resistance among grape cultivars. Specifically, bioassay

of grapevine leaves with culture filtrates showed that their phytotoxicities were active and host-selective. The sensitive range of grapevines to culture filtrates was consistent with the host range to the pathogen. It showed the same pattern as the assay results in apple with AM-toxins produced by *Alternaria mali* and pear leaves with AK-toxins produced *A. kikuchiana* (4, 10, 11, 16). Quantitative differences in resistance against toxins were observed among cultivars in plants and these differences were correlated with resistance to the pathogens (1).

The screening procedure using culture filtrates for grape anthracnose resistance is accurate, economical, and labor-saving in terms of selecting resistant grapevine cultivars. These data for determining anthracnose resistance will be very useful in grape breeding program for developing disease resistant grape cultivars.

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