Screening for *Alternaria brassicicola* resistance in the *Brassicaceae*: Bio-assay optimization and confocal microscopy insights into the infection process

Marzena Nowakowska, Małgorzata Wrzesińska, Piotr Kamiński, Marcin Nowicki, Małgorzata Lichocka, Michał Tartanus, Elżbieta U Kozik

Heavy losses incited yearly by *Alternaria brassicicola* on the vegetable *Brassicaceae* – have prompted our search for sources of genetic resistance against the resultant disease, dark leaf spot. We optimized several parameters to test the performance of the plants under controlled conditions to this disease, including leaf age and position, inoculum concentration, and incubation temperature. Using these optimized conditions, we screened a collection of 38 *Brassicaceae* cultigens with two methods (detached leaf and seedlings). Our results show, that either method can be used for the *A. brassicicola* resistance breeding, while the plant's genotype was crucial in determining its response to the pathogen. The bio-assays for *Alternaria* resistance were more effective than the field tests, and resulted in identification of two interspecific hybrids that might be used in breeding programs. Confocal microscopy analyses of the leaf samples provided novel insights into the pathogen mode of infection: Direct epidermal infection or stomatal attack were dependent on plants' resistance against *A. brassicicola*. Further, the actin network of the host cells reorganized around the papillas deposited under the pathogen's appressorium. Papilla composition is predicted to be important in determining the plant's resistance.

Screening for *Alternaria brassicicola* resistance in the *Brassicaceae*: Bio-assay optimization and confocal microscopy insights into the infection process.

Marzena Nowakowska[1], Małgorzata Wrzesińska[1], Piotr Kamiński[1], Marcin Nowicki[1], Małgorzata Lichocka[2], Michał Tartanus[3], Elżbieta U. Kozik[1]

[1] Department of Genetics, Breeding, and Biotechnology of Vegetable Crops, Research Institute of Horticulture, Skierniewice, Poland

[2] Laboratory of Confocal and Fluorescence Microscopy, Institute of Biochemistry and Biophysics - Polish Academy of Sciences, Warszawa, Poland

[3] School of Informatics, State Higher Vocational School in Skierniewice, Skierniewice, Poland

Corresponding Authors:

Elżbieta U. Kozik[1], Marcin Nowicki[1]

[1] Department of Genetics, Breeding, and Biotechnology of Vegetable Crops, Research Institute of Horticulture, Skierniewice, Poland

Email address: elzbieta.kozik@inhort.pl, marcin.nowicki@inhort.pl

Abstract

Heavy losses incited yearly by *Alternaria brassicicola* on the vegetable *Brassicaceae* – have prompted our search for sources of genetic resistance against the resultant disease, dark leaf spot. We optimized several parameters to test the performance of the plants under controlled conditions to this disease, including leaf age and position, inoculum concentration, and incubation temperature. Using these optimized conditions, we screened a collection of 38 *Brassicaceae* cultigens with two methods (detached leaf and seedlings). Our results show, that either method can be used for the *A. brassicicola* resistance breeding, while the plant's genotype was crucial in determining its response to the pathogen. The bio-assays for *Alternaria* resistance were more effective than the field tests, and resulted in identification of two interspecific hybrids that might be used in breeding programs. Confocal microscopy analyses of the leaf samples provided novel insights into the pathogen mode of infection: Direct epidermal infection or stomatal attack were dependent on plants' resistance against *A. brassicicola*. Further, the actin network of the host cells reorganized around the papillas deposited under the pathogen's appressorium. Papilla composition is predicted to be important in determining the plant's resistance.

Introduction

Cabbages and edible brassicas feed humans worldwide (FAOSTAT data), placing 5th in the global production as a major vegetable crop. Dark leaf spot of brassicas (also referred to as black spot; (Brazauskienė et al. 2011; Conn et al. 1990; Deep & Sharma 2012; Scholze & Ding 2005) or Alternaria blight (Kumar et al. 2014; Meena et al. 2004; Shrestha et al. 2005)) is caused by *Alternaria* fungi (*A. brassisicola* [Schw.] Wiltsh, *A. brassicae* [Berk.] Sacc., *A. raphani* [Groves & Skolko], and *A. alternata* [Fr.] Kreissler (Bock et al. 2002; Köhl et al. 2010; Kubota et al. 2006; Kumar et al. 2014). The disease is the major bottleneck in the production of cultivated oilseed crops, Chinese cabbage, head cabbage, broccoli, cauliflower, and other important crops from this family regardless of location. It leads to 15% to 70% losses, mainly by infection of seeds and seedlings, but also of the edible produce (Kumar et al. 2014; Nowicki et al. 2012b; Shrestha et al. 2005). Production of the vegetable brassicas is mainly affected by *A. brassicicola* and *A. brassicae* (Kumar et al. 2014; Michereff et al. 2012; Nowicki et al. 2012b), while the oleiferous seed crops are mainly affected by *A. brassicae*. The dark leaf spot disease is particularly common in tropical or subtropical regions, but also threatens the *Brassicaceae* production in areas with high humidity and frequent rainfall (Humpherson-Jones & Phelps 1989). In Poland, this disease mainly impacts the late and mid-season cultivars of head cabbage, grown for storage or sauerkraut processing (reviewed in (Nowicki et al. 2012b)). Poland ranks 5th or 6th in global production of these crops (FAOSTAT), the yearly threats of *Alternaria* spp. (Kasprzyk et al. 2013) dealing yield losses, and heavy protective fungicide usage (Nowicki et al. 2012b) are economically important issues.

Both main pathogens, *A. brassicicola* and *A. brassicae*, infect host plants at all developmental stages. Typical disease symptoms – dark brown spots/lesions with characteristic concentric circumferences, often with a yellowish chlorotic halo – appear on leaves, stems, and siliques. Under conducive conditions, the colored spots develop a layer of brown-black conidial spores. The enlarging necrotic lesions drastically reduce the photosynthetic efficiency, hasten plants' senescence, and lead to collapse and death of plants under high pathogen pressure. Such symptoms generate several sources of *Brassicaceae* crops losses caused by black spot: Damping-off of seedlings, spotting of leaves of cabbages, blackleg of heads of cabbages (head cabbage and Chinese), and spotting/browning of cauliflower curds and broccoli florets (reviewed in Kumar et al. 2014; Nowicki et al. 2012b).

In order to augment integrated pathogen control, resistant *Brassicaceae* crops are needed. Although significant efforts have been made, to date no *Alternaria*-resistant cultivars exist, and the resistant wild *Brassicaceae* plants do not cross well with the domesticated ones (Hansen & Earle 1997). For planning of breeding programs designed to develop disease-resistant cultivars, it is important to find sources of Alternaria resistance in the *Brassica oleracea* germplasm, including white head cabbage or cauliflower. A successful breeding program also relies on methods for distinguishing genetic differences in resistance early in the plant development.

The main goal of our study was to search for sources of *A. brassicicola* resistance in the *Brassicaceae* germplasm. To address this issue, we developed rapid methods for evaluation of *A. brassicicola* resistance in *Brassica oleraceaea* under controlled conditions. This study aimed to compare the detached leaf and seedlings bio-assays regarding important variables such as age of leaf, leaf position, inoculum concentration, and incubation temperature. All of these factors influence the dark leaf spot severity. Both methods were also compared with the results of the field assessment of Alternaria resistance across a broad collection of germplasm. Moreover,

confocal investigations were employed on the double-stained samples of inoculated leaves, for novel investigations of the subcellular processes accompanying the *A. brassicicola* infection of plants differing in susceptibility.

Materials and methods

Plant and pathogen material

Brassicaceae germplasm used in this study included cultivars, breeding lines, interspecific hybrids, landraces, and wild accessions from the germplasm collection at the Research Institute of Horticulture (InHort; Skierniewice, Poland), collectively referred to as cultigens (Table 1). Ten days after sowing, plants were transplanted into ∅ 10-cm plastic pots containing a peat substrate Kronen-Klasmann. Seedlings were grown on benches in a greenhouse at 21/16°C (day/night) and 10 h of light, fertilized and watered in accordance with the accepted practice for the species.

Three *A. brassicicola* isolates (X2038, X2039, X2040) used in this study were obtained from Geves – Snes National Seed Testing Station, France. Thirteen isolates were collected in recent years from *Brassicaceae* crops symptomatic for dark leaf spot, grown in various regions of Poland (2011). Pathogen identity was confirmed by microscopic observations of morphological characteristics. Stock cultures of *A. brassicicola* were maintained on standard Potato Dextrose Agar (PDA) media at 4 °C. For the preparation of inoculum, each isolate was incubated on PDA at 24±1 °C in the dark. Conidia of 10-days old cultures were washed off the plate with sterile distilled water and filtered through two layers of cheesecloth to remove the remaining mycelium. The conidial suspension was then shaken and supplemented with 0.1% agar solution. The inoculum concentration was determined by three-time measurements with a haemocytometer using a stereoscopic microscope Nikon Eclipse E200, and final inoculum concentrations were adjusted according to the protocols used in particular tests described below.

Pathogenicity and aggressiveness of all isolates were checked on the detached leaves of a susceptible cv. Kamienna Głowa (PNOS Ożarów, Poland). Leaves were detached with scissors from 45-days old plants and immediately placed on wet cellulose wadding in plastic boxes. Conidial suspension (40 µl) at the $10^5 \times ml^{-1}$ was placed on the center of the adaxial side of each leaf. The boxes with inoculated leaves were covered with glass to maintain stable and high RH (>85%) and placed in the growth chamber at 25 °C in darkness. Disease symptoms were assessed in two independent experiments, based on the size of necrotic lesions at 7 dpi. All 16 tested isolates were pathogenic, despite differences in their aggressiveness (Suppl. Fig. 1). For the subsequent bio-assays, six most aggressive isolates (X2038, X2039, X2040, IW1, IW6, IW11) were mixed in equal proportions, as they expressed similar virulence checked separately, in the detached leaf assay on 10 cultigens (data not published).

Experiments conditions

Bio-assay optimization

Experiments were conducted under controlled environment conditions in the growth chambers of InHort. These assays included detached leaf and seedlings bio-assays, described below. We evaluated the influence of several variables on the disease severity in cultigens with various levels of susceptibility to *A. brassicicola*: Inoculum concentration, leaf age and position, incubation temperature, and testing method (detached leaf; seedlings).

