

Barrage formation is independent from heterokaryon incompatibility in *Verticillium dahliae*

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Abstract Barrage formation has been traditionally used for the assessment of mycelial compatibility in many fungi and has often been assumed to represent a non-self recognition phenotype that is directly associated with vegetative incompatibility in these organisms. In this work, the optimal growth conditions for large-scale studies of barrage formation in the asexual fungus *Verticillium dahliae* were determined, and they were used for the analysis of a diverse collection comprising 69 isolates of *V. dahliae* and related species. Barrage formation was very frequent on a defined complete agar medium within *V. dahliae* and between species of the genus. However, it was not correlated with the classification of *V. dahliae* isolates into Vegetative Compatibility Groups (VCGs) (based on the standard method using complementary *nit* mutants), as it was recorded at high frequencies both within and between *V. dahliae* VCGs. The high overall frequency of barrage formation demonstrated the presence of a higher level of mycelial incompatibility in *V. dahliae* than heterokaryon incompatibility assessed by forcing complementary *nit* mutants to form heterokaryons under selective conditions. The possible association of barrage formation with morphological characteristics of the fungal

colonies was investigated, and a negative correlation of frequency and intensity of barrages with the isolates' capacity for pigment production was detected.

Keywords Barrage formation · Heterokaryon incompatibility · Mycelial compatibility groups · Mycelial interactions · Non-selective compatibility tests · Vegetative compatibility

Introduction

Verticillium dahliae Kleb. is a soil-borne phytopathogenic ascomycete that causes vascular wilt in hundreds of plants and billions of dollars in annual crop losses worldwide. The longevity of its microsclerotia in the soil, its vascular habitat, its limited host specialization, and the shortage of resistant host germplasm, have hindered control of the disease. A thorough understanding of the population biology of the fungus is hopefully predicted to facilitate disease management (Pegg and Brady 2002; Klosterman et al. 2009).

Given the asexual nature of *V. dahliae*, the well established parasexual cycle is perceived as the only means of exchange of genetic information between members of this species (Hastie 1964; Puhalla and Mayfield 1974; Typas and Heale 1978). However, even from the first relevant studies, it was evident that when different strains of the fungus come in contact, the formation of viable heterokaryons is frequently prevented by incompatibility systems (Heale 1966; Typas and Heale 1976), resulting in the subdivision of

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the species into compatibility groups. Through the years, a general assumption was made, i.e., that *V. dahliae* isolates belonging to different compatibility groups are genetically isolated and, thus, may represent clonal lineages that possibly differ in physiology and pathogenicity (Leslie 1993; Katan 2000). Different methods have been employed for the characterization of *V. dahliae* populations regarding vegetative compatibility, including: pairing of complementary auxotrophic mutants requiring amino acids, purines/pyrimidines or vitamins (Typas and Heale 1976; Clarkson and Heale 1985); morphological mutants in the pigment biosynthesis pathway (Puhalla 1979; Puhalla and Hummel 1983); and nitrate-nonutilizing (*nit*) mutants (the compatibility data produced by complementation tests of appropriate mutants of fungal strains are generally referred to as heterokaryon incompatibility results) (Joaquim and Rowe 1990, 1991; Strausbaugh et al. 1992; Katan 2000; Bhat et al. 2003; Jiménez-Díaz et al. 2006; Papaioannou et al. 2013b). Due to the relative ease of selection of spontaneous *nit* mutants on chlorate-amended growth media, the latter technique has been by far the most widely used method during the last two decades for the classification of *V. dahliae* isolates into five main Vegetative Compatibility Groups (VCGs); of these, VCGs 1, 2, and 4 have been further subdivided depending on the vigour of complementation (Joaquim and Rowe 1990; Bhat et al. 2003). More recently, considerable progress has been made in the elucidation of molecular and phylogenetic relations between VCGs and their subgroups, revealing a previously unanticipated complexity in the relations within VCGs and between subgroups, e.g., VCGs 2 and 4 were shown to consist of molecularly distinct subgroups with complex inter-VCG relationships, and VCG subgroup 2B has been clearly demonstrated to be polyphyletic (Collado-Romero et al. 2006, 2008; Martin 2010; Papaioannou et al. 2013a, b).

Another approach that has been followed for the population-level assessment of vegetative compatibility in many fungi is the analysis of barrage (or demarcation or aversion zone or line) formation, which represents a type of non-self recognition. Based on the study of barrage formation between wild-type colonies, fungal isolates can be assigned to mycelial compatibility groups (Leslie 1993; Smith et al. 2006). This method is based on the general assumption that vegetatively incompatible isolates of a fungal species form a barrage at the interface where confronting hyphae of colonies

