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

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Abstract

Heavy losses incited yearly by *Alternaria brassicicola* on the vegetable *Brassicaceae* – have prompted our search for sources of genetic resistance against the pathogen and 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 *A. brassicicola* resistance were run under more stringent conditions 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 data into the pathogen mode of infection: Direct epidermal infection or stomatal attack were related to plants' resistance against *A. brassicicola* among the cultigens tested. Further, the actin network of the host cells reorganized around the papillas deposited under the pathogen's appressorium.

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 the *Brassicaceae* 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 by Nowicki et al. 2012b). Poland ranks 5th or 6th in global production of these crops (FAOSTAT), and thus, the yearly threats of *Alternaria* spp. (Kasprzyk et al. 2013) causing yield losses, necessitating 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 contributed, 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 dark spot severity. Both testing 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 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 to 2014). 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-day 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-day old plants and immediately placed on wet cellulose wadding in plastic boxes. One drop of conidial suspension (40 µl) at $10^5 \times ml^{-1}$ 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 based on the size of necrotic lesions at 3 and 7 dpi, in two independent experiments. All 16 tested isolates were pathogenic, despite differences in their aggressiveness (Suppl. Fig. 1). For the subsequent bio-assays, the six most aggressive isolates (X2038, X2039, X2040, IW1, IW6, IW11) were mixed in equal proportions, as they had similar virulence levels 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-day 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 experiments 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-day 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 positions, collected from 55-day old plants. Detached leaves were inoculated with conidia suspension (10^5 conidia × ml^{-1}) and incubated as described above. We conducted two independent experiments, 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-day 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 the 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-day old plants. The severity of disease symptoms on seedlings was evaluated after inoculating 45-day 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). Sub-pool of these cultigens was subjected for additional field trials in 2015.

The soil type was a pseudopodsolic over loamy sand (1.5% organic matter, pH 6.5). The tested plants were grown 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 the 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 isolate X2039 (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 MgC_{l2},) 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 50 mM PBS pH 7.4 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

Data from the independent experiment sets of laboratory bio-assays were compared pairwise, for each variable undergoing optimization. Statistical analyses indicated similarity of both sets (F-tests always resulted in $p > 0.74$; indicated in Fig. 1 to 4; two-way ANOVA results showing the same significance levels for singular variables and their interactions), which permitted combing the data from both sets for analyses presented below. As each variable to be optimized (Fig. 1 to 4) included at least one resistant cultigen, this deviated the recorded values from normality, hence, neither the separate datasets nor the combined dataset were checked for normality.
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 (confidence level 0.95), with post-hoc Tukey's Honestly Significant Difference (HSD; $\alpha = 0.05$) analyses. Values of F and p for each ANOVA analyses are reported along the respective data.

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. Due to lack of significance ($F = 0.578$; $p = 0.63$) for the interaction cultigen $\times$ inoculum concentration, main effects of both factors were investigated separately with one-way-ANOVA and post-hoc Tukey tests. Disease severity increased as the inoculum concentration increased above 10^3 conidia $\times$ ml^{-1} on all tested cultigens except *S. alba*, regardless of their susceptibility to *A. brassicicola* (Fig. 1). Our data shows lack of significant differences between the disease severity recorded at the two highest conidial concentrations ($F = 2.854$, $p = 0.093$). 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*. Based on the data, either of the two highest inoculum concentrations can be used, and the subsequent bio-assays employed the 10^5 conidia $\times$ ml^{-1} inoculum concentration.

Experimental results indicated that leaf age ($p < 0.001$) and genotype ($p < 0.001$), as well as their interaction ($p < 0.001$) 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. (for cultigens: $p < 0.001$; for 35/45-day old and older: $p < 0.001$; for interaction cultigen $\times$ age: $p < 0.05$). Since the DSI of PGH08C and PGH33P lacked significant differences from each other at 45- or 55-day old, the ranking of the cultigens resistance would not be affected by selecting leaves at these stages. For all further assays, leaves from 45-day old plants were used, to reduce the length of experiments.