Four inoculum concentrations (10^3, 10^4, 5×10^4, 10^5 conidia × ml^{-1}) were tested for severity of disease symptoms in a detached leaf assay using four cultigens of *Brassicaceae* plants (PGH01C, PGH05I, PGH08C, *Sinapis alba*), each showing a different susceptibility to the pathogen as per our initial experiments. The 3rd or 4th fully expanded leaves taken from 35-days old plants of each cultigen were placed immediately in plastic boxes lined with moist tissue paper, and hand-sprayed over the leaf upper (adaxial) surface with a conidia suspension (see above) until completely covered. The boxes containing the inoculated leaves were covered with glass to maintain stable high RH (>85%) and then placed in a growth chamber at 25 °C in darkness. Each inoculum concentration was tested in a series of two independent replications with 22 leaves per cultigen.

To assess the disease severity on leaves from plants at different ages, seeds were sown at 10-days intervals and two fully expanded leaves at the 3rd and 4th leaf position on a plant were collected from 35-, 45- and 55-days old plants. Disease development as relevant to the leaf position on a plant was determined using two fully expanded leaves attaching at the first and second, third and fourth, fifth and sixth leaf position, collected from 55-day old plants. Detached leaves were inoculated with conidia suspension (10^5 conidia × ml^{-1}) and incubated as described above. The experiment was conducted in two independent replicates, each consisting of two boxes containing 18 leaves (one leaf position per one box) for each of the four studied cultigens (PGH01C, PGH05I, PGH08C, and PGH33P).

The influence of incubation temperature (18, 22, 25, and 30 °C) on disease intensity in the five studied cultigens (PGH01C, PGH05I, PGH08C, PGH33P, PGH34K) was evaluated using the seedlings tests and the detached leaf assay. At 35-days old, the seedlings were inoculated with conidial suspension (10^5 conidia × ml^{-1}) until run-off, using a hand-sprayer. Inoculated plants were covered with polythene bags to preserve high humidity, and incubated in a growth chamber (25 °C day/night, 12 h photoperiod). We performed a similar study using the leaves (3rd and 4th) detached from 35-day old plants, using the methods described above. The experiment was designed in two independent replicates. The number of seedlings or leaves used in the test differed for each cultigen and for each incubation temperature due to availability of plant materials (n=12 to 20), in particular for the interspecific hybrids (n=10 to 20), and was considered in the post-hoc statistical analyses.

To compare two methods of inoculation, 38 cultigens of *Brassicaceae* plants (Table 1) were evaluated with the detached leaf and seedlings tests. Head cabbage line 'PGH23K' was used as the susceptible control, and *S. alba* and *Camelina sativa* were used as the resistant controls.

In the detached leaf assay, the disease severity was examined on two leaves (3rd, 4th true leaves) detached from 45-days old plants. The severity of disease symptoms on seedlings was evaluated on the 45-days old seedlings. The experiment was conducted as two replications of independent sets, and each cultigen was represented by 15 to 20 seedlings/leaves depending on the material availability within each cultigen.

Field evaluations

A collection of 23 cultigens including *B. oleracea* (head cabbage, cauliflower), *B. rapa* (Chinese cabbage), *B. napus,* and interspecific hybrids at various degrees of homosygosity, with diverse morphological and agrobotanical characteristics, was evaluated in 2012 at the experimental field area (Department of Genetics, Breeding, and Biotechnology, InHort).

The soil type was a pseudopodsolic over loamy sand (1.5% organic matter, pH 6.5). The tested plants were developed from seeds in the greenhouse in the mid-May. Three weeks-old seedlings were planted in the field, with 50 cm between plants in the row and 60 cm between the rows. The design was a randomised complete block with three replications. Plots consisted of 10 plants in a single row. Fertilisation, irrigation, and pest control followed the current recommendations for cabbage production. No fungicides were applied during the vegetation period, for evaluation of resistance of the cultigens.

Disease ratings

The degree of infection on detached leaves or seedlings was assessed four days after inoculation. The field trials were assessed gradually from the mid-September until the mid-October, when plants reached maturity. Disease intensity was rated using 0 – 5 scale: 0 = no spots and no chlorosis on the investigated plant organ, 1 = disease symptoms visible on up to 10% area of the investigated plant organ, 2 = disease symptoms visible on 11% to 25% area of the investigated plant organ, 3 = disease symptoms visible on 26% to 50% area of the investigated plant organ, 4 = disease symptoms visible on 51% to 75% area of the investigated plant organ, 5 = disease symptoms visible on more than 76% area of the investigated plant organ.

The disease severity index (DSI) was calculated for each cultigen as a mean of the ratings determined for the seedlings/leaves, respectively, similar to other studies of this pathosystem (Hansen & Earle 1997, Doullah et al. 2006).

At the beginning of this study, such arithmetically biased methods of assessment of Alternaria dark leaf spot severity were employed, and keep on being used until this day (Conn et al. 1990; Deep & Sharma 2012; Doullah et al. 2006; Hong & Fitt 1995; Köhl et al. 2010; Mazumder et al. 2013; Rashid et al. 2011; Scholze & Ding 2005; Sharma et al. 2002; Sharma et al. 2004), although more accurate methods were developed (Brazauskienė et al. 2011; Meena et al. 2011; Shrestha et al. 2005). Moreover, the relative disease intensity on the cultigens undergoing testing (and, hence, their inferred resistance) would be kept, irrespective of the scale used for such an assessment.

Cultigens with a DSI of 0 to 1 were classified as highly resistant, those with a DSI of 1.1 to 2 as moderately resistant, and those with an index of 2.1 to 5 as cultigens with various levels of pathogen susceptibility.

Microscopic visualization of the infection process

Samples of tested cultigens (susceptible: PGH33P, PGH12P, ‘Kamienna Głowa’ and moderately resistant: PGH05I; Table 2) were taken using a paper punch (∅5 mm; at least 10 samples per stage and per cultigen) at 0 to 4 days from plants being inoculated with the isolate 2039 (seedlings assay). Ethanol-cleared samples were re-hydrated by soaking in decreasing ethanol solutions (100, 75, and 50% ethanol, v/v). The conventional dual-stain followed a previously described protocol (Nowicki et al. 2012a): Re-hydrated samples were soaked in 0.05% trypan blue (w/v; CarlRoth Poland, aq) overnight at room temperature, then washed three times with distilled water (5 min, room temperature), and finally soaked in 0.05% aniline blue (w/v; Sigma-Aldrich Poland) in 150 mM KH_2PO_4, pH 9 for 3-4 h, RT. The samples then were de-stained in 150 mM KH_2PO_4, pH 9, three times for 15 min, and mounted with water for microscopic observations.

The protocol for visualization of actin filaments was established after testing several available methods (Chang & Nick 2012; Kobayashi et al. 1997; Langenberg 1978; Maisch & Nick 2007; Miklis et al. 2007; Olyslaegers & Verbelen 1998; Opalski et al. 2005; Vitha et al. 2000). Based on the results of this initial study, we chose to follow the method of Olyslaegers & Verbelen (1998) with minor modifications. Samples were fixed immediately after collection, in mixture of 1% formaldehyde (prepared freshly; w/v), 0.05% glutaraldehyde (v/v), 2% glycerol [v/v], and 1% DMSO [v/v] in fixation buffer (50 mM PBS, pH 6.9, 1 mM EDTA, 2 mM MgCl2,) for 30 min at RT. After washing with the fixation buffer (no aldehydes), excess aldehydes were quenched with freshly prepared $NaBH_4$ (aq), and samples were permeabilized with Triton X-100 in fixation buffer (30 min, RT). After three washes with fixation buffer (pH 7.4; 5 min, RT), samples were soaked overnight (4 °C; in darkness) with 0.66 µM phalloidin-rhodamine (Sigma-Aldrich Poland) in 50 mM PBS pH 7.4, and 0.05% (w/v) aniline blue. Samples were then washed with 100 mM PBS pH 6.9 and mounted with water for microscopic observations.
Confocal laser scanning microscopy was performed using a Nikon C1 microscope equipped with solid-state and diode lasers. Image acquisition of dual trypan blue - aniline blue stained samples was performed in a sequential mode to avoid spectral cross-talk. For fluorescence excitation of trypan blue, 488 nm of Sapphire solid-state laser (Coherent) was used in a single track mode. Fluorescence was collected through a filter block with a 650 nm LP emission filter. For the aniline blue channel, the 408 nm excitation line of a diode laser was used in a single track mode. Fluorescence was collected through a filter block with a 513-530 nm BP emission filter. Fluorescence of rhodamine was induced using 543 nm He/Ne laser and collected through 605/675 nm emission filter. Z-series images were collected at 0.7-1 µm intervals through the specimens. All images were processed using EZ C1 Free-Viewer (v.3.90; http://nikon-ez-c1-freeviewer.software.informer.com/); the digital quantifications were performed using ImageJ (Abràmoff et al. 2004). Rendering of the 3D papilla structures from microscopic Z-stacks was done with Blender ver.2.74 and GIMP ver.2.8.2.

Statistical analyses

Computation and data comparisons were performed using MS Excel 2007 and R (ver. 3.2.0). Descriptive statistics employed calculation of means, standard deviations (SD), medians, quartiles, etc. Data comparisons were performed by one- or two-way-ANOVA, with post-hoc Duncan's or Tukey's Honestly Significant Difference (HSD) analyses.

Results

Bio-assay optimization

Disease resistance in a breeding program is best tested in the field, under natural pathogen infection. In contrast to this method, growth chamber tests can often be fast, efficient, and high-throughput. Therefore, we attempted optimization of the seedlings and detached leaf assays under controlled conditions for the brassicas – *A. brassicicola* pathosystem, varying the inoculum concentration, age of leaf, leaf position, and incubation temperature. We later assessed the reliability of these two methods, by comparing their results with data from field assays.

For optimization of the inoculum concentration, four concentrations (10^3, 10^4, 5×10^4, and 10^5 conidia $\times$ ml^{-1}) were used. Disease severity increased as the inoculum concentration increased on all tested cultigens, regardless of their susceptibility level to *A. brassicicola* (Fig. 1). Our data shows lack of significant differences between the disease severity recorded at the two highest conidial concentrations ($F = 2.8536$, $p = 0.09322$). The critical inoculum concentration appeared to be 5×10^4 conidia $\times$ ml^{-1}, since only here and under higher concentration, the pathogen evoked modest disease symptoms in the resistance standard *S. alba*. In order to maintain high efficiency and stringency of selection, the subsequent bio-assays employed the 10^5 conidia $\times$ ml^{-1} inoculum concentration.

