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Identification and characterization of pathotypes in *Puccinia horiana*, a rust pathogen of *Chrysanthemum* x *morifolium*

Mathias De Backer · Hossein Alaei · Erik Van Bockstaele · Isabel Roldan-Ruiz · Theo van der Lee · Martine Maes · Kurt Heungens

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Abstract *Puccinia horiana* is the causal agent of chrysanthemum white rust or Japanese rust. This microcyclic autoecious rust has a quarantine status and can cause major damage in the commercial production of *Chrysanthemum* x *morifolium*. Given the international and often trans-continental production of planting material and cut flowers of chrysanthemum and the decreasing availability of registered fungicides in specific regions, breeding for resistance against *P. horiana* will gain importance and will need to involve the appropriate resistance genes for the pathotypes that may be present. As pathotypes have not been well characterized in this system, the main objective was to build an international collection of isolates and screen these on a large collection of cultivars to identify different pathotypes. Using a robust and high throughput bioassay, we tested 36 selected cultivars with 22 individual single-pustule isolates of *P. horiana*. The isolates originated from three different continents over 4 different collection years and included some isolates from cultivars previously reported as resistant. In most cases the bioassays resulted in a clear scoring of interaction phenotypes as susceptible or resistant, while in several cases consistent intermediate phenotypes were found, often on specific cultivars. Twenty-four of the cultivars gave a differential interaction phenotype profile. All isolates produced a unique profile, infecting a minimum of 4 and a maximum of 19 differential cultivars. Based on the Person analysis of these profiles, this pathosystem contains at least seven resistance genes (and seven avirulence genes), demonstrating the highly complex race structure in this pathosystem.

Keywords Avirulence · Bioassay · *Chrysanthemum x morifolium* · Elicitors · Resistance · Rust

M. De Backer · H. Alaei · E. Van Bockstaele · I. Roldan-Ruiz · M. Maes · K. Heungens (✉)
Institute for Agricultural and Fisheries Research (ILVO),
Plant Sciences Unit,
Burg. Van Gansberghelaan 96 bus 2,
9820 Merelbeke, Belgium
e-mail: kurt.heungens@ilvo.vlaanderen.be

H. Alaei
Faculty of Agriculture, Department of Crop Protection,
Vali-e-Asr Rafsanjan University,
518 Rafsanjan, Iran

E. Van Bockstaele
Faculty of Bioscience Engineering,
Department of Plant Production, Ghent University,
Coupure links 653,
9000 Ghent, Belgium

T. van der Lee
Plant Research International,
Biointeractions and Plant Health,
Droevendaalsesteeg 1,
6708 PB Wageningen, the Netherlands

Introduction

Puccinia horiana Hennings is an autoecious microcyclic rust fungus (Pucciniales) that causes Chrysanthemum

white rust. The teliospores are normally formed on the lower side of the leaves and can germinate *in situ* into a promycelium without a period of dormancy (Firman and Martin 1968; Kapooria and Zadoks 1973). On every promycelium an average of two basidiospores are formed (Kapooria and Zadoks 1973), which are the mobile and infective propagules. The optimal conditions for development of the pathogen are a high relative humidity and cool temperatures (17–20°C) (Firman and Martin 1968). Under these conditions, symptoms appear 7–10 days post infection as chlorotic spots that develop teliospores after 14–18 days. Leaf wetness and a high relative humidity are essential for basidiospore formation, survival, and infection (Firman and Martin 1968; Zandvoort et al. 1968).

P. horiana can infect more than 10 *Chrysanthemum* species (Hiratsuka 1957; Punithalingam 1968), but it is especially known as a pathogen of the commercially important *Chrysanthemum* x *morifolium*. This species is grown for the production of cut flowers, potted plants and garden chrysanthemums, in numerous varieties and flowering forms. The turnover of chrysanthemum on the Dutch flower auctions in 2008 was €332 × 10^6 for cut flowers and €30 × 10^6 for potted plants, making it one of the most important florist species (Anon. 2009). The cut flower varieties are mostly produced in Japan, the Netherlands, Italy and Colombia and are usually grown in greenhouses or in plastic tunnels (Spaargaren 2002). Multiflora chrysanthemums (garden mums) are usually grown outdoors (Tierens 2007).

Puccinia horiana was first detected in Japan in 1895 (Hennings 1901; Hiratsuka 1957), from where it spread to China and South Africa (Priest 1995). Since 1963 it has been reported in England and other European countries (Baker 1967). Currently, the pathogen has been reported in most Chrysanthemum-growing areas (Whipps 1993; EPPO 2004), where it can cause significant economic loss if not controlled properly. It is classified as a pathogen of quarantine importance for the European and Mediterranean Plant Protection Organization (EPPO), the Interafrican Phytosanitary Council (IAPSC), the Junta del Acuerdo de Cartagena (JUNAC) (currently "Comunidad Andina", (CAN)) and the North American Plant Protection Organization (NAPPO) (EPPO 2004).

As soon as *P. horiana* was reported as a major problem for chrysanthemum growers in the UK and Germany, the presence of resistance in local host cultivars was studied (Stark and Stach 1965; Baker 1967; Dickens 1968). Dickens (1968) evaluated the resistance of 37 cultivars with an isolate from cv. Favourite. He observed resistance in the cvs Princess Anne and Rival's Rival, which were reported as susceptible in the study of Baker (1967). Cv. Princess Anne was also reported as susceptible in Sweden (Nilsson 1964), Denmark (Anon. 1964) and Norway (Gjaerum 1964). Dickens (1971) showed that cv. Favourite did not display any sign of infection after inoculation with an isolate from cv. Mayford Perfection. An inoculation study on 270 cultivars by Martin and Firman (1970) showed comparable discrepancies with the previously described studies. Wojdyla (1999) also described some discrepancies in susceptibility between his study and other inoculation experiments. A study in which six Japanese isolates were inoculated on 40 cultivars involved the first systematic testing of multiple isolates on multiple hosts, and revealed differential interaction phenotype profiles for several of the cultivars used (Yamaguchi 1981). A more recent study performed in Mexico on five cultivars with 16 Mexican isolates from four different regions showed variance in virulence, but did not clearly describe pathotypes (Velasco et al. 2007). These data all suggest the presence of pathotypes (physiological races), most likely as a result of gene-for-gene type of interactions (Flor 1956; Flor 1971; Stukenbrock and McDonald 2009).