We observed significant effects of leaf position ($p < 0.001$), genotype ($p < 0.001$), and their interaction ($p < 0.001$) 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. For cultigens PGH05I and PGH08C, the DSI lacked significant differences between the oldest leaves (1st and 2nd leaves) and middle ones (3rd and 4th leaves), whereas for the other two cultivars, we recorded significantly higher DSI on the oldest leaves, compared with the younger stages tested. Based on these results, we chose testing the 3rd and 4th leaves in the subsequent experiments, as to limit the susceptibility groups that arise.

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). ANOVA analyses indicated significance for temperature $\times$ cultigen interactions for both, leaf and seedling tests ($p = 0.001$, and 0.038, respectively). In both tests, three out of five tested cultigens showed no significant differences between 22, 25, and 30 °C. Moreover, all cultigens in both tests showed no differences between

25 and 30 °C. 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, exceeding slightly the DSI for either 22 °C or 30 °C. For the detached leaf tests (Fig. 3A), analysis of variance showed that incubation temperature had no effect on disease severity for most tested cultigens ($p > 0.05$). The significant differences between various incubation temperatures were apparent only for the line PGH33P ($F = 27.8526$, $p < 0.001$). In case of other cultigens, there was either no significant difference among temperatures (PGH01C: $p > 0.05$; PGH05I: $p > 0.05$; PGH08C: $p > 0.05$), or significant differences in symptom severity were recorded only between 18 °C and 25 °C (PGH34K: $p < 0.001$). Based on our data, the *A. brassicicola* selection can be conducted under 22 or 25 °C with either testing method. For subsequent testing, we chose the 25 °C as the assay temperature.

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* ($F = 2.2445$. $p = 0.134$). It is important to note, that the DSI of only four cultigens (PGH11P, PGH12P, PGH19K, PGH28K) exhibited significant differences ($p < 0.05$; results of separate pairwise comparisons of these four cultigens) between the seedlings assay and the detached leaf assay. Correlation analysis on the mean DSI for each cultigen in the leaf and seedling bio-assays (0.921) indicated that either method may be used for preliminary screening.

Analysis of variance revealed a significant effect of the cultigen ($p < 0.001$), driving the impact of the interaction of cultigen × testing method ($p < 0.001$) 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*. Based on the statistical model generated by the two-way ANOVA, genotype had a higher effect on the disease intensity ($p < 0.001$) than the testing method used ($p < 0.001$), driving the interaction of both factors ($p < 0.001$). For most cultigens, the DSI under field conditions was lower than observed in the laboratory tests, with the exception of a few cultigens. Lower DSI 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. Correlation analyses between the field results and either leaf or seedling bio-assays (respectively, 0.408; 0.244) confirmed this result (only cultigens analyzed under all three testing methods were included here; Table 1). Thus, regardless of the results of the laboratory screens, resistance breeding must be confirmed under field epidemics.

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 plant (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 (moderately) 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 observed (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 lacked 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 ($p = 0.005$; $n = 15$). These differences are unlikely to be explained by increased number of stomata in this resistant cultigen (Table 2), as even including the increased stomata density in this line into the χ^2 test only slightly weakens the result ($p = 0.07$ for 11 stomatal *vs*. 4 direct penetration attempts). Papilla deposition or size lacked statistical differences in relation to plant's susceptibility level (Fig. 4 and papilla sizes data not shown; PGH12P: 119.62±44.58 μm^2; PGH05I: 188.25.25±71.25 μm^2; $p = 0.09$). Taking into account lack of differences in papilla size or deposition pace, the above 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, and was confirmed at 4 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. Based on the microscopic data, we posed a trend of *A. brassicicola* infection mode, depending on the plant's resistance level. 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 above the threshold of 10^3 conidia × ml^{-1} (with the exception of the resistant *S. alba*). Significantly higher disease intensity was recorded for the highest concentration of conidia (10^5 × ml^{-1}), compared with the two lowest inoculum loads used. 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). Additionally, King (1994) also 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 × ml^{-5} of *A. brassicicola.* Inoculum concentrations comparable with those used in our study (10^5 conidia × 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). As our studies showed lack of DSI differences between 22 and 25 °C, either temperature may be used for evaluating germplasm's resistance.