interact (various types of barrages have been described for different fungi, usually involving a central darkly pigmented area and/or a thicker and higher mycelial layer), whereas compatible pairs are typically predicted to grow into each other without any obvious morphological alteration (Esser and Meinhardt 1984; Worrall 1997). In contrast to inhibition zones that are free of hyphae due to the secretion and sensing of inhibitory molecules, the barrage formation requires hyphal fusion and cytoplasmic mixing between the hyphae of the incompatible confronted colonies, leading to abnormal or even lethal fusions (Esser 2006). Barrage formation has been regarded as conceptually equivalent to heterokaryon incompatibility (Leslie 1993), and the two phenomena have been often considered to be different manifestations of the same vegetative incompatibility function. However, different reports have reached contradictory conclusions regarding the concordance between heterokaryon incompatibility and barrage formation. A high correlation has been observed in *Podospora anserina* and *Fusarium graminearum* (Rizet 1952; McCallum et al. 2004), but discrepancies between the two tests have been reported in other cases. In *Neurospora crassa*, barrage formation is not associated with four tested heterokaryon incompatibility genes (Micali and Smith 2003), and a perfect correlation between the two phenomena is also absent in *Sclerotinia sclerotiorum* (Ford et al. 1995; Jo et al. 2008). A similar inconsistency between barrage formation and heterokaryon incompatibility was also demonstrated for one of the six known vegetative incompatibility loci of *Cryphonectria parasitica*, indicating that the two phenomena are not different manifestations of the same process (Smith et al. 2006).

The purposes of the present study were: (i) to test barrage formation in *Verticillium* species for the first time, under different growth conditions, and to determine the optimal conditions for a large-scale study; (ii) to analyze a diverse collection of isolates of *V. dahliae* and relative species regarding barrage formation; (iii) to compare mycelial compatibility of *V. dahliae* isolates based on barrage formation with their heterokaryon compatibility classification according to the traditional *nit* mutants-based complementation procedure; and (iv) to test the possibility of an association between mycelial compatibility behaviour and the isolates' capacity for pigmentation and other morphological characteristics.

Table 1 *Verticillium* isolates used in this study, with code names, VCGs, original hosts, and geographic origins (sources)

Isolate code	VCG ^a	Host	Origin (source) ^b
<i>V. dahliae</i>			
T9	1A (1)	cotton	USA, CA (A)
V44	1A (1)	cotton	USA, TX (A)
V138I	1A (2)	cotton	Spain (B)
cotVd16	1A (3)	cotton	Turkey (C)
cotVd47	1A (3)	cotton	Turkey (C)
V607I (R04)	1B (4)	green ash	USA, MN (B)
V661I (328 v-1)	1B (4)	cotton	Greece (B)
V666I (347 v-1)	1B (4)	cotton	Greece (B)
PH	2A (1)	pistachio	USA, CA (A)
V320I	2A (4)	cotton	USA, CA (B)
egpVd23	2A (5)	eggplant	Turkey (C)
ep8	2A (6)	eggplant	Israel (D)
31 V	2A (7)	melon	Greece (E)
115	2B (1)	cotton	Syria (A)
Ls.17	2B (8)	lettuce	USA, CA (F)
V357I (JY)	2B (4)	cotton	China (B)
V16	2B (9)	potato	Germany (G)
cotVd18	2B (3)	cotton	Turkey (C)
123-3	2B (10)	olive	Greece (H)
Cf.38 ^c	2B (11, 12)	chili pepper	USA, CA (F)
V702I	2Ba (13)	artichoke	Spain (B)
423-1	2B, 2A (10)	pistachio	Greece (H)
20 V	2B, 2A (7)	tomato	Greece (E)
530-1	2B, 2A (10)	pepper	Greece (H)
128 V	2B, 1, 4A (7)	pepper	Greece (E)
25 V (SS4)	2AB, 1, 4A (7)	cotton	USA (E)
70–21	3 (14)	pepper	USA, AZ (A)
PCW	3 (1)	pepper	USA, CA (A)
BB	4A (1)	potato	USA, ID (A)
131-M	4A (15)	potato	USA (B)
171-4A	4A (15)	potato	USA (B)
V830	4A (9)	potato	USA, OH (G)
Dvd-E6	4A (16)	eggplant	Canada (I)
S39	4B (1)	soil	USA, OH (A)
V304I (cot120)	4B (2)	cotton	Israel (B)
V684I	4B (13)	artichoke	Spain (B)

Table 1 (continued)

Isolate code	VCG ^a	Host	Origin (source) ^b
157	4B (17)	potato	USA, OR (I)
V39	4B (9)	sunflower	Germany (G)
1 V	4B (7)	cotton	Greece (E)
435-1	4B, 4A (10)	tomato	Greece (H)
456-1	4B, 4A (10)	dill	Greece (H)
457-5	4B, 4A (10)	spinach	Greece (H)
Ca.83	6 (11)	bell pepper	USA, CA (F)
Ca.146	6 (11)	bell pepper	USA, CA (F)
Ca.148	6 (11)	bell pepper	USA, CA (F)
Cf.162 ^c	HSI ^d (11, 12)	chili pepper	USA, CA (F)
V13	HSI (9)	cotton	Spain (G)
Ud1-4-1 ^c	–	Japanese spikenard	Japan (J)
TO-0 ^c	–	tomato	Japan (J)
CA26 ^c	–	cabbage	Japan (J)
<i>V. albo-atrum</i>			
220	–	alfalfa	UK (K)
HP	–	potato	Japan (J)
Ms.102	–	alfalfa	USA, PA (F)
P10	–	hop	Germany (L)
T2	–	hop	Slovenia (L)
<i>V. longisporum</i>			
81	–	oilseed rape	Denmark (G)
CA10	–	cabbage	Japan (J)
Dk-1	–	Japanese radish	Japan (J)
G19	–	oilseed rape	Germany (K)
K2	–	oilseed rape	Russia (M)
<i>V. tricorpus</i>			
15	VTR-5 (18)	soil	Israel (D)
18	VTR-5 (18)	soil	Israel (D)
19	VTR-7 (18)	potato	Israel (D)
22	VTR-7 (18)	potato	Israel (D)
<i>V. nubilum</i>			
278,734	–	potato	UK (IMI)
<i>Gibellulopsis nigrescens</i> (formerly <i>V. nigrescens</i>)			
9	VN-5 (18)	potato	Israel (D)
14	VN-5 (18)	<i>Graminae</i> (potato field)	Israel (D)
18	VN-10 (18)	soil	Israel (D)