Resistance to host-specific pathogens that can overcome the plant basal resistance, such as rusts, depends on the presence or absence of avirulence (Avr) genes in the pathogen and the corresponding resistance (R) genes in the host. These avirulence factors are often effector molecules that play a role in the infection process (Bent and Mackey 2007). In such interactions, the Avr gene product (recently referred to as an elicitor) can interact with the product of an R gene in the host to start a cascade of host defense reactions, which often leads to an hypersensitive reaction (HR-reaction) and ultimately, resistance (Staskawicz et al. 1995). In rusts, specific proteins secreted by haustoria into the host cell have been shown to be elicitors (Catanzariti et al. 2007).

Flor (1956) showed that in gene-for-gene type of resistance interactions, multiple resistance genes can be present in the host, whose products each react with the products of corresponding Avr genes in the pathogen. In such ideal pathosystems with n R genes

(and n Avr genes), 2^n different host genotypes (cultivars) can produce differential reactions with 2^n different pathogen genotypes (pathotypes). Some pathosystems involving gene-for-gene relations have been studied in detail using genetic and molecular analysis. The number of R and Avr genes present in these pathosystems has been determined, pending the discovery of new pathotypes or differential cultivars (Ellis et al. 2007; Bolton et al. 2008). In several cases, this number can be determined even if not all cultivars and pathotypes are known, via analysis of the proportions of resistance-breaking pathotypes and the proportions of susceptible cultivars in an incomplete set of cultivars and pathotypes (Person 1959). Especially if more than three resistance genes are present, multiple isolates need to be tested on multiple cultivars to obtain reliable proportions.

Considering the increasing international importance of Chrysanthemum and its planting material and the increasing restriction of chemical fungicide use in some regions, breeding for resistance to a relevant set of pathotypes is becoming more important. Since most published inoculation studies with *P. horiana* used only a limited number of isolates, we developed a robust and efficient screening assay that is compact, easy, and reliable. Using this assay we tested an international collection of isolates on a set of cultivars for which anecdotal reports of differential reactions had been noted by some of the main chrysanthemum-breeding companies. These data are used to determine the minimum number of Avr (and R) genes involved in this pathosystem and to identify isolates with different specificity that can be exploited in R gene identification in resistance breeding programs.

Materials and methods

Fungal isolates

A total of 22 isolates of *P. horiana* were collected on commercially-grown *Chrysanthemum* x *morifolium* plants between 2003 and 2009 (Table 1). The main selection criterion was a wide geographic distribution, including isolates from different continents. For the Belgian isolates, the geographic origin surveyed most intensively due to our location, we included collection year as an extra factor. For isolates collected after 2005, breeders were asked to preferentially provide symptomatic plant material from cultivars that were previously reported as resistant, which increased the probability of encountering new pathotypes. Diseased

Table 1 Isolates of *Puccinia horiana* with isolate code, origin, collection year and source

Isolate code	Origin	Collection year	Source[a]
BE1	Belgium, region Ghent	2003	ILVO
BE2	Belgium, region Ghent	2003	ILVO
BE3	Belgium, region Ghent	2003	ILVO
BE4	Belgium, region Ghent	2005	ILVO
BE5	Belgium; region Mechelen	2007	Gediflora
BE6	Belgium; region Mechelen	2008	Gediflora
CO1	Colombia; region Boyaca	2008	ICA
CO2	Colombia; region Cundinamarca	2008	ICA
FR1	France	2007	BBV
FR2	France	2008	BBV
GB1	United Kingdom	2005	FERA
GB2	United Kingdom	2005	FERA
GB3	United Kingdom	2005	FERA
JP1	Japan, region Hiroshima	2008	Deliflor
JP2	Japan, region Hiroshima	2008	Deliflor
JP3	Japan, region Tochigi	2009	Deliflor
MY1	Malaysia, region Cameron Highlands	2008	Dekker Breeding
MY2	Malaysia	2008	Dekker Breeding
NL1	the Netherlands	2006	PD
NL2	the Netherlands	2008	RVZ
PL1	Poland	2006	INSAD
US1	USA (Massachusetts)	2008	USDA APHIS PPQ

[a] Samples obtained via: ILVO: Institute for Agricultural and Fisheries Research, Merelbeke, Belgium / Gediflora, Staden-Oostnieuwkerke, Belgium / ICA: Instituto Colombiano Agropecuario, Bogota, Colombia / BBV: Bretagne Biotechnologie Végétale, Saint Pol de Léon, France / FERA (formerly CSL): The Food and Environment Research Agency, York, United Kingdom / Deliflor, Maasdijk, the Netherlands / Dekker Breeding, Hensbroek, the Netherlands / PD: Plant Protection Service, Wageningen, the Netherlands / RVZ: Royal Van Zanten, Rijsenhout, the Netherlands / INSAD: Research Institute of Pomology and Floriculture, Skierniewice, Poland / USDA APHIS PPQ: United States Department of Agriculture—Animal and Plant Health Inspection Service—Plant Protection and Quarantine.

plant material was used to inoculate fresh cuttings of the susceptible cvs Medonia, Taliedo or cv. 29 (see "Cultivars" section) as described by Alaei et al. (2009). Single pustule cultures of each isolate were established before conducting pathotype tests. All the isolates were maintained by tri-weekly transfer onto fresh and rust-free cuttings of cvs Medonia and Taliedo as described by Alaei et al. (2009), except the Colombian isolates, which were maintained on cv. 29, but using the same transfer schedule. Because of the quarantine status of *P. horiana*, the pathogen was only handled in laboratories and growth chambers that fulfill the biosafety rules for quarantine plant pathogens.