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-day old plants did not show any leaf spot symptoms of *A. brassicicola*, in contrast to the 45- and 60-day old plants being very susceptible. In our studies, the intensity of symptoms gradually increased from the 35-day 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 assay efficacies of the selection process 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 among the *Brassicaceae* germplasm. 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 tests under 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 epidemiological 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 epidemiological 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), hence the need to repeatedly test the resistance in the field to prevent escapes. 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. Yet, in case of limited germplasm pool, cauliflower being the case in this study, such moderate resistance recorded in the field may prove the only resort for the subsequent breeding.

We decided to use a mixture of pathogen isolates in equal proportions for screening our collection of *Brassicaceae* cultigens, by selecting the isolates highest in aggressiveness as per 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). Common difficulties currently experienced in studying this pathosystem 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). Moreover, using a mixture of isolates in the laboratory bioassay would likely mimic the field condition, and help make conclusions of the field data.

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*. All the remaining cultigens (head cabbage, cauliflower, Chinese cabbage, rape) displayed various levels of susceptibility. This is in agreement with related 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 the genetic control over moderate *A. brassicicola* resistance in two inter-specific hybrid 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. Also McRoberts & Lennard (1996) diligently tested the host and non-host reactions of susceptible plant materials on an array of Alternaria species; it could be their use of susceptible lines that resulted in comparatively low proportion of stomatal attempted infections. In contrast, we studied the infection mode 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 PGH05I necessitated infection by the stomata. This observation needs to be confirmed on a larger array of cultigens, particularly because we only used one (moderate) resistant cultigen in the microscopic studies. 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. It is in agreement with McRoberts & Lennard (1996), who also reported rapid primary resistance response in both host and non-host systems, but also claimed callose deposition *per se* relatively unimportant to the outcome of the resistance response. Thus, also 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.

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Table 1(on next page)

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

[a]Cultigens' commercial names or codes used during breeding at InHort. [b]Cultigens' DSI±SD in the seedlings test (T-sd), detached leaf test (T-lf), and in the field under natural epidemics (T-field) in years 2012 and 2015. Experimental details are described in the Materials and methods section. (–) : not determined. HSD (Tukey) for T-sd and T-pl (two-way-ANOVA): 0.707. HSD (Tukey) for T-field (one-way-ANOVA): 1.521. Correlation analyses resulted 0.921 for T-sd *vs*. T-lf; 0.408 for T-field2012 *vs*. T-lf; and 0.244 for T-field2012 *vs*. T-sd. [c]Vegetation period from planting to harvest maturity. [d]Species and genetic information (when available) are listed out.