Experimental results indicated that leaf age ($F = 28.396$, $p = 1.707e^{-07}$) and genotype ($F = 14.990$, $p = 3.092e^{-09}$), as well as their interaction ($F = 11.363$, $p = 3.775e^{-07}$) had significant effects on disease intensity levels among the tested cultigens (Fig. 2A). Irrespective of cultigen susceptibility to *A. brassicicola*, older leaves exhibited more symptoms than younger leaves. In all four cultigens tested, we observed the lowest disease intensities when inoculated leaves were taken from 35-days old plants. Inoculated leaves derived from plants 10 days older resulted in a significant increase of disease symptoms (Fig. 2A) for all cultigens (for cultigens: $F = 5.9254$, $p = 0.0006421$; for plant ages 35/45-days old: $F = 18.1081$, $p = 2.961e^{-05}$; for interaction of both factors: $F = 3.0232$, $p = 0.0302953$). Cultigens PGH01C and PGH05I lacked significant differences in disease development between the leaves taken from 45– and 55–days old plants (age: $F = 0.7438$, $p = 0.39188$;). On the other hand, the remaining two cultigens PGH08C and PGH33P showed only slightly significant differences in disease intensities between each other ($F = 4.0833$, $p = 0.04649$), but their respective DSIs increased significantly from 45 to 55 days old ($F = 11.1940$, $p = 0.00142$). Therefore, to maintain efficient and stringent conditions, we used 45-days old seedlings for subsequent experiments.

We observed significant effects of leaf position ($F = 109.474$, $p < 2.2e^{-16}$), genotype ($F = 96.433$, $p < 2.2e^{-16}$), and their interaction ($F = 103.316$, $p < 2.2e^{-16}$) on disease intensities among the tested cultigens (Fig. 2B). We observed that for all cultigens, the oldest detached leaves (1st and 2nd leaves) displayed more intense disease symptoms compared with the younger ones (with significant differences for the 3rd and 4rd true leaves in PGH01C and PGH33P: $F = 20.1915$, $p = 1.714e^{-05}$; with significant differences for the 5th and 6rd true leaf in all cultigens tested: $F = 94.254$, $p < 2.2e^{-16}$) (Fig. 2B). The effect of leaf age was diminished in the less infected cultigens (PGH05I, PGH08C), but significant differences existed between the 3rd to 4th leaves and the 5th and 6rd true leaves ($F = 45.590$, $p = 3.921e^{-14}$). For subsequent experimentation used for comparison of the testing methods, we used the 3rd to 4th leaf stage, to ensure stringent and high efficiency conditions.

Our data on the seedlings and detached leaf bio-assays under temperatures 18, 22, 25, or 30 °C, suggest a significant effect of this parameter on disease intensity, regardless of the *A. brassicicola* susceptibility of the cultigen (Fig. 3). In both tests, disease severity peaked at 25 °C in all five cultigens. In the seedlings tests, the DSI values for 25 °C differed significantly from a lower DSI noted for 18 °C regardless of the cultigen tested ($F = 24.3821$, $p = 2.094e^{-06}$), exceeding slightly the DSI for either 22 °C or 30 °C ($F = 5.5458$; $p = 0.019$). For the detached leaf tests (Fig. 3A), analysis of variance showed that incubation temperature had no effect on disease severity for most tested cultigens ($F = 0.1602$, $p = 0.689168$). The significant differences between various incubation temperatures were apparent only for the line PGH33P ($F = 27.8526$, $p = 7.402e^{-07}$). In case of other cultigens, there was either no significant difference among temperatures (PGH01C: $F = 0.2367$, $p = 0.6276$; PGH05I: $F = 1.8276$, $p = 0.17958$; PGH08C: $F = 2.5365$, $p = 0.1143$), or significant differences in symptom severity were recorded only between 18 °C and 25 °C (PGH34K: $F = 26.6842$, $p = 2.733e^{-05}$). We continued to use temperature 25 °C for the subsequent bio-assay comparisons to provide stringent conditions, to enable selection of the best-performing cultigens.

Comparison of two laboratory testing methods

Disease severity was compared in 38 cultigens of brassicas employing two bio-assays (seedlings and detached leaf assays; Table 1). Comparison of these assays (two-way-ANOVA) indicated that the two methods were not significantly different in evaluating the cultigen's resistance against *A. brassicicola* ($F = 2.2445$. $p = 0.1342$). It is important to note, that the DSIs of only four cultigens (PGH11P, PGH12P, PGH19K, PGH28K) exhibited significant differences ($p < 0.05$) between the seedlings assay and the detached leaf assay.

Analysis of variance revealed a significant effect of the cultigen ($F = 478.8214$; $p < 2.2e^{-16}$) and the interaction of cultigen × testing method ($F = 5.9677$; $p < 2.2e^{-16}$) on the disease severity. From the 38 cultigens tested using both methods, two cultigens PGH05I and PGH25I displayed the lowest disease symptoms (DSI = 1.65±0.7 and 1.90±0.8, respectively), followed by PGH34K (2.12±0.6) and PGH28K (2.70±1.0). The remaining cultigens showed various degrees of susceptibility to *A. brassicicola* in both, the detached leaf assays and the seedlings assays. From the two resistance standards tested, *S. alba* showed barely any disease symptoms, while *C. sativa* remained free of the dark leaf spot symptoms.

Field evaluations and test cross-comparison

Twenty three *Brassicaceae* cultigens were tested in the field and showed a broad range of dark leaf spot severity (Table 1). The lowest DSI values were obtained for 'Sława z Enkhuizen' (0.8±0.3), 'Kilagreg F_1' (1.3±0.6), and PGH24K (1.4±0.1). Five other cultigens (PGH36K, PGH05I, PGH23K, PGH35C, and PGH22K) expressed slightly higher, but still relatively low disease severity (from 1.8 to 2.2). The highest disease severity (4.4) was observed for 'Bilko F_1', PGH12P, and 'Kamienna Głowa'. Ratings for the remaining cultigens tested in the field ranged from 2.7 to 3.3, representing a rather narrow range.

When comparing the bio-assays results with field data (Table 1), it was apparent, that 14 cultigens differed in response to *A. brassicicola*. Genotype had a higher effect on the disease intensity ($F = 6.3155$; $p < 2.2e^{-16}$) than the testing method used for evaluation ($F = 11.8785$; $p = 7.532e^{-06}$), driving the interaction of both factors ($F = 5.7477$, $p < 2.2e^{-16}$). Better field than

laboratory performance seems to be the stronger effect, as it occurred in more cases and in more intensity than the opposite. Lower DSIs in the field than in the bio-assays were observed for majority of cultigens tested (PGH09K; PGH22K; PGH23K; PGH24K; PGH30K; 'Kilagreg F_1'; PGH36K; PGH35C; 'Sława z Enkhuizen', PGH02C, PGH03C, PGH04C, PGH17K, PGH19K), while only PGH05I showed inverse behavior, with worse field than laboratory performance, yet of small effect in terms of the biological impact.

In summary, we optimized several parameters important for standardizing two bio-assays of *Brassicaceae* cultigens with relation to their response to *A. brassicicola* inoculation. In the course of this study, we confirmed the inverse relationship between leaf age and *A. brassicicola* resistance. Comparison of both testing methods (seedlings and detached leaf) with the field data using a broad array of cultigens indicated that (i) under controlled conditions, the genotype rather than the testing method underlies the cultigen's resistance; (ii) the stringent conditions of our optimized laboratory screens allowed to reliably distinguish the resistant cultivars from the susceptible ones; (iii) field assays confirmed the particularly good performance of the line PGH05I, despite minor DSI differences.

Microscopic analyses of the A. brassicicola infection process

After optimizing the main parameters for the bio-assays, we sampled the ongoing experiments for comparative microscopic analyses of the infection process in plant materials differing in response to *A. brassicicola*. Confocal microscopic analyses of dually stained samples visualized the subcellular events following the pathogen inoculation.

Staining of chitin in the pathogen cell wall with trypan blue, and of callose deposited in the host plant papillas with aniline blue, visualized the interactions between the pathogen and the host plants. Various stages of pathogen infection (germinating conidia, aerial hyphae growth, epidermal and stomatal penetrations, establishment of haustoria and secondary hyphae) together with the plant's defense against infection (papilla depositions) were recorded (Fig. 4). These analyses showed that *A. brassicicola* invades leaf surface by means of both, direct penetration and entry growth stomata. Detailed counting of the penetration events suggested a novel hypothesis on the relationship between plant's susceptibility status and the penetration mode assumed by the pathogen. The susceptible plants tested were predominantly infected by direct hyphae growth or penetration through appressorium, with only occasional stomatal infection (line PGH12P; $p = 0.0014$; $n = 43$), or with no significant preference to the infection mode (PGH33P, 'Kamienna Głowa'; Table 2). Contrastingly, the defenses raised by the line PGH05I with the lowest DSI, necessitated the pathogen to penetrate through the stomata instead ($p = 0.011$; $n = 10$). Papilla deposition or size lacked statistical differences (PGH12P: 119.62±44.58 μm^2; PGH05I: 188.25.25±71.25 μm^2) in relation to plant's susceptibility level ($F = 3.258$; $p = 0.09$). This observation suggests the varying papilla composition as one of the factors responsible for the pathogen penetration mode, and – hence – plant's resistance.

Involvement of host plant's actin filaments in generating the defense response (papilla deposition) to *A. brassicicola* infection was studied afterwards. Phalloidin labeled with rhodamine effectively stained the actin filaments of both, the plant cells and *A. brassicicola* hyphae. From the seven tested protocols of actin staining, we only succeeded with one method (see the Methods section) with slight modifications. Further attempts at modifications of the staining procedure, such as exchange of buffering or chelating agents, failed to improve the stain.

Taken together, this suggests that effective staining of actin networks requires extensive experimentation, depending on the analyzed species/pathosystem.

Based on our observations, pathogen inoculation resulted in rapid reorganization of the subcellular actin filaments networks, in terms of localization and densities. While the inoculated plants showed strands of filaments running across the cells without particular order, this changed drastically already at 2 dpi. The developing papilla served as focal point for actin filaments network, enclosing the papilla in a cocoon (Fig. 4). Although we failed to reliably calculate the high densities of the filaments in plants differing in response to *A. brassicicola*, our observations confirm the involvement of actin filaments in plant's response to pathogen attack.

Taken together, our microscopic studies helped visualize the *A. brassicicola* infection process in plants differing in response to this pathogen. Novel data on lack of time or size (quantitative) differences between hosts' papilla deposition in response to pathogen infection suggests qualitative differences in papillas composition in moderately resistant vs. susceptible plants. Dramatic reorganization of cell's actin filaments documents their participation in generating the defense responses irrespective of cultigen's resistance status.