Cultivars

A set of 36 Chrysanthemum test cultivars was selected based on the multi-year experience of four major Chrysanthemum breeding companies: Deliflor (Maasdijk, NL), Dekker Chrysanten (Hensbroek, NL), Fides (De Lier, NL), and Royal Van Zanten (Rijsenhout, NL). This set consisted of cultivars that are usually reported as susceptible (positive controls), cultivars assumed to be resistant (using inoculation tests with non-characterized isolates), and cultivars for which anecdotal reports of infections were reported to the breeders. Details of the test cultivars are given in Table 2. Mother plants of these cultivars were maintained by Fides. Upon request, 3-week-old rooted cuttings of 10–15 cm height were prepared in Grodan SBS 36/77 rockwool blocks (Roermond, the Netherlands) and transferred to our test facility where they were maintained in a greenhouse for maximum 1 week before inoculation. The cultivars can be requested from the respective companies for scientific purposes.

Bioassay

The first objective was to develop a compact and reliable screening assay. Given these criteria and the need for leaf wetness and a high humidity for successful infection, we developed an assay that could be conducted on relatively small plants inside closed plastic containers, several of which should easily fit in a controlled environment such as a growth chamber. Expanding on previously-described inoculation methods (Firman and Martin 1968; Yamaguchi 1981; Takatsu et al. 2000; Alaei et al. 2009), different inoculation techniques (suspending infected leaves from the lid of the container versus spraying of macerated leaves over the plants), different ways of suspending leaves (with tape versus with agar),

Table 2 Cultivars used, including cultivar number, plant type and source

Cultivar number	Type	Source[a]
1	Multiflora	Royal van Zanten
2	Cut flower	Deliflor
3[b]	Cut flower	Royal van Zanten
4	Cut flower	Fides
5	Cut flower	Deliflor
6	Cut flower	Deliflor
7	Cut flower	Deliflor
8	Cut flower	Deliflor
9	Cut flower	Deliflor
10	Cut flower	Deliflor
11	Cut flower	Deliflor
12	Cut flower	Deliflor
13	Cut flower	Dekker Chrysanten
14	Cut flower	Dekker Chrysanten
15	Cut flower	Dekker Chrysanten
16	Cut flower	Dekker Chrysanten
17	Cut flower	Dekker Chrysanten
18	Cut flower	Dekker Chrysanten
19	Cut flower	Dekker Chrysanten
20	Cut flower	Dekker Chrysanten
21	Cut flower	Fides
22	Cut flower	Fides
23	Cut flower	Fides
24	Cut flower	Fides
25	Cut flower	Fides
26	Cut flower	Fides
27	Pot flower	Fides
28	Multiflora	Fides
29	Cut flower	Royal van Zanten
30	Cut flower	Royal van Zanten
31	Pot flower	Royal van Zanten
32	Multiflora	Royal van Zanten
33	Multiflora	Royal van Zanten
34	Multiflora	Royal van Zanten
35	Multiflora	Royal van Zanten
36	Multiflora	Royal van Zanten

[a] Royal van Zanten (RVZ), Rijsenhout, the Netherlands / Fides, De Lier, the Netherlands / Deliflor, Maasdijk, the Netherlands / Dekker Chrysanten, Hensbroek, the Netherlands. [b] cv. 3=Alec Bedser

different plant sizes, different substrates (peat versus rockwool), and different plastic containers of different sizes were tested in preliminary assays. Eventually, we chose plastic containers of 46.5 cm length × 26.2 cm width × 26.0 cm height (Savic, Kortrijk, Belgium). Glass plates cut to size were used as covers during inoculum production. The inoculum of basidiospores was generated by approximately 36 heavily-infected whole leaves distributed evenly and stuck to the covers with their telia pointing downwards using 1% water agar as adhesive. To obtain the heavily-infected whole leaves used for the inoculation, 50 fresh cuttings placed in Grodan AO 36/40 rockwool slabs were used per plastic container. For pathotype screenings, which involved larger cuttings, the plants were placed in plastic trays (9 by 4 wells, cut out of Grodan SBS 36/77 trays) on the bottom of each container and covered by an inverted second container. The test chamber height was increased to allow for larger plants. Only 18 plants were used per tray, using alternate positions. In this way, we avoided shadow effects caused by larger leaves covering the smaller plants that could possibly have resulted in false negative results. The space was high enough to ensure homogeneous dispersal of the spores over the larger test plants and avoided that the plants reached the cover during the incubation period. To ensure a high relative humidity and a water film on the leaves, the cuttings, the inner sides of the plastic containers and the covers holding the inoculum were misted with demineralized water using a Preval Sprayer (Yonkers; NY; USA). A layer of approximately 1 cm of demineralized water was placed in the bottom of the plastic container, providing water for the plants and further contributing to a high relative humidity. After setup, the plastic containers were placed in a dark growth chamber at 17°C. Two days after the start of the inoculation (dpi), fluorescent light (Gro-lux® F58W/GRO-T8, Osram Sylvania, MA, USA) was provided during 16 h per day. In general, symptoms were easy to evaluate at 21 dpi, but a final evaluation was done at 28 dpi to include possible retarded symptoms.

To test the homogeneity and the dose of inoculum, three microscope slides were placed in each plastic container at the level of the cuttings during two separate screenings (using isolates CO2 and BE4). After 24 h, the slides were removed and the number of basidiospores in one square mm was counted at three arbitrarily chosen locations on each slide. Data were analyzed using ANOVA in Statistica 9.0 (Statsoft, OK, USA).

Before each assay, plastic containers and covers were always washed and subsequently sterilized with 70% ethanol. Trays were washed and subsequently sterilized in 0.5% sodium hypochlorite overnight.