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

Cultigen[a]	T-sd[b]	T-lf[b]	T-field2012[b]	T-field2015[b]	Veg. period [day][c]	Background[d]
PGH01C	3.1±0.8	2.9±0.8	–	–	60	cauliflower *Brassica oleracea* var. *botrytis* DC
PGH02C	3.4±0.6	4.1±0.7	2.7±0.6	–	70	cauliflower *Brassica oleracea* var. *botrytis* DC
PGH03C	4.6±0.7	4.4±0.6	3.3±0.7	–	60	cauliflower *Brassica oleracea* var. *botrytis* DC
PGH04C	4.1±0.7	4.4±0.6	3.3±0.6	–	60	cauliflower *Brassica oleracea* var. *botrytis* DC
PGH05I	1.6±0.7	1.7±0.7	1.9±0.5	1.7±0.3	–	interspecific *B. oleracea* × *B. napus* S_5/BC_2
PGH06I	3.1±1.1	3.2±0.7	–	–	–	interspecific *B. oleracea* × *B. napus* S_5/BC_2
'Bilko F_1'	4.2±0.6	4.6±0.6	4.3±0.5	4.5±0.4	70	Chinese cabbage *Brassica rapa* var. *pekinensis*
PGH08C	3.1±1.2	3.4±1.0	–	–	85	cauliflower *Brassica oleracea* var. *botrytis* DC
PGH09K	4.3±0.7	4.6±0.5	2.3±0.6	–	120	head cabbage *Brassica oleracea* var. *capitata*
PGH10P	3.1±0.5	3.0±0.0	–	–	55	swede *Brassica napus*
PGH11P	3.6±0.5	2.6±0.5	–	–	55	swede *Brassica napus*
PGH12P	3.7±0.6	4.8±0.5	4.7±0.6	–	55	Chinese cabbage *Brassica rapa* var. *pekinensis*
PGH13R	3.2±0.6	3.5±0.8	3.3±0.6	3.1±0.4	-	seedrape *Brassica napus*
PGH14R	4.0±0.8	4.1±0.9	3.0±0	4.5±0.1	-	seedrape *Brassica napus*
PGH15R	3.3±0.6	3.3±0.7	3.0±0	4.7±0.1	-	seedrape *Brassica napus*
PGH16K	3.0±0.7	3.8±0.9	3.3±0.6	–	90	head cabbage *Brassica oleracea* var. *capitata*
PGH17K	4.6±0.5	4.5±0.7	3.0±0	–	90	head cabbage *Brassica oleracea* var. *capitata*
PGH18K	3.7±0.5	4.1±0.7	3.7±0.6	–	90	head cabbage *Brassica oleracea* var. *capitata*
PGH19K	3.8±0.9	4.4±0.7	4.3±0.6	–	90	head cabbage *Brassica oleracea* var. *capitata*
PGH20K	4.0±0.6	3.7±0.9	3.3±0.6	–	90	head cabbage *Brassica oleracea* var. *capitata*
'Sława z Enkhuizen'	3.9±0.8	4.4±0.5	0.8±0.3	0.9±0.2	62	head cabbage *Brassica oleracea* var. *capitata*
PGH22K	3.7±0.8	4.2±0.7	2.3±0.6	2.3±0.4	110	head cabbage *Brassica oleracea* var. *capitata*
PGH23K	3.7±0.6	4.3±0.6	2.0±0	2.4±0.1	110	head cabbage *Brassica oleracea* var. *capitata*

PGH24K	3.3±0.8	3.9±0.6	1.4±0.1	–	90	head cabbage *Brassica oleracea* var. *capitata*
PGH25I	2.0±1	1.8±0.6	–	–	-	interspecific *B. oleracea* × *B. napus* S_5/BC_2
PGH26K	3.4±0.6	3.7±0.7	3.1±0.6	–	120	head cabbage *Brassica oleracea* var. *capitata*
PGH27I	3.9±0.7	3.8±0.4	–	–	-	interspecific *B. oleracea* × *B. napus* S_5/BC_2
PGH28K	2.1±0.7	3.3±0.8	2.7±0.6	–	120	head cabbage *Brassica oleracea* var. *capitata*
PGH29K	3.1±0.7	3.7±0.7	3.3±0.3	–	120	head cabbage *Brassica oleracea* var. *capitata*
PGH30K	3.5±0.6	3.5±0.6	2.7±0.5	3.0±0.3	110	head cabbage *Brassica oleracea* var. *capitata*
'Kamienna Głowa'	4.0±0.4	4.3±0.8	4.4±0.3	–	120	head cabbage *Brassica oleracea* var. *capitata*
'Kilagreg F_1'	3.7±0.7	3.9±0.8	1.3±0.6	1.2±0.3	65	head cabbage *Brassica oleracea* var. *capitata*
PGH33P	3.2±0.8	3.5±0.8	–	–	60	Chinese cabbage *Brassica rapa* var. *pekinensis*
PGH34K	2.1±0.5	2.2±0.6	–	–	85	head cabbage *Brassica oleracea* var. *capitata*
PGH35C	3.3±0.7	2.8±0.7	2.3±0.4	2.4±0.1	85	cauliflower *Brassica oleracea* var. *botrytis* DC
PGH36K	3.9±0.9	4.3±0.6	1.7±0.4	–	70	head cabbage *Brassica oleracea* var. *capitata*
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]Cultigens' commercial names or codes used during breeding at InHort.