DISCUSSION

Reliable methods for accurate evaluation of genotypes for pathogen resistance are an important issue for breeding programs. Therefore, we standardized and compared two methods for evaluating resistance of *Brassicaceae* plants against *A. brassicicola*. Impacts of incubation temperature, inoculum concentration, leaf age and position on disease severity were analyzed on *Brassicaceae* cultigens.

Our data indicate increased disease severity with an increase of inoculum concentration. Significantly higher disease intensity was recorded for the two highest concentrations of conidia (5×10^4 and $10^5 \times ml^{-1}$). Similar results were obtained for the effects of inoculum concentrations on *B. rapa* tested with *A. brassicicola* (Doullah et al. 2006) and *B. napus* tested with *A. brassicae* (Hong & Fitt 1995). In contrast, King (1994) noticed no significant differences in disease intensity for *B. oleracea* var. *capitata* and *B. napus* inoculated with 2.3×10^4, 3.7×10^5, and 5×10^4 conidia $\times ml^{-5}$ of *A. brassicicola.* Inoculum concentrations comparable with those used in our study (10^5 conidia $\times ml^{-1}$) or higher were successfully employed for evaluation of cultigens with various level of *Alternaria spp.* resistance (Gupta et al. 2013; Köhl et al. 2010; Mazumder et al. 2013; Scholze & Ding 2005; Tohyama & Tsuda 1995).

Incubation temperature was important in our study for evoking disease symptoms regardless of the plant material tested, particularly in the detached leaf assays. Optimized assay temperature of 25 °C resulting in the highest disease severity irrespective of apparent cultigen's susceptibility, is in agreement with other studies of this pathosystem, or of the related *A. brassiceae* (Doullah et al. 2006; Gupta et al. 2013; Hong & Fitt 1995; Kennedy & Graham 1995; Mazumder et al. 2013; Rashid et al. 2011; Sharma et al. 2002; Su'udi et al. 2011; Zală et al. 2014).

Using the detached leaf method, we observed that disease intensity scores were correlated with the leaf age, irrespective of the cultigen's apparent resistance/susceptibility. Our data are in agreement with other previous reports, where the older leaves of *Brassicaceae* plants are more susceptible to infection by *A. brassicicola* than younger leaves. Such "age-conditioned susceptibility" (Domsch 1957) was recorded in nearly all *Alternaria*-host pathosystems, including the oleiferous *Brassicaceae* crops, and their main pathogens – *A. brassicae* and *A. brassicicola* (Allen et al. 1983; Deep & Sharma 2012; Doullah et al. 2006; Hong & Fitt 1995; Rotem 1998; Saharan & Mehta 2002). Despite this observation being generally agreed on, differences exist how the specific leaf ages influence disease severity. For instance, Deep & Sharma (2012) reported that the younger plants of susceptible cauliflower at 15 and 30-days old plants did not show any leaf spot symptoms of *A. brassicicola*, in contrast to the 45- and 60-days old plants being very susceptible. In our studies, the intensity of symptoms gradually increased from the 35-days old plants, as they got older. Such differences in disease development might result from the inoculation techniques used or, more likely, differences in pathogen aggressiveness or the genetic resistance of the plant materials. The environment may also play an important role in such investigations, especially since our tests were conducted under controlled conditions in the growth chambers, while the experiments of Deep and Sharma (2012) employed greenhouse conditions. It is generally accepted that even small changes in the environmental factors of a bio-assay may be critical for identification and categorization of susceptible or resistant genotypes (Kozik & Sobiczewski 2000).

It may be possible, as suggested by others (Horsfall & Dimond 1957), that susceptibility to necrotrophic pathogens, such as Alternarias, may result from the low sugar levels in older plants. The relationship between plant or leaf age and disease development has also been attributed to

the amount of epicuticular wax on the leaf surface, which decreased with increasing leaf age (Conn & Tewari 1989). In our previous studies, the intensity of disease symptoms on the 1st and 2nd leaves of cauliflower and white cabbage plants infected by *A. brassicicola* did not depend on wax presence. But, removing the epicuticular wax resulted in higher disease intensity when testing the 3rd and younger leaves (data not published).

The parameters optimized in our study were then used to enhance the stringency and efficiency of both evaluation methods to compare efficacies across a broad collection of cultigens. The main effect of genotype on disease severity was more significant than differences between the two inoculation methods. Therefore, either the detached leaf or seedlings test could be used as reliable tools to evaluate *A. brassicicola* resistance in germplasm accessions of the brassicas. These observations are in agreement with those of Doullah et al. (2006), who found a strong positive correlation between the detached leaf test and the seedling test using 56 cultivars of *B. rapa*. They also recommended the detached leaf method for primary screening and selection within *B. rapa* accessions resistant against *A. brassicicola*, before final tests under natural field infection. The advantage of the detached leaf method over the seedlings test is a possibility for resistance evaluation in large populations within the germplasm collection. The same conclusions might be drawn from our results on disease assessment of *Brassicaceae*, in particular *B. oleracea*. Other authors have also found the detached leaf method to be simple, easy, and fast for evaluation of *Alternarias* resistance in cabbage and cauliflower (Sharma et al. 2004). Similar outcome was presented for related pathosystems, where the detached leaf inoculation was the most efficient and reliable technique of four studied methods for screening of *A. brassicae* resistance in rape seed and mustard (Vishvanath & Kolte 1999).

The unexpected outcome of this study was the differences between the evaluations using controlled conditions tests and field assessments of our cultigens. For cultigens showing less intense disease symptoms in the field than in the controlled conditions, the differences may have arisen from the particularly stringent conditions employed for the bio-assays. On the other hand, the cultigen PGH05I performed better under artificial inoculation, scoring only slightly worse under natural epiphytotic conditions. This might indicate the presence of other stress factors: Local differences in growing conditions or climate (Hong & Fitt 1995; Scholze 2002; Shrestha et al. 2005), suboptimal developmental stage upon pathogen incidence, or presence of other pathogens under natural epiphytotic conditions, in particular the opportunistic ones such as *A. alternata* (Kubota et al. 2006; Michereff et al. 2012; Tohyama & Tsuda 1995). Under natural infection in the field, the conidia concentration may be low and plants may escape infection (Sharma et al. 2002). Further, the length of vegetation period of the tested cultigens of cabbage also plays an important role in the susceptibility to *A. brassicicola* in the field. This hypothesis was confirmed by our results for the early cultigens such as 'Sława z Enkhuizen', 'Kilagreg F1", and PGH24K, showing lower infestation symptoms in the field, while late cultigens such as PGH09K or PGH30K were more susceptible. Comparatively, higher resistance of the interspecific hybrid PGH05I was recorded after 120 days of vegetation, and therefore was independent of the plant age. The Chinese cabbage cultigens (PGH07P, PGH33P, PGH12P) and two lines of swede (PGH10P, PGH11P) were susceptible in the field irrespective of their short vegetation period. Due to these and other factors influencing the severity, field assays with or without inoculation may be inappropriate for evaluation of germplasm at the early stage of resistance breeding (Sharma et al. 2002). It is worth noting, however, that the disagreements of disease intensity observed here, mostly do not influence the apparent cultigen's resistance, i.e., cultigens classified as "moderately resistant" in the bio-assays do not change their status to

"susceptible" under the field trials. Rather, most changes in this respect occurred for the group of cultigens classified as susceptible in the bio-assays but moderately resistant in the field. This, however, would have only a minor impact on the study's outcome, as the susceptible cultigens would have been discarded in the stringent preliminary laboratory screens, at the early stages of selection.

We decided to use a mixture of pathogen isolates in equal proportions for screening our collection of *Brassicaceae* cultigens, due to lack of differences in virulence in the preliminary assays. Other scholars of this pathosystem have used isolates with contrasting pathogenicity towards the host plants (Cho et al. 2006; Pochon et al. 2013; Su'udi et al. 2011). Alternatively, and similar to our approach, plants with varying reactions to the pathogen were used for experimentation (Doullah et al. 2006; Mazumder et al. 2013; Meena et al. 2011; Sharma et al. 2002). Testing a modest local collection of *A. brassicicola* and *A. brassicae* isolates from infected cauliflower plants indicated differences among three cultivars in a detached-leaf assay (Deep & Sharma 2012). The difficulties currently experienced deal in particular with lack of pathogen/testing standardization described above and lack of pathogen resistance sources among the cultivated cultigens (Kumar et al. 2014; Nowicki et al. 2012b; Sharma et al. 2002).

Several attempts have been made to discover the sources of high level resistance against *A. brassicicola* or *A. brassicae*, but until now no such materials have been identified among the cultivated species of the Brassica genus (reviewed in Kumar et al. 2014; Nowicki et al. 2012b). High levels of resistance against these pathogens have been reported in the wild relatives of Brassica inside and outside the tribe *Brassicaceae* (reviewed in Kumar et al. 2014). Our study on assessment of *A. brassicicola* resistance among 36 cultigens including mainly *B. oleracea* (18 head cabbage, 3 Chinese cabbage, 6 cauliflowers), but also 4 interspecific crosses and 5 *B. napus* accessions, revealed lack of high *A. brassicicola* resistance, when compared with the most resistant plants of *C. sativa* and *S. alba*. But, it is of note, that the two interspecific hybrids (PGH05I, PGH25I) showed moderate resistance against *A. brassicicola* in the bio-assays. All the remaining cultigens (head cabbage, cauliflower, Chinese cabbage, rape) displayed various levels of susceptibility. This is in agreement with other studies (Cherukuri et al. 2009), that also pointed out lack of true source of resistance against *A. brassicae* among the *B. oleracea, B. campestris, B. nigra, B. juncea, B. napus and B. carinata* accessions. Interestingly, an assessment of *A. brassiceae* resistance among 38 cultigens (Sharma et al. 2002) indicated that vegetable brassicas (cauliflower, cabbage, and broccoli) were comparatively less susceptible than the cultivated oilseed brassicas. Our bio-assays data prove that the high level of genetic control over *A. brassicicola* resistance in exists two cultigens (PGH05I, PGH25I). Therefore, these might be promising sources of *A. brassicicola* resistance in brassicas breeding programs.