Pathotype screening

The 22 isolates were separately inoculated on the 36 test cultivars (Tables 1, 2). Inoculum was prepared for each isolate of *P. horiana* using fresh cuttings of cvs Medonia, Taliedo, and in case of the Columbian isolates, cv. 29, as described above. The single-pustule cultures were increased to approximately 150 infected cuttings during three to four three-week cycles to obtain a sufficient number of infected leaves. Per isolate, three cuttings (replicates) of each of the 36 test cultivars were inoculated. Each set of 36 test cultivars was randomly distributed over two plastic containers (18 cultivars per container). The screenings were conducted between July 2007 and December 2009. To test the reproducibility of the results and account for possible effects due to differences in the physiological status of the host, nine isolates were screened twice, usually separated by several months.

Disease scoring

Plants were scored according to Table 3. If no teliospores were observed, the interaction was rated

Table 3 Interaction phenotype scoring and interpretation

Interaction phenotype score	Number of cuttings with infection score[a]			Interaction phenotype interpretation
	2	1	0	
0	0	0	3	resistant
1	0	1	2	resistant
2	0	2	1	resistant
3	0	3	0	resistant
4	1	2	0	susceptible (S)
5	2	1	0	susceptible (S)
6	3	0	0	susceptible (S)

[a] See Materials and methods for scoring system

Table 4 Interaction phenotype score (See Table 3) of 22 isolates of *Puccinia horiana* (columns; see Table 1) on 36 *Chrysanthemum* x *morifolium* cultivars (rows; see Table 2). Isolates with two sets of numbers (upper and lower) were tested twice, at different time points

Cultivar number	Isolates																					Comm.[a]
	BE1	BE2	BE3	BE4	BE5	BE6	CO1	CO2	FR1	FR2	GB1	GB2	GB3	JP1	JP2	JP3	MY1	MY2	NL1	NL2	PL1	US1	
1	3	3	3	2	2	2	1	5	3	4	4	6	6	5	6	6	1	4	6	6	3	6	
				3	4	3	0	3			3			6			5					6	
2	6	6	6	6	6	6	4	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	
				6	6	6	6	6			6			6			6					6	
3	6	6	3	6	3	6	4	6	5	0	6	4	6	0	1	6	6	6	6	6	3	6	
				6	3	3	3	6			6			3			6					6	
4	1	2	3	2	3	3	0	0	2	3	0	3	3	1	3	3	0	0	3	6	3	5	V, W
				3	0	4	0	3			6			4			0					6	
5	0	6	6	0	0	6	0	0	6	6	6	0	6	0	0	0	6	0	6	6	6	2	
				0	0	6	0	0			6			0			6					1	
6	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
				0	0	0	0	0			0			0			0					0	
7	3	1	3	3	2	3	0	1	4	2	3	2	3	0	0	1	6	6	6	0	3	0	V, W
				3	3	3	0	3			3			0			6					0	
8	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
				0	0	5	0	0			0			0			0					0	
9	0	0	0	0	6	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
				0	6	6	0	0			0			0			0					0	
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
				0	0	0	0	0			0			0			0					0	
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
				0	0	0	0	0			0			0			0					0	
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
				0	0	0	0	0			0			0			0					0	
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	
				0	0	0	0	0			0			0			0					0	
14	6	6	6	6	6	6	5	0	6	6	6	6	6	6	6	6	6	6	6	6	6	6	
				6	6	6	5	0			6			6			6					6	
15	4	0	3	3	2	5	0	1	3	6	2	2	3	4	6	3	0	0	6	0	3	3	W, Z
				3	0	5	0	3			5			6			3					3	
16	3	3	6	6	3	5	0	1	2	0	3	6	6	6	6	6	6	2	6	6	6	6	Z
				6	0	6	0	3			6			6			6					6	
17	3	3	6	3	0	0	0	0	0	0	0	3	2	6	6	3	0	0	6	6	2	2	Z
				3	0	4	0	0			4			6			0					6	
18	0	0	0	0	6	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
				0	6	6	0	0			0			0			0					0	
19	0	0	3	0	0	0	0	2	0	6	0	0	0	0	0	0	6	0	6	0	0	0	

				0	0	0	0	3			0			0			6					0		
20	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	6	6	6	6	0	0		
				0	0	0	1	3			0			0			6					0		
21	0	0	0	0	6	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
				0	6	6	0	0			0			0			0					0		
22	0	0	3	0	0	0	0	0	0	6	4	0	0	0	0	0	6	0	6	6	0	0	W, X	
				0	0	0	0	0			0			0			6					0		
23	0	0	0	0	0	0	3	6	0	0	0	0	0	0	0	1	6	6	6	6	0	2		
				0	0	0	5	6			0			0			6					0		
24	0	3	3	0	0	6	0	0	6	6	4	0	5	0	0	0	6	0	6	6	2	0		
				1	0	6	0	0			6			0			6					0		
25	2	4	3	0	0	2	0	0	2	2	0	0	0	0	3	5	0	0	1	0	2	0	Y	
				3	0	6	0	0			1			3			0					0		
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
				0	0	0	0	0			0			0			0					0		
27	3	3	6	6	3	5	0	3	3	0	5	6	3	0	0	0	6	6	6	0	4	0	W, X	
				3	3	6	0	3			4			0			6					0		
28	0	0	0	0	0	0	0	2	0	0	3	0	3	0	1	3	0	3	6	3	0	5		
				0	0	0	0	2			3			3			3					6		
29	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6		
				6	6	6	6	6			6			6			6					6		
30	3	0	0	6	0	0	0	0	0	0	0	6	0	6	6	3	0	1	6	6	0	2	Y	
				1	0	0	0	0			1			6			0					6		
31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	6	6	0	3		
				0	0	0	0	1			3			6			0					3		
32	0	0	0	0	2	2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0		
				0	3	3	0	0			0			0			0					0		
33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
				0	0	0	0	0			0			0			0					0		
34	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
				0	0	4	0	0			0			0			0					0		
35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
				0	0	0	0	0			0			0			0					0		
36	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	4	0	0	1		
				0	0	0	0	0			0			0			0					0		