Table 1 (continued)

Isolate code	VCG ^a	Host	Origin (source) ^b
19	VN-11 (18)	soil	Israel (D)

^aData on “bridging” behavior (i.e., complementation to varying degrees with tester strains of more than one VCG subgroups) were available for several *V. dahliae* isolates that were included in this study; these secondary VCG interactions are provided after commas. The reference of studies reporting the VCG assignment of each isolate is provided in brackets, as follows: 1 = Joaquim and Rowe 1990; 2 = Korolev et al. 2001; 3 = Dervis et al. 2008; 4 = Collado-Romero et al. 2006; 5 = Dervis et al. 2009; 6 = Korolev et al. 2000; 7 = Elena and Paplomatas 1998; 8 = Papaioannou & Typas, unpublished data; 9 = Zeise and von Tiedemann 2001; 10 = Papaioannou et al. 2013b; 11 = Bhat et al. 2003; 12 = Papaioannou et al. 2014; 13 = Jiménez-Díaz et al. 2006; 14 = Joaquim and Rowe 1991; 15 = mentioned by Collado-Romero et al. 2008 as unpublished results; 16 = Dobinson et al. 1998; 17 = Dobinson et al. 2000; 18 = Korolev and Katan 1999

^b Provided by: A = R. Rowe, OARDC, The Ohio State University, USA; B = M. Jiménez-Gasco, The Pennsylvania State University, USA; C = S. Dervis, University of Mustafa Kemal, Turkey; D = T. Katan, The Volcani Center, Israel; E = E. Paplomatas, Agriculture University of Athens, Greece; F = K. Subbarao, University of California, Davis, USA; G = A. von Tiedemann, University of Göttingen, Germany; H = E. Ligoixigakis, Plant Protection Institute, N.AG.RE.F., Greece; I = K. Dobinson, University of Western Ontario, Canada & Agriculture and Agri-Food, Canada; J = T. Usami, Chiba University, Japan; K = J. Heale, University of London, UK; L = S. Radišek, Slovenian Institute for Hop Research and Brewing, Slovenia; M = O. Strunnikova, All-Russian Research Institute for Agricultural Microbiology, Russia; IMI = International Mycological Institute, UK (presently Centre for Agricultural Bioscience International, CABI, UK)

^c These isolates were initially assigned to VCG 6 by Bhat et al. (2003). The VCG classification of them was later re-examined by Papaioannou et al. (2014), and the revised classification is provided in this Table

^d HSI = heterokaryon self-incompatible

^e Reported to bear the MAT1-1-1 mating type idiomorph (Usami et al. 2009)

Materials and methods

Fungal isolates

Monoconidial cultures from a total of 65 *Verticillium* isolates (50 *V. dahliae*, 5 *V. albo-atrum*, 5 *V. longisporum*, 4 *V. tricorpus*, and 1 *V. nubilum* representative strains), as well as 4 isolates of *Gibellulopsis nigrescens* – formerly *V. nigrescens*—from various hosts and geographic origins were employed in this study (Table 1). Members of all known VCGs of

V. dahliae were included in this collection. All isolates were stored as conidial suspensions in Czapek-Dox broth supplemented with 20 % sterile glycerol, at –80 °C. Actively growing cultures were obtained prior to each experiment by spreading aliquots of the frozen conidial suspensions on PDA plates (Scharlau, Barcelona, Spain) and incubating at 24 °C, in the dark, for 5 days.

Growth media and culture conditions for the assessment of barrage formation

Various combinations of complete (PDA, PCA, CM) and minimal (Czapek-Dox) agar media (described by Typas and Heale 1976), with different light conditions during incubation (a 12-h ambient-light photoperiod or continuous darkness), were tested for the determination of those conditions that favour the production of discrete and reproducible mycelial interactions between confronted hyphae of wild-type isolates. For each pairing, two isolates were point-inoculated (from the edge of 1-week-old colonies grown on CM agar) at a specified distance between inocula on the agar surface, and the plates were incubated at the specified conditions for 10 days. Different distances in the range of 0.5–3.0 cm between fungal inocula were tried in preliminary tests and assessed for the reproducibility of the results. Initial experiments demonstrated that up to five different isolates could be inoculated at a cruciform pattern and tested on the same plate (Fig. 1), with individual pairings never interfering with each other under these conditions. After the end of the incubation period, barrage formation was recorded, and the intensity of each barrage was estimated in an arbitrary 1–3 ordinal scale (Fig. 1). Class 0 corresponded to compatible interactions, characterized by the gradual intermingling of the confronting colonies with no apparent morphological differentiation. Each pairing was scored in at least two replicates, and self-pairings were included as controls in all experiments.