[b]Cultigens' DSI±SD in the seedlings test (T-sd), detached leaf test (T-lf), and in the field under natural epidemics (T-field) in years 2012 and 2015. Experimental details are described in the Materials and methods section. (–) : not determined. HSD (Tukey) for T-sd and T-pl (two-way-ANOVA): 0.707. HSD (Tukey) for T-field (one-way-ANOVA): 1.521. Correlation analyses resulted 0.921 for T-sd *vs*. T-lf; 0.408 for T-field2012 *vs*. T-lf; and 0.244 for T-field2012 *vs*. T-sd.

[c]Vegetation period from planting to harvest maturity.

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

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 ($p = 0.63$), main effects of both factors were investigated separately with one-way-ANOVAs and post-hoc Tukey HSD tests ($\alpha=0.05$). Capital letters denote the post-hoc grouping for inoculum concentration (HSD: 0.51); small letters – post-hoc grouping for cultigen of significantly different reaction to *A.brassicicola* (HSD: 0.323). Presented data comes from two independent experiment sets, with F-test showing no differences between them ($p = 0.97$), and ANOVA results showing very close significance levels between either data set for each variable studied.

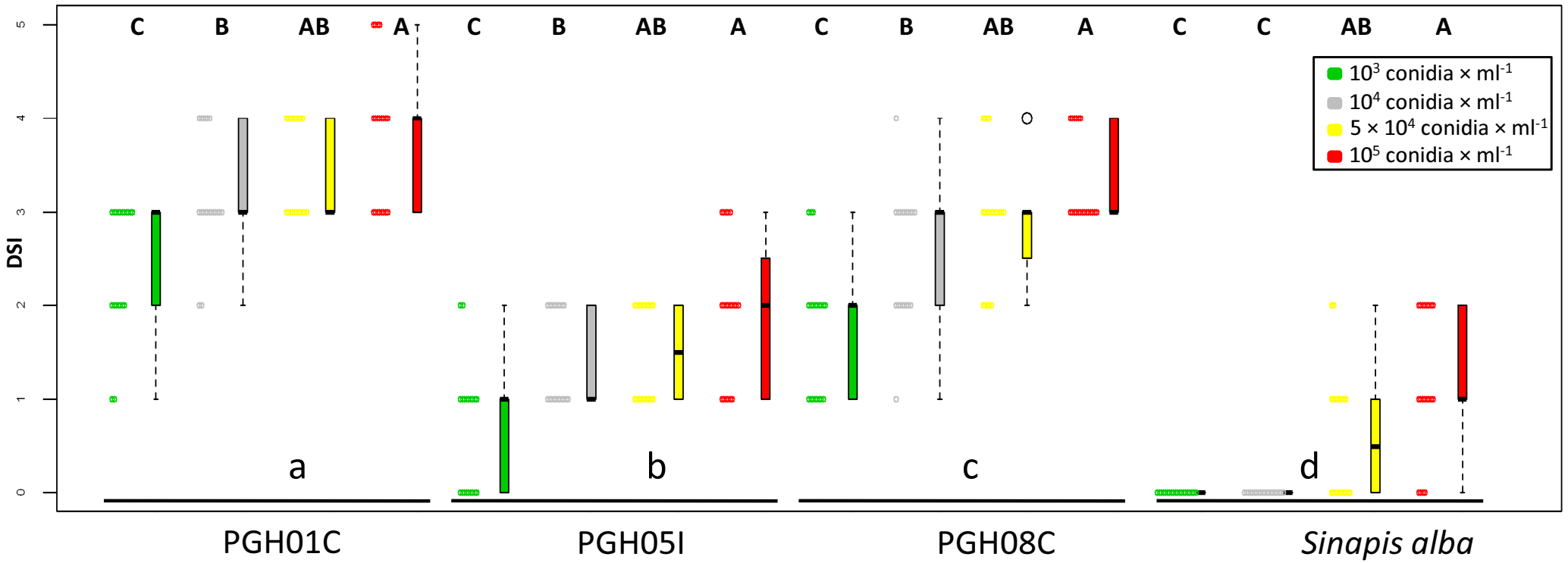

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Figure 2(on next page)