[a] Comments: (V) infection on stipules; (W) infection on leaf tips; (X) infection on upper side of the leaf; (Y) symptoms limited to minor puncture-like lesions, only visible in counter light, and no formation of teliospores; (Z) retarded symptom expression (teliospore formation after 4 weeks instead of 2–3 weeks)

"0". If plants contained more than 10 well-developed pustules per leaf, the interaction was rated "2". A score of "1" was given to interactions if only a few pustules on a few leaves were observed. Interactions with a score of "1" included plants that only showed infection on the stipules, the leaf tips, or the upper side of the leaf. In case macroscopic evaluation of a lesion was difficult, leaves were checked microscopically for the presence of teliospores. The scores of the three replicate interactions were added together, resulting in an overall interaction phenotype score per cultivar ranging from 0 to 6 (Table 3). The interaction phenotypes were reduced to two groups to allow more detailed analysis. Interaction phenotypes were rated "susceptible" (the given isolate was considered "virulent" on the given cultivar) with a score of 4 or more or "resistant" (the given isolate was considered "avirulent" on the given cultivar) with a score of 3 or less. For the nine isolates that were screened twice on the set of cultivars, interaction phenotypes with a score of at least 4 in one of the two replicate tests were rated "susceptible". The choice of the cutoff value was validated by analysis of frequency distribution of the interaction phenotype scores (See Results and Discussion sections).

Analysis of interaction phenotype profiles

After classification of each interaction as susceptible versus resistant, the cultivar-by-isolate interaction phenotype matrix was sorted based on decreasing virulence of the isolates (number of cultivars they were able to infect) and increasing susceptibility of the cultivars (number of isolates they are susceptible to). To determine the minimum number of resistance genes involved in this pathosystem, the sorted matrix was analyzed according to two methodologies. The first analysis was based on the fact that in an ideal gene-for-gene system with a total of n R genes (and n Avr genes), cultivars lacking x R genes can be infected by 2^x pathotypes that contain up to x Avr genes. The number of cultivars that can be infected by 2^x pathotypes is dependent on the number of R genes in the system and follows a binomial distribution (n!/((n-x)!*x!)) (Person 1959). The second analysis of the pathosystem was based on the geometric rule (Person 1959), which implies that for any two cultivars or pathotypes, the number of specific R genes or Avr genes missing in both cultivars or pathotypes (y) results in 2^y common susceptible interactions. Examination of the number of common susceptible interactions therefore allows determination of the number of commonly missing Avr or R genes. For example, if one cultivar is infected by eight pathotypes and the other by 16, and there are four pathotypes that infect both cultivars, then the first cultivar lacks three R genes, the second one lacks four R genes, and two specific R genes are lacking in both cultivars.

Results

The new bioassay allowed the simultaneous screening of 18 large cuttings on a surface area of approximately 0.12 m^2. Inoculation using infected leaves attached to the cover of the plastic container produced consistent and uniform infection in preliminary experiments, in contrast to inoculation with macerated leaf suspensions (data not shown). The use of rockwool as a substrate allowed cleaner and easier handling of the plants, and resulted in few to no problems with infections due to *Botrytis* compared to peat-based potting media. The basidiospore inoculum dose at the level of the plants varied from 20 to 170 spores mm^{-2}, with an average (± SD) of 78.5±29.2 spores mm^{-2}, a lower quartile of 58 spores mm^{-2}, and an upper quartile of 93.5 spores mm^{-2}. No significant difference (P=0.32) was observed between the two isolates (71.6±12.5 spores mm^{-2} for plastic containers inoculated with CO2 versus 85.5±29.4 spores mm^{-2} for plastic containers inoculated with BE4). Within each isolate, the difference in spore dose between the plastic containers (6 per isolate) was also not significant, except for the two most differing plastic containers of isolate BE4 (115.8±39.5 spores mm^{-2} versus 34.1±16.4 spores mm^{-2}; P=0.026). Within plastic containers, several statistically significant differences were observed, but on average, the SD between the different sampling points within a plastic container was only 19.2 spores mm^{-2}.

The results of the screening are presented in Table 4. A total of 82.5% of the interaction phenotypes showed either no symptoms (61.8% had a score of 0) or severe symptoms (20.7% had a score of 6). The third most common interaction phenotype was a score of 3 (8.7%), resulting from a consistent scoring of minor symptoms (1+1+1) on the three replicate plants. Such consistent minor symptoms were mostly observed on specific cultivars: 61% of

score 3 was given to 6 cultivars (cvs 1, 4, 7, 15, 27, 28). Only 8.8% of the interaction phenotypes had variable scoring between replicates of the same experiment, but a score of 0 as well as a score of 2 was never observed together.

In general, the results of the nine isolates that were tested twice were consistent. Using the scoring system of ≤ 3 for a resistant and ≥ 4 for a susceptible interaction phenotype, consistent classification was obtained in 302 out of the 324 combinations (93.2%) that were repeated. With isolates BE4, BE6, GB1, and JP1 larger variation was observed, frequently on the same cultivars, e.g., cvs 1, 4, 17 and 30. If omitting these more variable isolates and cultivars, consistent classification was obtained in 169 out of 172 cases (98.3%). However, as these few variable interactions may represent a biologically interesting phenomenon and as their phenotype did not affect the main conclusions (see further), they were included in further analyses.

Eight cultivars (cvs 6, 10, 11, 12, 26, 32, 33, 35) showed complete resistance against all isolates tested and two cultivars (cvs 2, 29) showed overall susceptibility. There were three other groups of cultivars that showed the same interaction phenotype profile for all isolates: cvs 9, 18 and 21, cvs 8 and 34, and cvs 13 and 36. None of the 22 isolates showed an identical infection profile on the set of 36 cultivars so each of them represents a different pathotype, and will be referred to as such.