Our microscopic observations of the *A. brassicicola*-host interaction resulted in visualization of the pathogen cycle including: germinating conidia, aerial hyphae formation and growth, infection structures (appressoria and haustoria), and development of secondary hyphae. Simultaneously, we observed the defense responses in host plants' leaves: A drastic reorganization of actin networks, deposition of papillae, cell death upon colonization, and a limitation of pathogen spread by callose deposition around the infected cell(s). Some authors (Pochon et al. 2013; Sharma et al. 2014) reported on *A. brassicicola* infection (penetration) routes depending on a given isolate's aggressiveness, but agreed on both direct (epidermal) and stomatal penetration possible for the *Alternarias* in several pathosystems. In particular, Sharma et al. (2014) claimed an aberrant behavior of the least aggressive isolate by only using the direct penetration. In contrast, we observed such preference in relation to host plant's resistance in cultigens showing

different reactions to inoculation with *A. brassicicola*. While the highly susceptible cultigens were readily infected by direct epidermal penetration, the host defenses present in the moderately resistant cultigen necessitated infection by the stomata. Rapid subcellular changes upon perceived pathogen attack were further underscored by strong evidence of actin networks engaging in defense responses, tightly surrounding the developing papilla – irrespective of host plant's resistance. In the light of our findings, further research on papilla composition in plants of contrasting *A. brassicicola* response might shed more light on the background of molecular mechanisms of resistance in either cultigen. Investigation of plant hormones' interplay (jasmonic acid, abscisic acid, and salicylic acid) (Mazumder et al. 2013; Su'udi et al. 2011) and their influence on host plant's susceptibility/resistance is of particular import for comprehension of the subcellular defense mechanisms in the pathosystem studied here.

Conclusions

Genotype of the *Brassicaceae* cultigens studied in this project played a crucial role in determining their resistance against *A. brassicicola*, irrespective of two phytotron testing methods employed. From the bio-assay variables undergoing optimization, the developmental stage of the materials tested had the largest influence on disease severity, in agreement with the accepted "age-conditioned susceptibility" for Alternaria blight. Inoculum concentration, followed by incubation temperature, also influenced disease severity. All parameters affected the reaction of the plant to the pathogen, irrespective of their resistance status. Reactions to the pathogen lacked major differences under the two phytotron methods used, but several cultigens performed comparatively better in the field, suggesting that we applied particularly stringent conditions for either detached leaf or seedlings tests. Two interspecific hybrids with promising levels of *A. brassicicola* resistance were identified from among the pool of 38 cultigens included in this study, with potential for further resistance breeding and phytopathological studies. Microscopic visualization of the infection process in cultigens differing in susceptibility levels helped formulate a novel hypothesis on differences in pathogen infection mode being related to host plant's resistance. Also, papilla composition is likely an important factor in the resistance of the host plant, with actin networks participating in generating the defense responses. Our bio-assays and microscopic data contribute a material advancement in the economically important cabbage-*A. brassicicola* pathosystem.

564 Table 1. Cultigens used in this study. Species, genetic background (if known), vegetation period
565 [days], and DSI±SD in the seedling, detached leaf, and field assays are listed.

Cultigen[a]	**T-sd**[b]	**T-lf**[b]	**T-field**[b]	**Vegetation period [day]**[c]	**Background**[d]
PGH01C	3.1±0.8	2.9±0.8	–	60	cauliflower
PGH02C	3.4±0.6	4.1±0.7	2.7±0.6	70	cauliflower
PGH03C	4.6±0.7	4.4±0.6	3.3±0.7	60	cauliflower
PGH04C	4.1±0.7	4.4±0.6	3.3±0.6	60	cauliflower
PGH05I	1.6±0.7	1.7±0.7	1.9±0.5	–	interspecific *B. oleracea* × *B. napus* S_5 after BC_2
PGH06I	3.1±1.1	3.2±0.7	–	–	interspecific *B. oleracea* × *B. napus* S_5 after BC_2
'Bilko F_1'	4.2±0.6	4.6±0.6	4.3±0.5	70	Chinese cabbage
PGH08C	3.1±1.2	3.4±1.0	–	85	cauliflower
PGH09K	4.3±0.7	4.6±0.5	2.3±0.6	120	head cabbage
PGH10P	3.1±0.5	3.0±0.0	–	55	swede
PGH11P	3.6±0.5	2.6±0.5	–	55	swede
PGH12P	3.7±0.6	4.8±0.5	4.7±0.6	55	Chinese cabbage
PGH13R	3.2±0.6	3.5±0.8	3.3±0.6	-	seedrape
PGH14R	4.0±0.8	4.1±0.9	3.0±0	-	seedrape
PGH15R	3.3±0.6	3.3±0.7	3.0±0	-	seedrape
PGH16K	3.0±0.7	3.8±0.9	3.3±0.6	90	head cabbage
PGH17K	4.6±0.5	4.5±0.7	3.0±0	90	head cabbage
PGH18K	3.7±0.5	4.1±0.7	3.7±0.6	90	head cabbage
PGH19K	3.8±0.9	4.4±0.7	4.3±0.6	90	head cabbage
PGH20K	4.0±0.	3.7±0.	3.3±0.6	90	head cabbage

	6	9			
'Sława z Enkhuizen'	3.9±0.8	4.4±0.5	0.8±0.3	62	head cabbage
PGH22K	3.7±0.8	4.2±0.7	2.3±0.6	110	head cabbage
PGH23K	3.7±0.6	4.3±0.6	2.0±0	110	head cabbage
PGH24K	3.3±0.8	3.9±0.6	1.4±0.1	90	head cabbage
PGH25I	2.0±1	1.8±0.6	–	-	interspecific *B. oleracea* × *B. napus* S_5 after BC_2
PGH26K	3.4±0.6	3.7±0.7	3.1±0.6	120	head cabbage
PGH27I	3.9±0.7	3.8±0.4	–	-	interspecific *B. oleracea* × *B. napus* S_5 after BC_2
PGH28K	2.1±0.7	3.3±0.8	2.7±0.6	120	head cabbage
PGH29K	3.1±0.7	3.7±0.7	3.3±0.3	120	head cabbage
PGH30K	3.5±0.6	3.5±0.6	2.7±0.5	110	head cabbage
'Kamienna Głowa'	4.0±0.4	4.3±0.8	4.4±0.3	120	head cabbage
'Kilagreg F_1'	3.7±0.7	3.9±0.8	1.3±0.6	65	head cabbage
PGH33P	3.2±0.8	3.5±0.8	–	60	Chinese cabbage
PGH34K	2.1±0.5	2.2±0.6	–	85	head cabbage
PGH35C	3.3±0.7	2.8±0.7	2.3±0.4	85	cauliflower
PGH36K	3.9±0.9	4.3±0.6	1.7±0.4	70	head cabbage
Camelina sativa	0.0±0.0	0.0±0.0	–	–	wild accession
Sinapis alba	0.4±0.2	0.6±0.7	–	–	wild accession

[a]Cultigen's commercial names or codes used during breeding of these materials at InHort are presented.

[b]Cultigen's DSI±SD in the seedlings test (T-sd), detached leaf test (T-lf), and in the field under natural epiphytosis (T-field). Details are described in the Materials and methods section. – : not determined. HSD (Tukey) for T-sd and T-pl (two-way-ANOVA): 0.707225. HSD (Tukey) for T-field (one-way-ANOVA): 1.520984

[c]Vegetation period from planting to harvest maturity.

573 [d]Species and genetic information (when available) are listed out.

575 Table 2. Stomatal infection and direct epidermal germination counts for the analyzed cultigens.

CULTIGEN[a]	**PGH05I**	**PGH33P**	**PGH12P**	**'Kamienna Głowa'**
DSI	1.6±0.7	3.2±0.8	3.7±0.6	4.0±0.4
DIRECT[b]	2	12	32	15
STOMATA[b]	13	6	11	15
χ^2 against 50%-50%[c]	**0.005**	0.157	**0.001**	1

576 [a]Cultigens were arranged according to their field performance (increasing DSI; see Table 1).
577 [b]Direct (epidermal) or stomatal penetration counts, based on the analyzed dual stained samples.
578 [c]Results of statistical analyses (*P* from the χ^2 test**)** of the pathogen's non-preference hypothesis.

ACKNOWLEDGEMENTS

Authors wish to recognize the excellent technical contribution of Ms. Marzena Czajka, Mrs. Krystyna Szewczyk, Mrs. Małgorzata Pakuła, and Mr. Ireneusz Werkowski into this study. Dr. Michael Havey (University of Wisconsin-Madison, USA) and Dr. Todd C. Wehner (North Carolina State University, USA) are gratefully acknowledged for critical reading of this manuscript. Dr. Dorothy M. Tappenden (Michigan State University and Lansing Community College, MI, USA) provided great editorial help with this manuscript.

CITED LITERATURE

Abràmoff MD, Magalhães PJ, and Ram SJ. 2004. Image processing with ImageJ. *Biophotonics international* 11:36-42.

Allen S, Brown J, and Kochman J. 1983. Effects of Leaf Age, Host Growth Stage, Leaf Injury, and Pollen on the Infection of Sunflower by A Iternaria helianthi.

Bock CH, Thrall PH, Brubaker CL, and Burdon JJ. 2002. Detection of genetic variation in *Alternaria brassicicola* using AFLP fingerprinting. *Mycological Research* 106:428-434.

Brazauskienė I, Petraitienė E, Brazauskas G, and Semaškienė R. 2011. Medium-term trends in dark leaf and pod spot epidemics in *Brassica napus* and *Brassica rapa* in Lithuania. *Journal of Plant Diseases and Protection*:197-207.

Chang X, and Nick P. 2012. Defence signalling triggered by Flg22 and harpin is integrated into a different stilbene output in *Vitis* cells. *PLoS One* 7:e40446-e40446.

Cherukuri SC, Plaha P, and Sharma R. 2011. Evaluation of some cultivated Brassicas and their related alien species for disease resistance. *Cruciferae Newsletter* 30: 18-22.

Cho Y, Davis JW, Kim K-H, Wang J, Sun Q-H, Cramer Jr RA, and Lawrence CB. 2006. A high throughput targeted gene disruption method for *Alternaria brassicicola* functional genomics using linear minimal element (LME) constructs. *Molecular plant-microbe interactions* 19:7-15.

Conn K, and Tewari J. 1989. Interactions of *Alternaria brassicae* conidia with leaf epicuticular wax of canola. *Mycological Research* 93:240-242.

Conn K, Tewari J, and Awasthi R. 1990. A disease assessment key for *Alternaria* black spot in rapeseed and mustard. *Disease des plantes Survey'au Canada* 70:19.

Deep S, and Sharma P. 2012. Host age as predisposing factor for incidence of black leaf spot of cauliflower caused by *Alternaria brassicae* and *Alternaria brassicicola*. *Indian Phytopathology* 65:71-75.

Domsch Kv. 1957. Die Raps-und Kohlschotenschwärze. *Zeitschrift für Pflanzenkrankheiten (Pflanzenpathologie) und Pflanzenschutz*:65-79.