Morphological characterization and microscopic observations

Growth rates were determined by measuring the perpendicular diameters of fungal colonies grown on CM agar at daily intervals, and the mean growth rates were calculated for the period of active growth until the end of the second week of incubation. Two weeks after

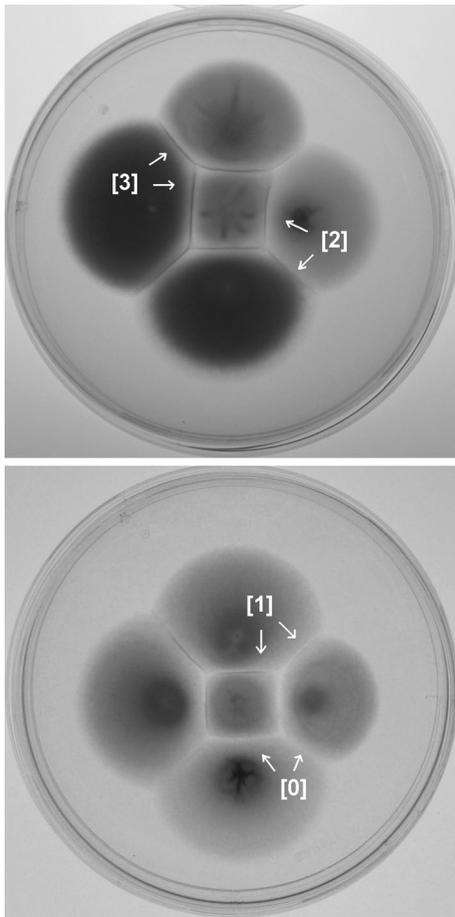


Fig. 1 Examples of barrage formation between colonies of several *V. dahliae* wild-type isolates on CM agar, at the end of the co-incubation period (10 days). The three-point (1–3) ordinal graduation (classes provided in *square brackets*) used for the assessment of the intensity of barrages is illustrated with representative examples (indicated with *arrows*). Class 0 corresponds to compatible interactions, characterized by the absence of barrages and the gradual intermingling of the compatible colonies with no apparent morphological differentiation; this process had not been completed for the majority of pairings by the end of the 10-day incubation period on CM agar, but was invariably verified with longer incubation for all tested compatible pairings

inoculation on CM agar, colonies were scored in a four-point ordinal graduation for hyphal density. A five-point scale was also used to assess pigment production at the bottom of the plates 3 weeks after inoculation. All morphological tests were performed in three replicates per parameter examined for each isolate.

For the live-cell imaging of undisturbed barrages at the microscopic level, representative incompatible pairings of *V. dahliae* isolates were performed on microscopy slides covered with thin layers of solidified

CM medium, along with compatible pairings which were used as controls. Inoculation and incubation of the slides were performed as described above for the standard barrage formation assays. After appropriate incubation, a drop of sterile water and a coverslip were placed on each slide, above the contact area of the confronting colonies. Alternatively, hyphae within the contact zone were subjected to double staining with the fluorescent dyes propidium iodide and fluorescein diacetate (Sigma-Aldrich Corporation, St. Louis, MO, USA) in order to determine dead or dying cells (Livanos et al. 2012). All samples were examined with a Zeiss Axioplan epifluorescence microscope with appropriate filters, and photos were captured with a Zeiss AxioCam MRc5 digital camera using AxioVs40 V4.40.0 software (Carl Zeiss AG, Oberkochen, Germany). All samples were checked for UV autofluorescence. The same microscope was used with a polarized light source for differential interference contrast (DIC or Nomarski) imaging of stained and non-stained samples.

Statistical analysis

Statistical tests were conducted with SPSS Statistics 20 software package (IBM Corporation, Armonk, NY, USA). When both normality of data and homogeneity of variances complied with the general assumptions for parametric tests, data were processed with *t*-test or one-way analysis of variance (one-way ANOVA) for dichotomous or multi-group variables, respectively. Post-hoc analysis between groups following ANOVA was accomplished with the Tukey HSD method. The non-parametric Mann–Whitney *U* test was used for comparisons of datasets not fulfilling any of the two criteria mentioned above.