A non-redundant matrix of 22 pathotypes by 24 cultivars (Table 5) was obtained after grouping of the cultivars with the same profile and reduction of the interaction phenotypes to susceptible vs. resistant (see Materials and methods). A cultivar listed in this table is interpreted in the sense of Person (1959), namely, a differential cultivar. In Table 5, the isolates (columns) were sorted based on decreasing number of virulent interactions and cultivars (rows) were sorted based on increasing number of susceptible interactions. This allowed further analysis of the data based on Person (1959), assuming that resistance in this pathosystems is indeed based on gene-for-gene type of interactions. Cultivars and isolates at the edges of this table are the most informative about the presence and absence of R genes and Avr genes, respectively. Cv. 6 was the universally resistant cultivar, resistant to all pathotypes used. On the other hand, cv. 2 was susceptible to all tested pathotypes. No pathotype tested infected all cultivars (the universally virulent isolate). Similarly, no isolate was observed that could only infect the universally susceptible cultivar (the universally avirulent isolate).

NL1 was able to infect the largest number of differential cultivars (19 out of 24). Other isolates that were able to infect a large number of cultivars were NL2, BE6 and MY1, with 14, 13 and 13 differential cultivars, respectively. Isolates BE1, BE5 and CO1 were the isolates that could infect the smallest number of differential cultivars (4). The remaining isolates were virulent on 5 to 12 cultivars, with a variable capacity to also produce minor symptoms (scores 1 to 3) on additional cultivars (Tables 4, 5).

We identified one cultivar (cv. 14) that was susceptible to 21 pathotypes. As the number of pathotypes a cultivar can be susceptible to is 2^x, this cultivar is susceptible to at least 32 pathotypes indicating that it is missing at least five ($2^5=32$) R genes. Based on the same rationale, NL2, BE6, MY1 and GB1 are able to infect at least 16 cultivars and are missing at least four Avr genes. Pathotypes NL2, BE6 and GB1 have 13, 9 and 11 susceptible interactions in common with NL1, respectively. Based on the geometric rule, this means they each have at least 16 susceptible interactions in common with NL1. In each case, there are also bidirectional differential interactions. As different pathotypes that each infect 16 cultivars can only have a maximum of eight susceptible interactions in common, pathotypes NL2, BE6 and GB1 have to infect at least 32 cultivars and lack at least five Avr genes. Based on the number of common susceptible interactions between MY1 and NL2 or GB1, we can also conclude that MY1 has to infect at least 32 cultivars. This brings the number of isolates infecting at least 32 cultivars to five, and these isolates have six cultivars in common that they can all infect. This situation can only be explained in an ideal system with seven or more R/Avr genes. Analysis of the number of common susceptible interactions in the most susceptible cultivars results in the same conclusion. With at least seven genes, the minimum number of possible pathotypes and cultivars is 128 ($=2^7$). Most of the possible pathotypes and differential cultivars are lacking from our available set and further determination of the number of R/Avr genes based on the geometric rule is therefore speculative. As a result, it was not possible to designate specific R genes and Avr genes to most cultivars and isolates, respectively, even though the minimum number of missing R genes and Avr genes

Table 5 Pathotypes of *P. horiana* (columns) and representative differential cultivars of *Chrysanthemum* x *morifolium* (rows) that were identified based on the observed interaction phenotype scores (Table 4) and the interaction phenotype interpretation (Table 3). Susceptible interactions are marked with "S". Cultivars were sorted increasingly based on the number of susceptible interactions. Pathotypes were sorted decreasingly based on the number of infecting cultivars. Presence (upper case letter) or absence (lower case letter) of specific Avr genes in the pathotypes and R genes in the cultivars is listed where possible. Avr/R genes labeled in italics were added based on the assumption of a single Avr gene in NL1 (see Results)

								Avr gene[a]	Pathotype																						
									NL1	NL2	BE6	MY1	GB1	US1	JP1	JP2	FR2	MY2	GB2	GB3	BE3	BE4	FR1	JP3	BE2	CO2	PL1	BE1	BE5	CO1		
								A/a	A	*a*	*a*		*a*	*a*	*a*										*a*	*a*				*a*			
								B/b	*b*		B																					
								C/c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	C	c	c	c	c		
								D/d	*d*					D																		
								E/e	*e*																				E			
								F/f	*f*																							
								G/g	*g*																							
Cultivar[b]	R gene[a] A/a	B/b	C/c	D/d	E/e	F/f	G/g																							# Susc.[c]	Min. # missing R genes
6	A	B	C	D	E	F	G																							0	0
8	*A*	b									S																			1	1
13	a								S																					1	1
9	*A*	b			e						S																	S		2	2
28	a			d					S					S																2	2
19	a								S			S					S													3	2
25	*A*	b									S													S	S					3	2
7	a								S			S						S					S							4	3
31	a								S	S					S	S														4	3
4	*A*	b		d						S	S		S	S	S															5	3
20	a		c						S	S		S						S								S				5	3
22	a								S	S		S	S				S													5	3
23	a		c						S	S		S						S								S			S	6	4
15	a	b							S		S		S		S	S	S										S			7	4
30	a			d					S	S				S	S	S			S			S								7	4
17	a	b		d					S	S	S		S	S	S	S					S									8	4
24	a	b							S	S	S	S	S				S			S			S							8	4
27	a	b							S		S	S	S					S	S		S	S					S			9	4
5	a	b							S	S	S	S	S				S			S	S		S		S		S			11	4
1	a		c	d	e				S	S		S	S	S	S	S	S	S	S					S		S		S		14	5
16	a	b		d					S	S	S	S	S	S	S	S		S	S	S	S			S			S			14	5
3	a	b	c	d					S	S	S	S	S	S				S	S	S		S	S	S	S	S		S		S	16	5
14	a	b	C	d	e				S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S		S	S	S	S	21	5
2	a	b	c	d	e	f	g		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	22	6
								# Susc.[c]	19	14	13	13	12	9	9	8	8	8	7	7	6	6	6	6	5	5	5	4	4	4		
								Min # missing Avr genes	5	5	5	5	5	4	4	4	4	4	4	4	4	3	4	4	3	3	3	3	3	3		

[a] The pathosystem is shown with seven genes, the minimum number involved based on the Person analysis of the data (see Results section).