Doullah M, Meah M, and Okazaki K. 2006. Development of an effective screening method for partial resistance to *Alternaria brassicicola* (dark leaf spot) in *Brassica rapa*. *European journal of plant pathology* 116:33-43.

Gupta P, Ravi I, and Sharma V. 2013. Induction of β-1,3-glucanase and chitinase activity in the defense response of *Eruca sativa* plants against the fungal pathogen *Alternaria brassicicola*. *Journal of Plant Interactions* 8:155-161.

Hansen LN, Earle ED. 1997. Somatic hybrids between *Brassica oleracea* L. and *Sinapis alba* L. with resistance to *Alternaria brassicae* (Berk.) Sacc. *Theoretical and Applied Genetics* 94(8):1078-1085.

623 Hong C, and Fitt BD. 1995. Effects of inoculum concentration, leaf age and wetness period on
624 the development of dark leaf and pod spot (*Alternaria brassicae*) on oilseed rape
625 (*Brassica napus*). *Annals of applied biology* 127:283-295.
626 Horsfall JG, and Dimond A. 1957. Interactions of tissue sugar, growth substances, and disease
627 susceptibility. *Zeitschrift für Pflanzenkrankheiten (Pflanzenpathologie) und*
628 *Pflanzenschutz*:415-421.
629 Humpherson-Jones F, and Phelps K. 1989. Climatic factors influencing spore production in
630 *Alternaria brassicae* and *Alternaria brassicicola*. *Annals of applied biology* 114:449-458.
631 Kasprzyk I, Sulborska A, Nowak M, Szymańska A, Kaczmarek J, Haratym W, Weryszko-
632 Chmielewska E, and Jędryczka M. 2013. Fluctuation range of the concentration of
633 airborne *Alternaria* conidiospores sampled at different geographical locations in Poland
634 (2010–2011). *Acta Agrobotanica* 66:65-76.
635 Kennedy R, and Graham A. 1995. Infection of oil-seed rape by *Alternaria brassicae* under
636 varying conditions of temperature and wetness. Proceedings of the 9th International
637 Rapeseed Congress, Cambridge, UK. p 603.
638 King SR. 1994. *Screening, selection, and genetics of resistance to Alternaria diseases in*
639 *Brassica oleracea*: Cornell University, August.
640 Kobayashi Y, Yamada M, Kobayashi I, and Kunoh H. 1997. Actin microfilaments are required
641 for the expression of nonhost resistance in higher plants. *Plant and cell physiology*
642 38:725-733.
643 Köhl J, Van Tongeren C, Groenenboom-de Haas B, Van Hoof R, Driessen R, and Van Der
644 Heijden L. 2010. Epidemiology of dark leaf spot caused by *Alternaria brassicicola* and
645 *A. brassicae* in organic seed production of cauliflower. *Plant pathology* 59:358-367.
646 Kozik EU, and Sobiczewski P. 2000. Response of tomato genotypes to bacterial speck
647 (*Pseudomonas syringae* pv. *tomato*). *Acta Physiologiae Plantarum* 22(3):243-246.
648 Kubota M, Abiko K, Yanagisawa Y, and Nishi K. 2006. Frequency of *Alternaria brassicicola* in
649 commercial cabbage seeds in Japan. *Journal of General Plant Pathology* 72:197-204.
650 Kumar D, Maurya N, Bharati YK, Kumar A, Kumar K, Srivastava K, Chand G, Kushwaha C,
651 Singh SK, and Mishra RK. 2014. *Alternaria* blight of oilseed Brassicas: A
652 comprehensive review. *African Journal of Microbiology Research* 8:2816-2829.
653 Langenberg W. 1978. Relative speed of fixation of glutaraldehyde and osmic acid in plant cells
654 measured by grana appearance in chloroplasts. *Protoplasma* 94:167-173.
655 Maisch J, and Nick P. 2007. Actin is involved in auxin-dependent patterning. *Plant Physiology*
656 143:1695-1704.
657 Mazumder M, Das S, Saha U, Chatterjee M, Bannerjee K, and Basu D. 2013. Salicylic acid-
658 mediated establishment of the compatibility between *Alternaria brassicicola* and
659 *Brassica juncea* is mitigated by abscisic acid in *Sinapis alba*. *Plant Physiology and*
660 *Biochemistry* 70:43-51.
661 Meena P, Meena R, Chattopadhyay C, and Kumar A. 2004. Identification of critical stage for
662 disease development and biocontrol of Alternaria blight of Indian mustard (Brassica
663 juncea). *Journal of phytopathology* 152:204-209.
664 Meena PD, Chattopadhyay C, Meena SS, and Kumar A. 2011. Area under disease progress curve
665 and apparent infection rate of *Alternaria* blight disease of Indian mustard (*Brassica*
666 *juncea*) at different plant age. *Archives of Phytopathology and Plant Protection* 44:684-
667 693.

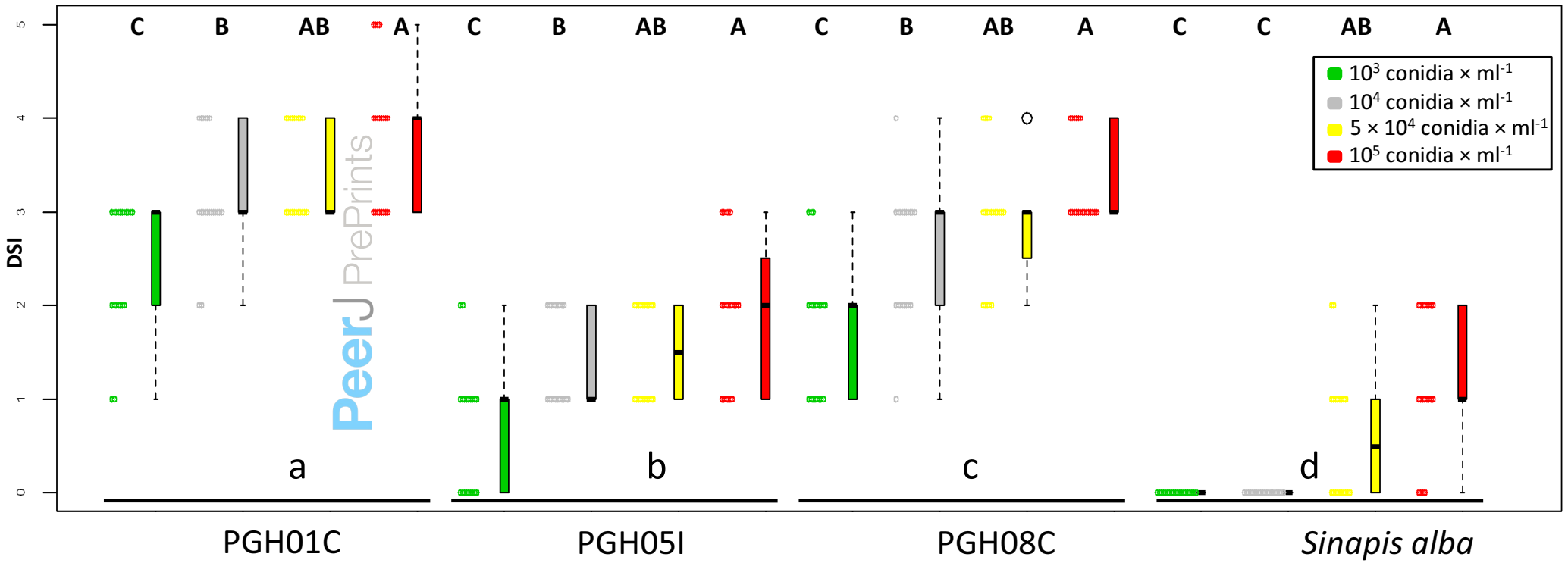
668 Michereff SJ, Noronha MA, Xavier Filha MS, Câmara MP, and Reis A. 2012. Survey and
669 prevalence of species causing *Alternaria* leaf spots on brassica species in Pernambuco.
670 *Horticultura Brasileira* 30:345-348.
671 Miklis M, Consonni C, Bhat RA, Lipka V, Schulze-Lefert P, and Panstruga R. 2007. Barley
672 MLO modulates actin-dependent and actin-independent antifungal defense pathways at
673 the cell periphery. *Plant Physiology* 144:1132-1143.
674 Nowicki M, Lichocka M, Nowakowska M, Kłosińska U, and Kozik EU. 2012a. A simple dual
675 stain for detailed investigations of plant-fungal pathogen interactions. *Vegetable Crops*
676 *Research Bulletin* 77:61-74.
677 Nowicki M, Nowakowska M, Niezgoda A, and Kozik E. 2012b. *Alternaria* black spot of
678 crucifers: Symptoms, importance of disease, and perspectives of resistance breeding.
679 *Vegetable Crops Research Bulletin* 76:5-19.
680 Olyslaegers G, and Verbelen J. 1998. Improved staining of F-actin and co-localization of
681 mitochondria in plant cells. *Journal of Microscopy* 192:73-77.
682 Opalski KS, Schultheiss H, Kogel K-H, and Hückelhoven R. 2005. The receptor-like MLO
683 protein and the RAC/ROP family G-protein RACB modulate actin reorganization in
684 barley attacked by the biotrophic powdery mildew fungus *Blumeria graminis* f. sp.
685 *hordei*. *The Plant Journal* 41:291-303.
686 Pochon S, Simoneau P, Pigné S, Balidas S, Bataillé-Simoneau N, Campion C, Jaspard E, Calmes
687 B, Hamon B, and Berruyer R. 2013. Dehydrin-like proteins in the necrotrophic fungus
688 *Alternaria brassicicola* have a role in plant pathogenesis and stress response. *PLoS One*
689 8:e75143.
690 Rashid M, Hossain I, and Khalequzzaman K. 2011. Effect of weather factors on inoculum
691 density and leaf spot development in radish seed crop infected with *Alternaria brassicae*.
692 *Bulletin of the Institute of Tropical Agriculture, Kyushu University* 34:43-47.
693 Rotem J. 1998. The biotic and physiological components of pathogenesis. *The Genus Alternaria;*
694 *Biology, Epidemiology, and Pathogenicity, The American Phytopathological Society, St*
695 *Paul, MN*:95-118.
696 Saharan G, and Mehta N. 2002. Fungal diseases of rapeseed-mustard. *Diseases of field*
697 *crops*:193-228.
698 Scholze P. 2002. In-vitro-germinability of conidia and differences in aggressivity in *Alternaria*
699 *brassicicola* isolates on brassicaceous vegetables (*Brassica oleracea* L.). *Pflanzenschutz*
700 *Berichte* 60:105-114.
701 Scholze P, and Ding Y. 2005. Manifestation of black spot disease (*Alternaria brassicicola*) in
702 intact leaves and detached leaf segments of cabbage plants grown in nutrient solutions
703 without N, P, K and Ca/Manifestierung der Schwarzfleckigkeit (*Alternaria brassicicola*)
704 auf intakten Blättern und isolierten Blattsegmenten von Kohlpflanzen, die mit
705 Nährlösungen ohne N, P, K und Ca ernährt wurden. *Zeitschrift für Pflanzenkrankheiten*
706 *und Pflanzenschutz/Journal of Plant Diseases and Protection*:562-572.
707 Sharma G, Kumar VD, Haque A, Bhat S, Prakash S, and Chopra V. 2002. *Brassica*
708 coenospecies: a rich reservoir for genetic resistance to leaf spot caused by *Alternaria*
709 *brassicae*. *Euphytica* 125:411-417.
710 Sharma P, Deep S, Bhati DS, Sharma M, and Chowdappa P. 2014. Penetration and infection
711 processes of *Alternaria brassicicola* on cauliflower leaf and *Alternaria brassicae* on
712 mustard leaf: A histopathological study. *Plant Pathology Journal* 13:100.