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 the Tukey tests post two-way-ANOVA (leaf age (A) or position (B) ×cultigen). HSD for leaf age×cultigen: 0.423. HSD for leaf position×cultigen: 0.555. Presented data comes from two independent experiment sets, with F-test showing no differences between them ($p = 0.993$ for leaf age; 0.986 for leaf position), and ANOVA results showing very close significance levels between either data set for each variable studied.

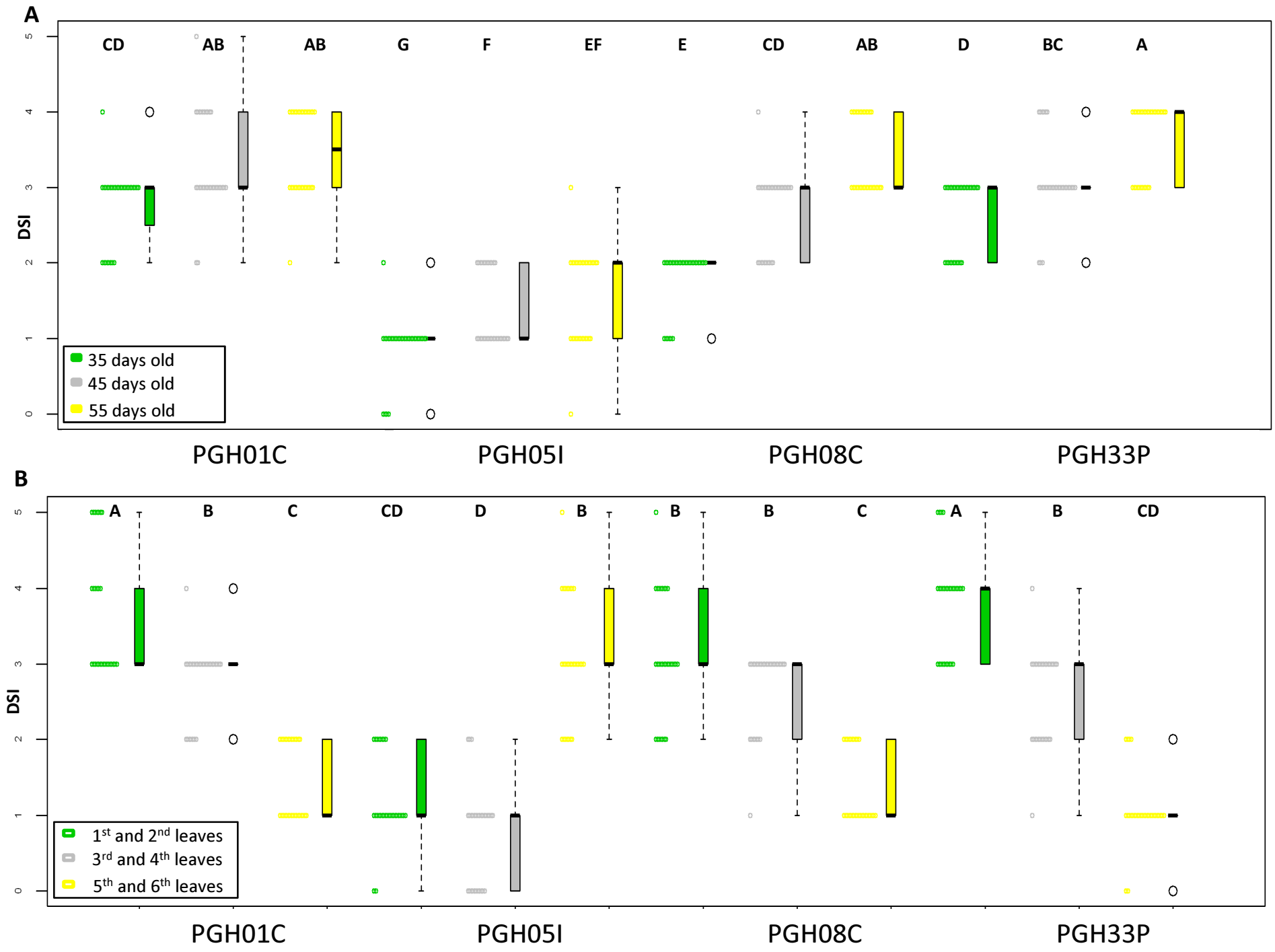