[b] Cvs 6, 10, 11, 12, 26, 32, 33, and 35 had the same interaction phenotype profile. Only the first cultivar is listed in the table as the representative cultivar. This was also the case for cvs 2 and 29, cvs 9, 18 and 21, cvs 8 and 34, and cvs 13 and 36.

[c] Number of susceptible interactions for each cultivar. Number of susceptible cultivars for each pathotype.

could be calculated (Table 5). Some cultivars show a unique interaction with a specific pathotype. Pathotype BE6 was the only one able to infect cv. 8, while only isolate NL1 was capable of infecting cv. 13. This indicates that cvs 8 and 13 each lack a specific but different R gene, the products of which interact with corresponding specific elicitors in these pathotypes. As these two pathotypes are both virulent on a large number of cultivars, they are very interesting in that these specific Avr genes probably represent the only Avr gene in the case of NL1 and one of the few Avr genes in the case of BE6. For NL1, this is because the resistance to this isolate in cvs 8, 9, 25 and 4 is probably generated by a single matching R gene, of which the Avr gene is lacking in BE6 (Table 5). As a result, BE6 is able to cause disease on these four specific cultivars. Pathotype CO2 is also very interesting in that it was the only one that could not infect cv. 14. Therefore, cv. 14 contains a single R gene that corresponds to an Avr gene that is only present in pathotype CO2. Two other Avr genes could be assigned specifically to isolates US1 and BE5 based on their differential interactions on cvs 28 and 9, respectively. Cv. 9 could only be infected by BE6 and BE5, suggesting this cultivar contains all available R genes except those for recognition of isolates BE6 and BE5. In a similar way, cv. 28 contains all available R genes except those for recognition of isolates NL1 and US1. Given the large number of possible combinations in a 128 by 128 matrix and the limited number of interactions available, further determination of the presence or absence of specific R genes in specific cultivars, and specific Avr genes in specific isolates is not possible using this approach.

Discussion

The above-described bioassay in closed plastic containers allowed testing at constant temperature, humidity and light conditions. We found these factors to be critical for consistently successful inoculation of the pathogen. Our average inoculum level of 78.5 basidiospores mm^{-2} is in accordance with the circa 70 spores mm^{-2} described by Yamaguchi (1981). The relatively small differences in inoculum level, the high inoculation density and the use of three replicates contributed to the robustness of the inoculation method. The interaction phenotypes were consistent over the three replicates in 91.2% of the cases. Also, results obtained with the nine isolates that were re-screened in a different period of the year were consistent. For most interactions, the timing of symptom development was as described previously (Firman and Martin 1968). However, for some cultivars it took a few days longer to develop teliospores (Table 4), which is why an additional scoring at 28 dpi was performed. The bioassay offered the possibility to simultaneously screen several isolates in three replicates on a relatively small surface area, such as that provided in a biosecurity growth chamber. At an evaluation rate of up to 216 cuttings per week we screened a total of 3348 cuttings, making it relatively high throughput.

Our focus was on the initial gene-for-gene type of recognition events: therefore, our scoring method aimed to evaluate the recognition events and not the amount of disease expression. We used an interaction phenotype scoring system that was relatively simple compared to the scoring scales that focus more on the relative leaf area covered with teliospores (Yamaguchi 1981; Wojdyla 1999; Barbosa et al. 2006) or the disease indexes derived from the infection level of separate leaves (Takatsu et al. 2000; Alaei 2008). For the analysis of the pathotypes involved, the interaction phenotype scores were reduced to susceptible or resistant interactions based on a clear frequency distribution. However, as in most bioassays, some intermediate phenotypes were observed. As soon as a cultivar showed a full-blown infection on at least one of the three replicate plants (a score ≥ 4) in at least one of the replicate tests, the cultivar was considered susceptible. This arbitrary threshold was based on the assumption that in such cases, recognition of the pathogen failed. This level was considered sufficiently stringent, as disease expression is not always complete, possibly due to the physiological status of the plants (Walters and Bingham 2007; Bolton 2009) or because expression of disease resistance is inherently variable in specific cultivars, as was observed with cvs 4, 17 and 30. Given the frequency distribution of the infection phenotypes, with few scores of 4, limited differences in outcome were observed if a more stringent infection phenotype cutoff (a score ≥ 5) was used. One exception is found for isolates JP1 and JP2 (data not shown). Replication in time also showed limited variability (6.8%), and was in half the cases linked to cultivars with variable

disease expression. These variable reactions were also linked to specific isolates (especially BE6 and GB1). However, we suspect that this variability may be more related to differences in environmental and host conditions between the experiments with these isolates than in inherent variability of the isolates themselves. The reason is that the general level of susceptibility fluctuated slightly between these replicate experiments. Under those conditions, a small infection phenotype is considered an incomplete expression of disease in the absence of an Avr/R match, instead of the result of an incomplete expression of defense after an Avr/R match. Therefore, preference is given to the more susceptible score, which is reflected in our scoring system. However, even if preference was here given to the resistant score for all the variable interactions, the main conclusion about the minimum number of genes involved in this system would hold, demonstrating the limited impact of these variable results and showing the robustness of the system.