Results

Mycelial interactions within and between *Verticillium* species

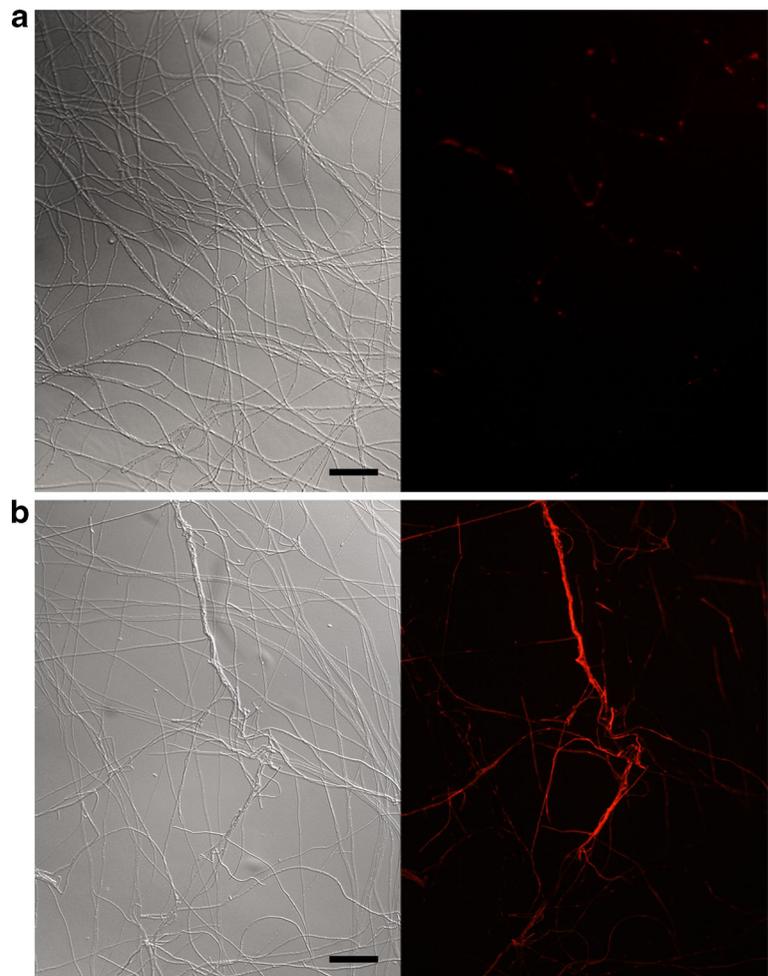
During an initial investigation of mycelial reactions between *Verticillium* wild-type isolates, a collection of 20 isolates of all species were confronted in all possible combinations, on different complete and minimal solid media, and incubated at 24 °C in the dark or under a 12-h ambient-light photoperiod. The formation of barrages was markedly influenced by both the growth

medium and the light during incubation (data not shown). Furthermore, the results depended considerably on the exact distance between inocula on the agar surface; the reproducibility of the method among independent repetitions of the pairings was not satisfactory when distances shorter than 1.5 cm were tested. The most reproducible results, with the most pronounced and discernible barrages, were obtained on CM agar plates, when inocula were placed 1.5 cm apart and incubated in continuous darkness for 10 days. Thus, these optimal growth conditions were consistently used in all subsequent experiments for the analysis of barrage formation in *Verticillium* species.

The 69 *Verticillium* isolates listed in Table 1 were paired in a total of 692 different intra- and inter-isolate pairings (at least two repetitions each; Online Resource 1) under the specific conditions mentioned above, and their mycelial reactions were recorded. Four reaction

types were generally observed for all *Verticillium* species: (i) the formation of a barrage at the contact line of the isolates, i.e., a relatively sharp distinct zone of thick mycelium, occasionally with accumulated dark pigment, of varying degrees of intensity (intensity was assigned in an arbitrary 1–3 scale, with 3 corresponding to the most pronounced barrages; examples are shown in Fig. 1); (ii) mutual intermingling of the colonies with no apparent morphological differentiation (compatible interactions; scored as 0); (iii) overgrowth of one colony by the other; and (iv) clear zones devoid of hyphae or consisting of thin mycelium between the confronted colonies. Under these conditions, the presumed barrages between incompatible wild-type colonies were microscopically found to consist of dense bundles of thickly-walled disorganized compartments, at the area where hyphae from the incompatible isolates had come into physical contact and repeatedly fused (Fig. 2). To

Fig. 2 Microscopic characteristics of hyphae within the contact zones of confronting colonies of *V. dahliae* isolates, in representative compatible (a) and incompatible (b) pairings (isolates 115 and Cf.38 for the compatible reaction; and Ls.17 and V16 for the incompatible one). For each pairing, a DIC micrograph of hyphae is provided on the left, and a photo of the same area of the sample produced with epifluorescence microscopy with propidium iodide staining, is shown on the right. Disorganized, thickly walled, and dead or dying hyphal compartments, which are thus stained with propidium iodide, predominate the contact zone of the incompatible pairing, in contrast to the compatible interaction. Scale bars = 50 μ m



identify dead or dying hyphal compartments, fluorescent staining with propidium iodide—which is not permeant to live cells – was employed and verified with fluorescein diacetate staining, used as a viability probe. The presumed nuclei of only few cells were stained with propidium iodide in all compatible pairings tested as controls, whereas brightly fluorescing dead cells predominated the contact zone of incompatible confronting colonies (Fig. 2). Occasionally, swollen hyphal cells resembling the initial stages of microsclerotia production were also observed along this line of contact, at the end of the incubation period, but mature microsclerotia were never detected. Although the gradual intermingling of compatible colonies was only partially completed for the majority of compatible pairs by the end of the 10-day incubation period on CM agar (examples are shown in Fig. 1), this introduced no compromise to the accuracy of the assay by visual inspection of plates. To confirm this, compatible pairings were incubated for longer incubation periods and invariably led to completely intermingled colonies, thus validating the accuracy of the initial assay.