713 Sharma P, Sharma S, and Sindhu M. 2004. A detached leaf technique for evaluation of resistance
714 in cabbage and cauliflower against three major pathogens. *Indian Phytopathology*
715 57:315-318.
716 Shrestha SK, Munk L, and Mathur SB. 2005. Role of weather on *Alternaria* leaf blight disease
717 and its effect on yield and yield components of mustard. *Nepal Agric Res J* 6:62-72.
718 Su'udi M, Kim MG, Park S-R, Hwang D-J, Bae S-C, and Ahn I-P. 2011. *Arabidopsis* cell death
719 in compatible and incompatible interactions with *Alternaria brassicicola*. *Molecules and*
720 *cells* 31:593-601.
721 Vitha S, Baluška F, Braun M, Šamaj J, Volkmann D, and Barlow PW. 2000. Comparison of
722 cryofixation and aldehyde fixation for plant actin immunocytochemistry: aldehydes do
723 not destroy F-actin. *The Histochemical Journal* 32:457-466.
724 Zală CR, Cristea S, Gruia L, and Manole S. 2014. Research on the biology of the *Alternaria*
725 *brassicae* fungus isolated from mustard. In: Mastorakis N, Mladenov V, Anisor N, Mijatovic I,
726 Dumitrascu D, and Erol A, editors. 3rd International Conference on Energy and Environment
727 Technologies and Equipment. Brasov, Romania. p 161-164.

Figure 1(on next page)

Impact of inoculum concentration on the disease intensity of investigated cultigens.

Raw data for disease intensity of each cultigen tested are presented as a series of stacked beeswarms in colors representing various inoculum concentrations tested (described in the legend), juxtaposed with respective boxplots (median is marked in black; box represent the interquartile range; whiskers extend to cover the rest of the data in each group; outliers are represented as empty white circles). Due to lack of significance for the interaction cultigen×inoculum concentration, main effects of both factors were investigated separately with one-way-ANOVA and post-hoc Tukey tests. Capital letters denote grouping for inoculum concentration (HSD: 0.5098796); small letters – grouping for cultigen of significantly different reaction to *A.brassicicola* (HSD: 0.3226193). n×inoculum concentration, main effects of both factors were investigated separately with one-way-ANOVA and post-hoc Tukey tests. Capital letters denote grouping for inoculum concentration (HSD: 0.5098796); small letters – grouping for cultigen of significantly different reaction to *A.brassicicola* (HSD: 0.3226193).

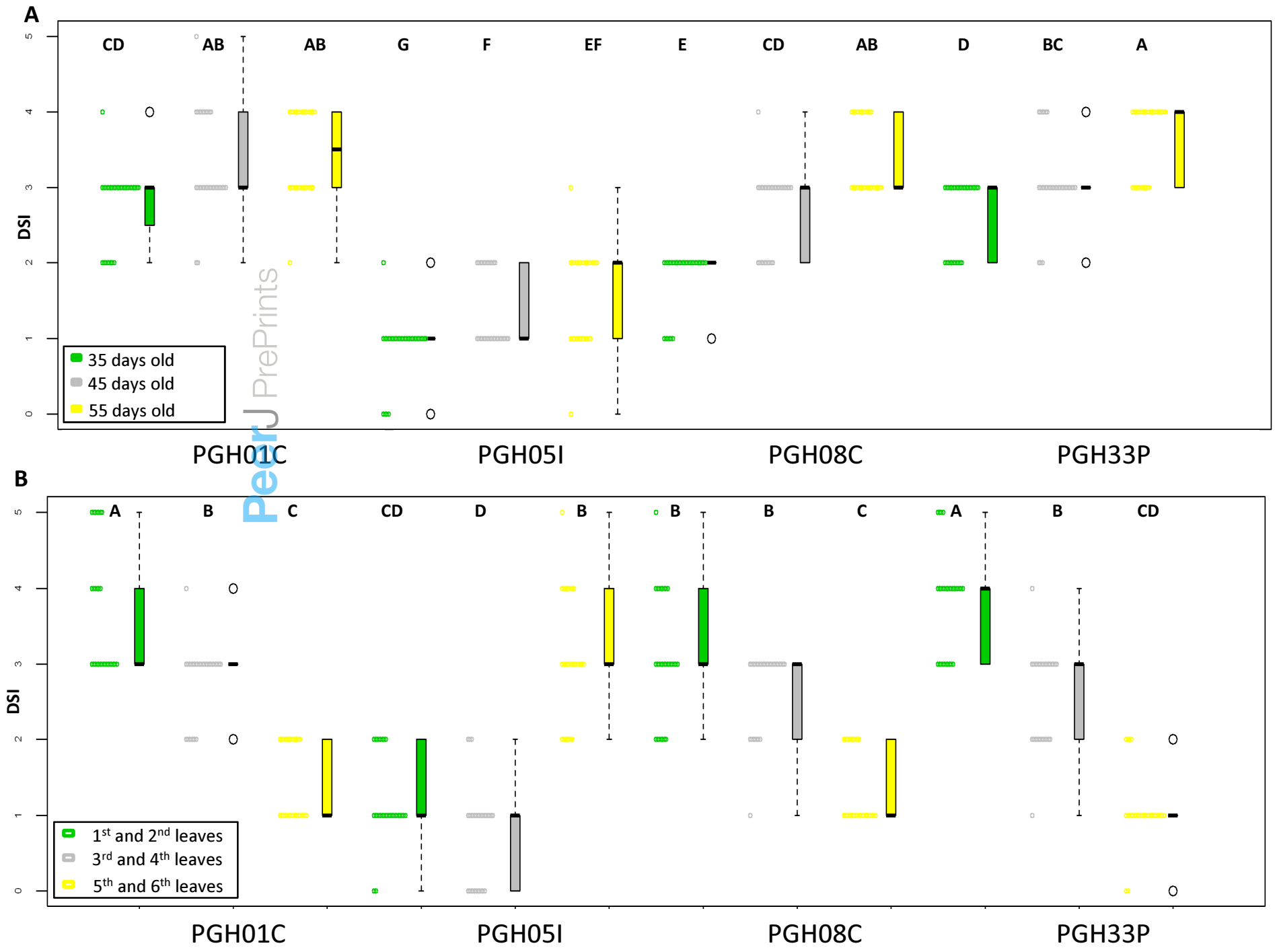


PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.1360v1 | CC-BY 4.0 Open Access | rec: 9 Sep 2015, publ: 9 Sep 2015

Figure 2(on next page)

Effect of leaf age (A) and position (B) on disease severity in chosen cultigens.

Raw data for disease intensity of each cultigen tested are presented as series of stacked beeswarms in colors representing leaf age tested or position tested (described in the respective legends), juxtaposed with respective boxplots (median is marked in black; box represent the interquartile range; whiskers extend to cover the rest of the data in each group; outliers are represented as empty white circles). Capital letters denote grouping according to two-way-ANOVA (leaf age (A) or position (B) ×cultigen) with post-hoc Tukey tests. HSD for leaf age×cultigen: 0.4233829. HSD for leaf position×cultigen: 0.55478. grouping for cultigen of significantly different reaction to *A.brassicicola* (HSD: 0.3226193). n×inoculum concentration, main effects of both factors were investigated separately with one-way-ANOVA and post-hoc Tukey tests. Capital letters denote grouping for inoculum concentration (HSD: 0.5098796); small letters – grouping for cultigen of significantly different reaction to *A.brassicicola* (HSD: 0.3226193).