Our results confirm the presence of pathotypes in *P. horiana* that can be explained by the gene-for-gene concept described by Flor (1956) and analyzed as described by Person (1959). These results should be confirmed by genetic studies, but preliminary analyses of resistance characteristics in progeny of a cross of a resistant and susceptible cultivar using isolates BE5, BE6, JP1, and NL1 indicate Mendelian inheritance of different R genes (data not shown). Although several studies have demonstrated the existence of differential reactions in the pathosystem of *P. horiana* and chrysanthemum, none have tested a comprehensive collection of isolates covering a broad geographical range on a large set of cultivars and tried to assess the race complexity of this pathosystem and the number of resistance genes involved (Baker 1967; Dickens 1968; Yamaguchi 1981; Wojdyla 1999; Velasco et al. 2007). The pathogenic variability shown in those studies was rather limited compared to the results obtained here, presumably due to the wider geographical origin of our isolates, the selection of isolates on cultivars that were previously reported as resistant, and the larger number of test cultivars used, including some on which anecdotal infections were reported.

The 22 isolates we tested represented 22 different pathotypes. Based on the number of isolates expressing susceptibility on a large number of cultivars and the number of cultivars commonly infected by these isolates, we could conclude that a minimum of seven genes are involved in this pathosystem. In a system with at least seven genes, five isolates infecting 32 cultivars or more can be found that have six susceptible interactions in common.

Complex gene-for-gene interactions have been found in other plant/rust interactions. In the flax/*Melampsora lini* pathosystem, 31 R alleles were located on 5 different loci, with two to 13 alleles per locus (Ellis et al. 2007). For *Puccinia triticina*, the most common leaf rust on wheat, most of the 60 currently known R genes were mapped to separate loci, distributed over the genome (Bolton et al. 2008). The large number of pathotypes in the chrysanthemum/white rust system surprised us as *P. horiana* is considered an asexual microcyclic rust.

The most virulent isolate in the present study was NL1. It may have adapted through mutation or via genetic exchange between different isolates, each lacking specific elicitors. As *P. horiana* is a microcyclic rust and microcyclic rust usually don't produce spermagonia and don't produce aecia, genetic exchange may have been generated asexually by other modes of dikaryotization that have been described (Ono 2002). Anastomosis of vegetative mycelia at an early stage of basidiospore infection can occur (Lindfors 1924; Walker 1928). If a similar genetic exchange can take place through fusion of basidiospore germ tubes or infecting mycelia in *P. horiana*, this may have resulted in the virulence spectrum observed. Alternatively, the sexual cycle may be hidden, as was found for other fungi such as *Aspergillus* (O'Gorman et al. 2009).

Although the data are limited due to the lack of many possible pathotypes and differential cultivars, they indicate relations between different isolates. The interaction phenotype profile of isolate CO1 fits into that of the more virulent isolate MY2, which fits into the MY1 profile, which in turn fits into the profile of NL1. Assuming that these observations can be extrapolated to the complete profile, this represents a geometric series (Person 1959). If so, this geometric series may be the result of stepwise and cumulative loss of Avr genes in their respective isolates, in which case these isolates would have dispersed internationally. Alternatively, isolates may independently have lost these specific Avr genes and become dominant due to selection pressure on large monocultures of chrysanthemum cultivars carrying the same specific R

genes. Genetic characterization of the different isolates with neutral markers such as AFLP or microsatellites may help differentiate between these hypotheses as it may reveal a common ancestry of specific isolates. Unfortunately, performing AFLP is difficult with *P. horiana,* because it is not easy to obtain sufficient amounts of non-contaminated DNA of this obligate pathogen and microsatellite markers have not yet been identified.

In general, most cultivars showed a clear presence or absence of symptoms. However, cvs 1, 4, 7, 15, 27 and 28 almost never exhibited complete resistance and still developed a few pustules. This indicates a quantitative aspect to the disease resistance in those cultivars, possibly due to suboptimal downstream defense reactions. As noted in Table 4, infection on these cultivars sometimes occurs exclusively on particular parts of the plant such as the leaf tips or the stipula. This could be due to incomplete resistance activation in those plants parts. Cvs 15 and 16 showed a slower development of symptoms compared to the other cultivars. Quantitative resistance genes with a minor impact may play a role in these cultivars (Poland et al. 2009). An interesting observation was also made about accidentally injured plants. While injury-free replicates of these cultivars showed complete resistance, injured plants showed clear infection and that only distally from the injury. This may indicate the requirement of an active vascular system in disease resistance.

Incorporation of resistance to *P. horiana* will gain importance in future chrysanthemum breeding due to the decreasing number of registered fungicides for this disease and an increase in the number of fungicide-resistant strains (data not shown). Also, control of the pathogen via lowering of the relative humidity is not always possible, such as in semi-covered chrysanthemum growing systems in the (sub) tropics. Even in heated greenhouses, this approach has been reduced due to the high cost of energy use (B. Brandwagt, personal communication).

The bioassay we developed allows relatively high-throughput screening of plantlets with one isolate or a combination of specific isolates. Given the increasingly international trade in cut flowers and the international and often trans-continental production of planting material, resistance breeding will need to involve as many R genes as possible. Ideally, the cultivars should be tested with as many as possible of the pathotypes containing a single Avr gene. Although the proportion of possible resistance-breaking pathotypes becomes smaller with additional R genes, a specific resistance-breaking pathotype may become important quantitatively due to selection pressure. Timely detection of all the pathotypes present in a region may help optimize R gene deployment. This could be achieved with a "pathotype detection network", consisting of non-fungicide-treated specific chrysanthemum cultivars that lack single or very few R genes. These cultivars could be placed and inspected at chrysanthemum growing facilities throughout the region in a similar way as is done for wheat rust detection (Kolmer 2005). Alternatively, molecular characterization of specific pathotypes via pathotype-specific markers would be an even faster way for pathotype detection and could make use of a single universally susceptible cultivar or air-trapped spores. However, as the Avr genes or linked markers have not been identified yet in *P. horiana*, this is not an option in the short term.

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