The formation of barrages was by far the most frequent reaction type on CM agar (observed in 80.4 % and 75.4 % of pairings within *V. dahliae* or considering all *Verticillium* species, respectively; Table 2), and, interestingly, it was also encountered in self-pairings with very high frequencies (90.7 % and 84.8 %, respectively). The rest of the reaction types were less frequently observed, with type (iii) being encountered mainly in a few inter-specific pairings. For comparison, 385 pairings of the same strain set (a total of 854 pairings taking repetitions into account) were performed on MM agar (Online Resource 1). A severe reduction in barrage formation was generally observed on MM (14.3 % for

all species), with reaction type (iv) being the most prevalent (41.8 %). Regarding reproducibility of the method that we used for the analysis of barrage formation, the vast majority of pairings yielded identical or very similar results between repetitions. To further validate this observation, 24 randomly selected pairings were repeated in 10 independent trials each; in all cases, the results were absolutely congruent regarding formation of barrages and 80–100 % (averaging 86.3 %) congruent regarding the assessment of barrage intensity (Online Resource 1).

Comparison of barrage formation and VCG classification in *V. dahliae*

Within *V. dahliae*, barrage formation (i.e., mycelial incompatibility) was compared with heterokaryon incompatibility on the basis of VCG classification using *nit* mutants. For this, the frequency and intensity of barrages within and among all VCGs of *V. dahliae*, in all possible combinations, were analyzed. Remarkably, no association of barrages with the traditional VCG system was found, with barrages being frequently formed in pairings of isolates belonging to either the same or different VCGs (Table 3). In addition, some isolates were compatible (i.e., they did not form barrages) with only one of a pair of compatible isolates, while others were compatible with both of mutually incompatible strains. These results hampered the classification of the isolates into clear-cut mycelial compatibility groups. Nevertheless, it was found that the frequency and vigour of barrage formation largely depended on the individual isolates tested, with extreme examples being isolates that tended to form barrages with all or most isolates, regardless of their VCG classification (e.g., *V. dahliae* isolates V16, Dvd-E6, and V13; Table 4), and others

Table 2 Frequency (%) and mean value of intensity (in an arbitrary 1–3 three-point scale; provided in brackets) of barrage formation on CM agar within and between *Verticillium* species (all individual pairings are listed in Online Resource 1)

species	<i>V. dahliae</i>	<i>V. albo-atrum</i>	<i>V. longisporum</i>	<i>V. tricorpus</i>	<i>V. nubilum</i>	<i>G. nigrescens</i>
<i>V. dahliae</i>	80.4 (1.9)	75.0 (1.8)	66.0 (1.9)	55.6 (1.9)	44.4 (1.5)	82.4 (1.5)
<i>V. albo-atrum</i>		64.3 (2.0)	45.5 (1.6)	75.0 (1.7)	0	100 (1.3)
<i>V. longisporum</i>			64.3 (1.5)	62.5 (1.5)	33.3 (1.5)	25.0 (2.0)
<i>V. tricorpus</i>				87.5 (1.7)	100 (1.0)	83.3 (1.4)
<i>V. nubilum</i>					100 (2.5)	0
<i>G. nigrescens</i>						60.0 (1.4)

Table 3 Frequency (%) and mean value of intensity (in an arbitrary 1–3 three-point scale; provided in brackets) of barrage formation on CM medium within and between VCGs of *V. dahliae*

(all individual pairings are listed in Online Resource 1; for clarity, only the primary VCG affinities of “bridging” isolates were used in this analysis)

VCG	1A	1B	2A	2B	3	4A	4B	6	HSI
1A	92.9 (2.2)	75.0 (1.8)	54.5 (2.0)	86.7 (1.9)	75.0 (1.8)	77.8 (1.9)	57.1 (2.0)	75.0 (2.3)	100 (2.5)
1B		100 (2.3)	33.3 (1.0)	66.7 (1.3)	100 (2.0)	100 (1.5)	100 (2.5)	100 (1.3)	0
2A			92.3 (2.4)	72.0 (1.8)	75.0 (1.6)	100 (2.1)	80.0 (1.6)	0	100 (2.8)
2B				82.6 (2.1)	66.7 (1.6)	100 (1.7)	71.4 (1.8)	100 (2.2)	92.3 (2.0)
3					100 (1.9)	100 (1.9)	75.0 (1.4)	ND ^a	100 (2.3)
4A						78.6 (2.2)	84.2 (2.1)	0	100 (2.1)
4B							83.3 (1.8)	75.0 (1.8)	83.3 (2.2)
6								100 (1.5)	100 (1.4)
HSI ^b									100 (2.2)

^aND = not determined^bHSI = heterokaryon self-incompatible

that mostly failed to generate any barrage (e.g., *V. dahliae* isolates cotVd47 and 131-M, and *V. longisporum* Dk-1).