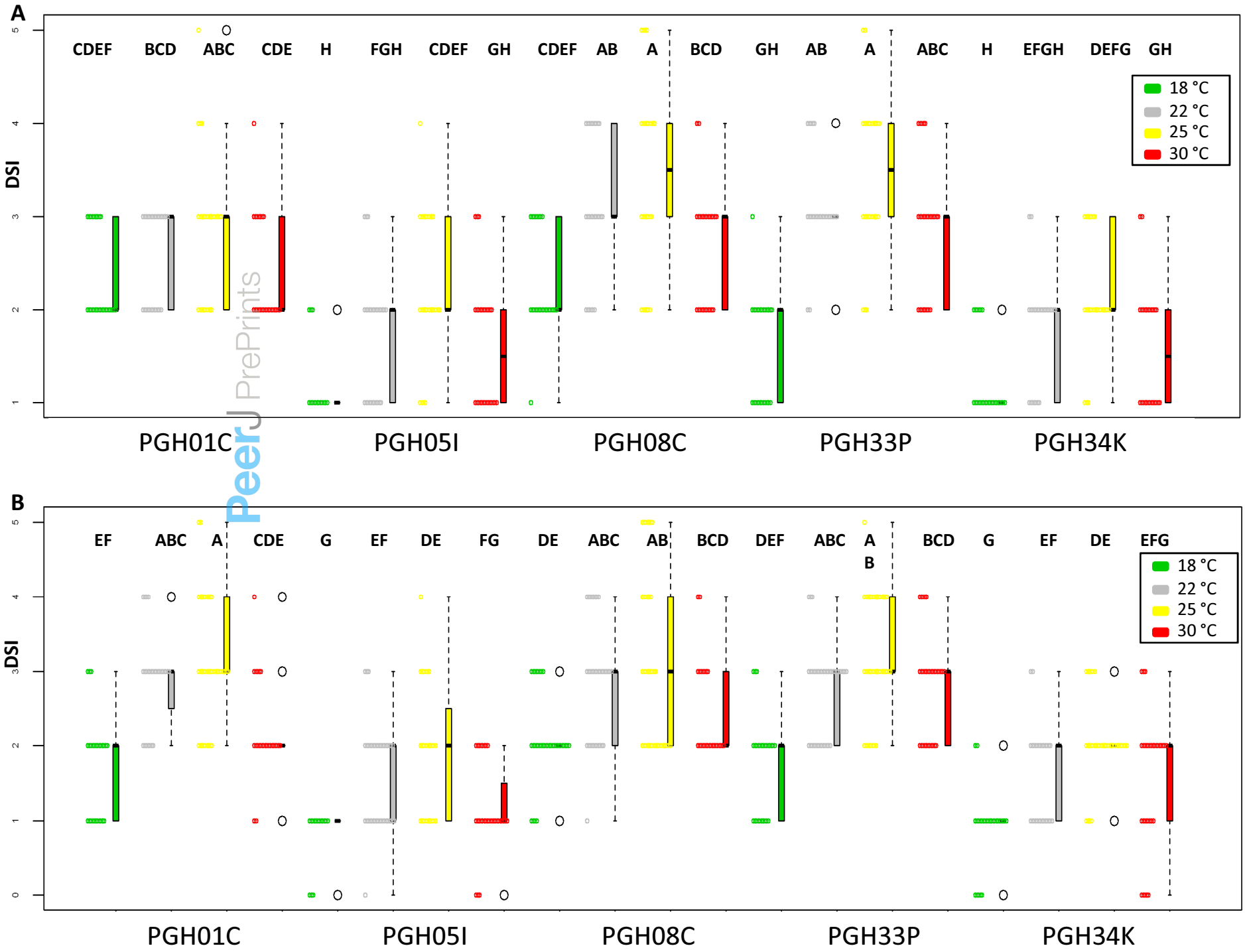


PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.1360v1 | CC-BY 4.0 Open Access | rec: 9 Sep 2015, publ: 9 Sep 2015

Figure 3(on next page)

Influence of incubation temperature on disease intensity in chosen cultigens.

(A) Detached leaf test. (B) Seedlings test. Raw data for disease intensity of each cultigen tested are presented as series of stacked beeswarms in colors representing the incubation temperature tested in either assay (described in the respective legends), juxtaposed with respective boxplots (median is marked in black; box represent the interquartile range; whiskers extend to cover the rest of the data in each group; outliers are represented as empty white circles). Capital letters denote grouping according to two-way-ANOVA (incubation temperature×cultigen) with post-hoc Tukey tests, for both kinds of assays. HSD for (A): 0.652232. HSD for (B): 0.6630485. d post-hoc Tukey tests. Capital letters denote grouping for inoculum concentration (HSD: 0.5098796); small letters – grouping for cultigen of significantly different reaction to *A.brassicicola* (HSD: 0.3226193).



4

Confocal microscopic analyses of the *A.brassicicola* – cabbage pathosystem.

Dually stained samples (trypan blue [red or blue channel] + aniline blue [green channel]: A,B,F; rhodamine-phalloidin [red channel] + aniline blue [green channel]: E,G,H; computer rendering of confocal dually stained Z-stacks: C,D) visualize the infection process. A: Successful infection of the leaves of susceptible cultigen PGH12P, upon growing through the deposited papilla (indicated by arrow). B: Hyphae made to extend, when did not succeed to grow through the papilla of the resistant cultigen ZYX. C: 3D rendering of a papilla representative for the susceptible cultigen PGH12P. D: 3D rendering of a papilla representative for the resistant cultigen PGH05I. Do notice, smaller number of stacked pictures generated a shorter papilla. E: Visualization of a successful infection of the susceptible cultigen PGH05I. Arrows from left to right denote: Germinating sporangium; successful direct penetration and overcoming plant's defences – development of haustoria and secondary hyphae; reaction of neighboring cells: strands of stained actin drive the deposition of callose cloak to prevent spread of the infection. F: Stomatal infection on the resistant cultigen PGH05I. G: Typical actin networks crossing cells of the uninfected leaves. H: Rapid polarization of the actin networks, tightly surrounding the papilla deposited beneath the perceived pathogen attack.

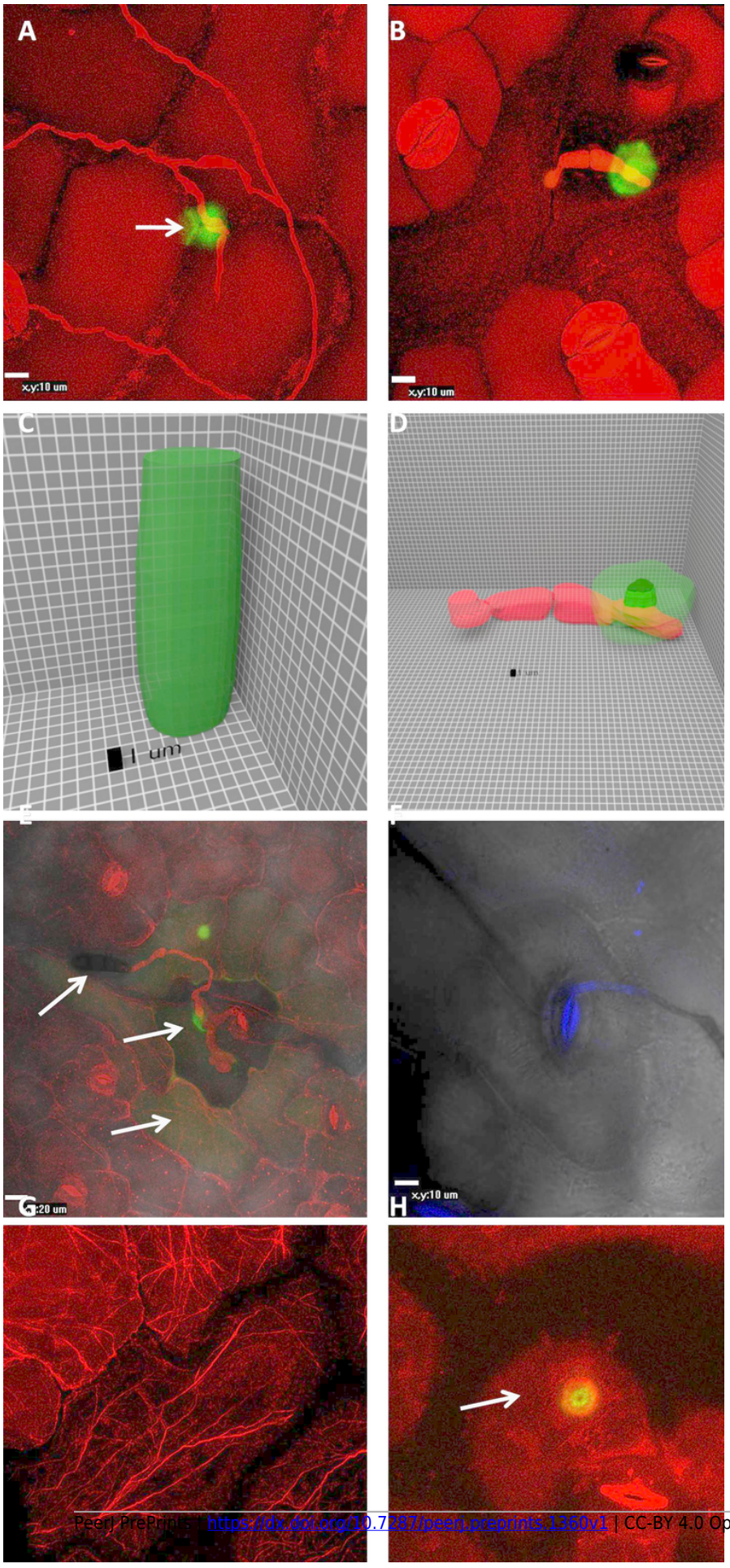
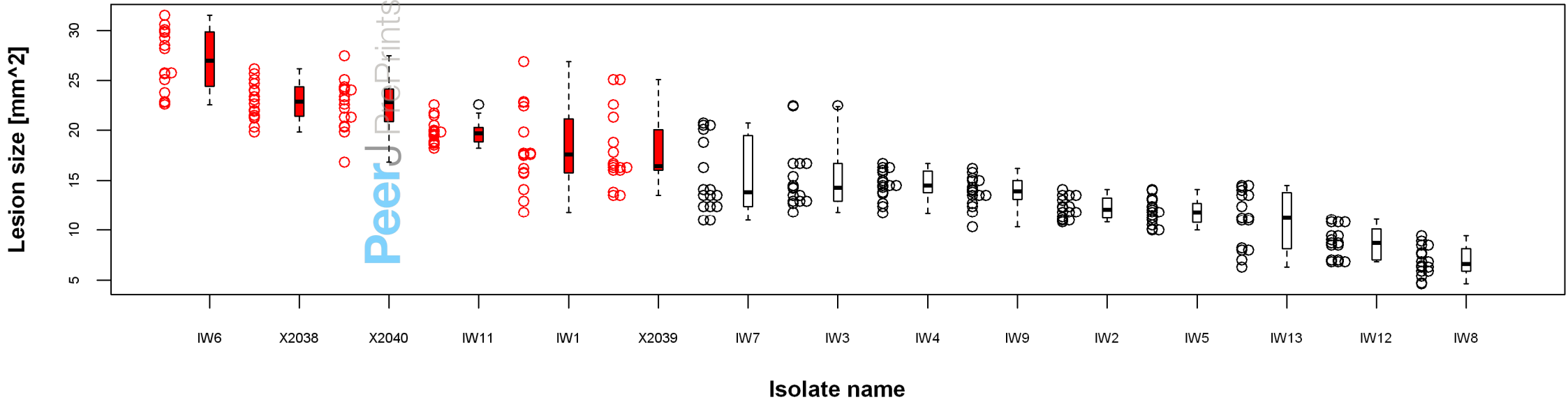


Figure 5(on next page)

Supplementary Fig.1: Aggressiveness of *A.brassicicola* isolate collection, expressed as lesion size [mm^2].

Bio-assays were performed on the detached leaves of *A.brassicicola*-susceptible 'Kamienna Głowa' cabbage, and evaluated at 7dpi (see Materials and Method section for details). Raw data for lesion sizes generated by each isolate tested are presented as series of stacked beeswarms, juxtaposed with respective boxplots (median is marked in black; box represent the interquartile range; whiskers extend to cover the rest of the data in each group; outliers are represented as empty white circles).Isolates colored red were used for inoculum mixture, used in all subsequent experimentation.



PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.1360v1 | CC-BY 4.0 Open Access | rec: 9 Sep 2015, publ: 9 Sep 2015