Correlation between mycelial compatibility and colony morphology

To test the possibility of a correlation between mycelial compatibility and colony morphology, a set of 26 isolates (including representative strains of all *V. dahliae* VCGs) were analyzed in terms of colony growth rate, hyphal density, and pigmentation on solid CM medium (Table 4). Barrage formation was not related to growth rate and hyphal density, but statistically significant differences were identified in both barrage frequency ($p=0.001$) and intensity ($p=0.008$) when isolates with slight and extended pigmentation ability were compared, with the former being more keen producers of barrages (Online Resource 2). When barrage formation in 328 pairings between these 26 isolates was analyzed in relation to the cumulative pigmentation capacity of each pair (Online Resource 3), three groups of pairs were identified (i.e., pairs with slight, moderate, and high total pigmentation, respectively). All of them were significantly different from each other regarding barrage intensity, with the same negative correlation between barrage formation and pigmentation potential (all differences were statistically significant at the $p=0.01$ level; Online Resource 4).

Discussion

In the present study, barrage formation in *Verticillium* was analyzed on a large scale for the first time, and it was shown to be overall very frequent (75.4 %) when tested on a complete agar medium. The high frequency observed in *V. dahliae* is in agreement with reports on other fungi (e.g., 70 % in *Botrytis cinerea* and up to 91.7 % in *Sclerotium rolfisii*; Korolev et al. 2008; Remesal and Navas-Cortés 2012). Our results demonstrated that mycelial reactions cannot be used to distinguish *Verticillium* species, as varying proportions of compatible interactions were recorded in almost all combinations of different species. This may be due to the common ancestry and close genetic relationships between *Verticillium* species, and it is in agreement with the study of barrage formation in *F. graminearum* in relation to *F. pseudograminearum* (Akinsanmi et al. 2008). The promotion of barrage formation in *Verticillium* species on a complete medium that is rich in carbon and nitrogen sources is in contrast to what was observed for *N. crassa*, where it was shown that a low-nitrogen minimal medium is required for efficient barrage formation (Micali and Smith 2003). This indicates that the genes involved in barrage formation in the two fungi are under different regulatory pathways.

No significant correlation of barrage formation with heterokaryon incompatibility was detected for any VCG of *V. dahliae*, with barrages being formed at substantial frequencies both within and among different VCGs. The

Table 4 Morphological characterization of selected *Verticillium* isolates and barrage formation frequency (%) and mean value of intensity (in an arbitrary 1–3 three-point scale; provided in brackets) of all different pairings in this study that involved each of the isolates (isolates of each species are sorted according to ascending pigmentation ability)

Isolate code	Growth rate ^a (cm/day)	Hyphal density ^b (1–4)	Pigmentation ^c (0–4)	Barrage formation frequency and intensity ^d
<i>V. dahliae</i>				
V320I	0.4	4	0	88.9 (2.3); 36
530-1	0.3	4	1	67.9 (1.6); 28
Cf.162	0.4	4	1	83.3 (1.8); 30
V44	0.3	1	2	82.4 (2.0); 34
Ls.17	0.5	4	2	93.3 (2.0); 30
V16	0.4	4	2	100 (2.1); 31
BB	0.4	2	2	90.3 (1.8); 31
Dvd-E6	0.5	4	2	97.0 (2.1); 33
V830	0.5	4	2	91.7 (1.8); 36
V13	0.5	4	2	95.0 (2.2); 40
T9	0.4	1	3	81.8 (2.0); 33
70–21	0.4	2	3	86.5 (1.8); 37
V684I	0.4	3	3	90.6 (1.8); 32
Ca.83	0.4	2	3	71.4 (1.4); 7
PH	0.4	3	4	60.6 (1.9); 33
V702I	0.4	4	4	70.4 (2.0); 27
25 V (SS4)	0.4	3	4	80.0 (1.4); 35
115	0.5	2	4	61.1 (1.3); 36
PCW	0.4	2	4	53.6 (1.5); 28
S39	0.4	3	4	69.0 (1.7); 29
CA26	0.4	3	4	62.9 (1.4); 35
TO-0	0.3	3	4	40.0 (1.3); 30
<i>V. albo-atrum</i>				
T2	0.4	3	1	97.0 (2.0); 32
Ms.102	0.4	2	3	45.2 (1.4); 31
<i>V. longisporum</i>				
K2	0.4	2	0	71.4 (1.7); 35
81	0.4	4	3	75.8 (2.0); 33

^aMean value of growth rate for the period of active growth of the colony on CM agar, until the end of the second week after inoculation

^bAssessed 2 weeks after inoculation on CM agar, in an arbitrary 1–4 scale

^cAssessed 3 weeks after inoculation on CM agar, in an arbitrary 0–4 scale

^dAssessed 10 days after inoculation of pairings, in an arbitrary 1–3 scale. For each isolate, the percent frequency of barrage formation is provided, followed by the mean intensity of formed barrages (in brackets), and – after the semicolon – the number of different pairings that involved each isolate and was considered in the calculations

frequent barrage formation even within VCGs indicates that mycelial compatibility is more restricted in *V. dahliae* populations than heterokaryon compatibility. The apparent lack of association implies that the two processes reveal genetic diversity of *V. dahliae* populations at different levels, and the diversity of the results (e.g., isolates that did not form barrages with anyone of mutually “incompatible” isolates, the presence of “super-compatible” and generally “incompatible” isolates) demonstrates that mycelial interaction is probably governed by multiple and putatively quantitative genetic factors. Similar data have also been reported for other fungi, e.g., *S. sclerotiorum*, *S. homoeocarpa*, *N. crassa*,

C. parasitica and *B. cinerea* (Ford et al. 1995; Micali and Smith 2003; Smith et al. 2006; Jo et al. 2008; Korolev et al. 2008), suggesting altogether that the traditionally presumed analogy between mycelial and heterokaryon incompatibility is not a general rule for ascomycetes.

An interesting finding in this work is that barrage formation in our experiments was frequent even in self-pairings of the tested *Verticillium* isolates, similarly with previous observations on other fungi, e.g., *F. oxysporum*, *F. graminearum*, *S. sclerotiorum*, and *Rosellinia necatrix* (Page 1961; Tariq et al. 1985; McCallum et al. 2004; Ikeda et al. 2011). Given that

the results we have obtained for the study of barrage formation in this work were overall highly reproducible, it is considered unlikely that the dark lines observed in self-pairings were due to random mycelial interactions rather than to genetically controlled recognition mechanisms. Similarly, the alternative hypothesis that these lines might merely correspond to the development of resting structures between the confronting colonies due to the local exhaustion of nutrients is also unlikely, since it was shown in our work that dead or dying hyphal compartments resulting from multiple lethal fusions predominated the contact area of incompatible pairings, and mature microsclerotia were never observed. Although the genetic basis of this phenomenon is still elusive, it could be hypothesized that the barrages observed in self-pairings are related to the phenomenon of partial or late heterokaryon compatibility that is frequently encountered in *V. dahliae* populations, even between complementary mutants of the same strain (Typas 1983; Joaquim and Rowe 1990, 1991; Daayf et al. 1995; Papaioannou et al. 2013b). The notion of incomplete compatibility in *Verticillium* is further supported by the recovery of heterokaryon self-incompatible isolates at considerable frequencies (Correll et al. 1988; Collado-Romero et al. 2006; Rataj-Guranowska 2006) and the previous finding that similar strains of other fungi have reduced but not absolutely abolished capacity to form hyphal anastomoses and viable heterokaryons under forced conditions (Correll et al. 1989; Hawthorne and Rees-George 1996).

Based on the observation of immature microsclerotia occasionally developing along the contact line of confronted colonies, we tested the hypothesis that an association might exist between barrage formation and main morphological characteristics of the isolates, mainly the capacity for pigmentation, which is known to be restricted to microsclerotia in *V. dahliae* (Pegg and Brady 2002). A negative correlation between the isolates' ability to accumulate dark pigments and the frequency and intensity of barrage formation was indeed detected, although most isolates require longer periods of incubation to fully express their pigmentation potential in their colonies' phenotype than the incubation period of 10 days used in our assay. It is presently not known whether this association would be significant in more extended collections of *Verticillium* isolates. However, it might be related to evidence from other fungi with coloured or dark mycelium in culture, such as members of the *Botryosphaeriaceae* and

Cryphonectriaceae, which tend to produce more pronounced barrages than fungi with light coloured mycelium (Burgess et al. 2009). These examples and the situation described in the present study for *Verticillium* indicate opposite correlations between barrage formation and pigmentation in different groups of fungi (i.e., a negative correlation for *Verticillium* and a positive one for *Botryosphaeriaceae* and *Cryphonectriaceae* members). Overall, these observations together suggest that barrage formation in fungi may be positively or negatively related to pigmentation ability and may thus indicate the presence of an unexplored putative link between mycelial compatibility behavior and morphogenesis of the fungal colony, which welcomes future study.

The extended incongruence that was demonstrated between barrage formation and heterokaryon incompatibility in *V. dahliae* suggests that the two phenomena are controlled by different—although possibly overlapping—genetic systems. Discrepancies between compatibility data for *V. dahliae* populations that were produced with the application of different methods have also been reported in the past. For example, a lower level of compatibility was revealed with the use of morphological mutants of *V. dahliae* isolates in non-forced tests than with the use of amino acid-, purine-/pyrimidine- or vitamin-requiring auxotrophic mutants in forced complementation trials, which generally demonstrated the existence of compatibility at a more pronounced level in populations of the fungus (Typas and Heale 1976; Puhalla 1979; Puhalla and Hummel 1983; Clarkson and Heale 1985). Even incompatible pairs of *Verticillium* isolates were successfully forced to “artificial” heterokaryon and diploid formation with the techniques of protoplast fusion and microinjection, thus denoting that at least part of the incompatibility barriers in *Verticillium* species are cell wall-related (Typas 1983). It could be speculated that the discrepancies observed between different methods for the assessment of vegetative incompatibility can be attributed—at least in part—to the fundamentally different criteria that are involved in the assessment of compatibility, i.e., formation of heterokaryons under different levels of selective pressure. Hastie and Heale (1984) have postulated that incompatibility barriers might probably be overcome to some extent under conditions of different selection pressure to permit hyphal fusions and the establishment of heterokaryons only under certain, forced conditions. If future research further substantiates this hypothesis, then both our

understanding of the population biology of *V. dahliae* and other fungi, and our methodological toolbox to unravel the compatibility behavior of these organisms, are predicted to advance significantly.

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