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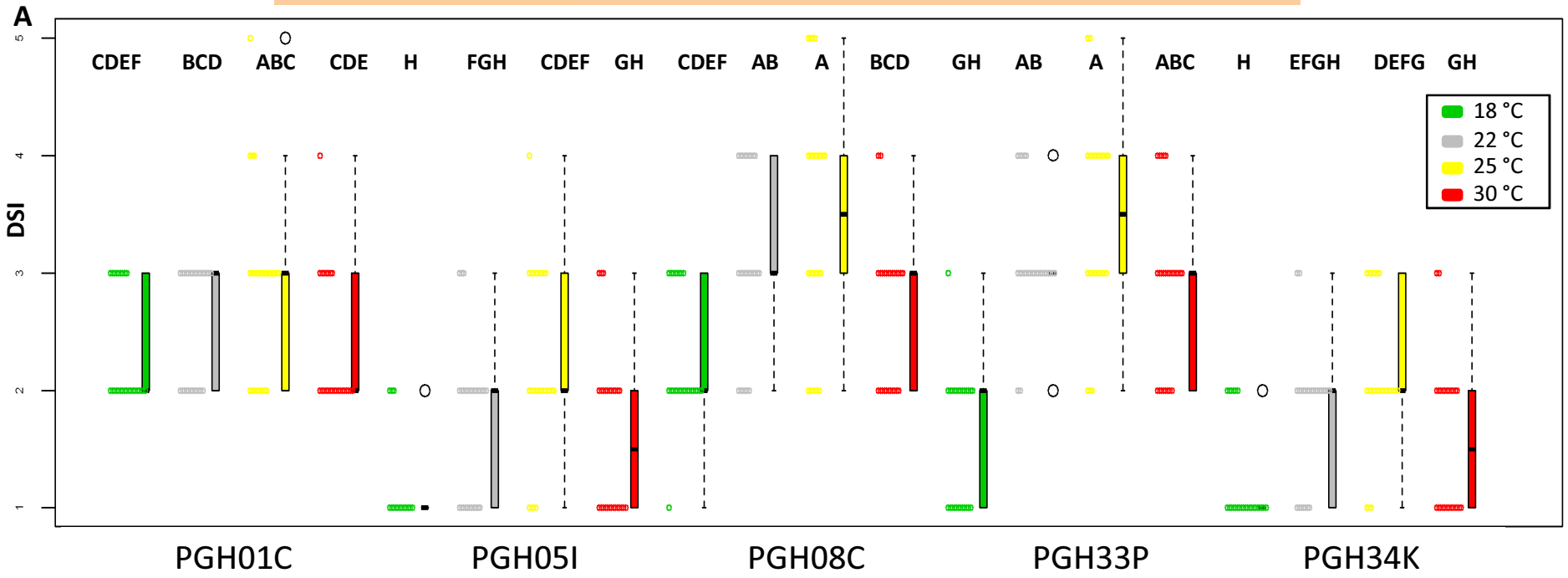
Figure 3(on next page)

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 the Tukey tests post two-way-ANOVA (incubation temperature×cultigen), for both kinds of assays. HSD for (A): 0.652. HSD for (B): 0.663. Presented data comes from two independent experiment sets, with F-test showing no differences between them ($p = 0.888$ for detached leaf test; 0.74 for seedlings test), and ANOVA results showing very close significance levels between either data set for each variable studied.



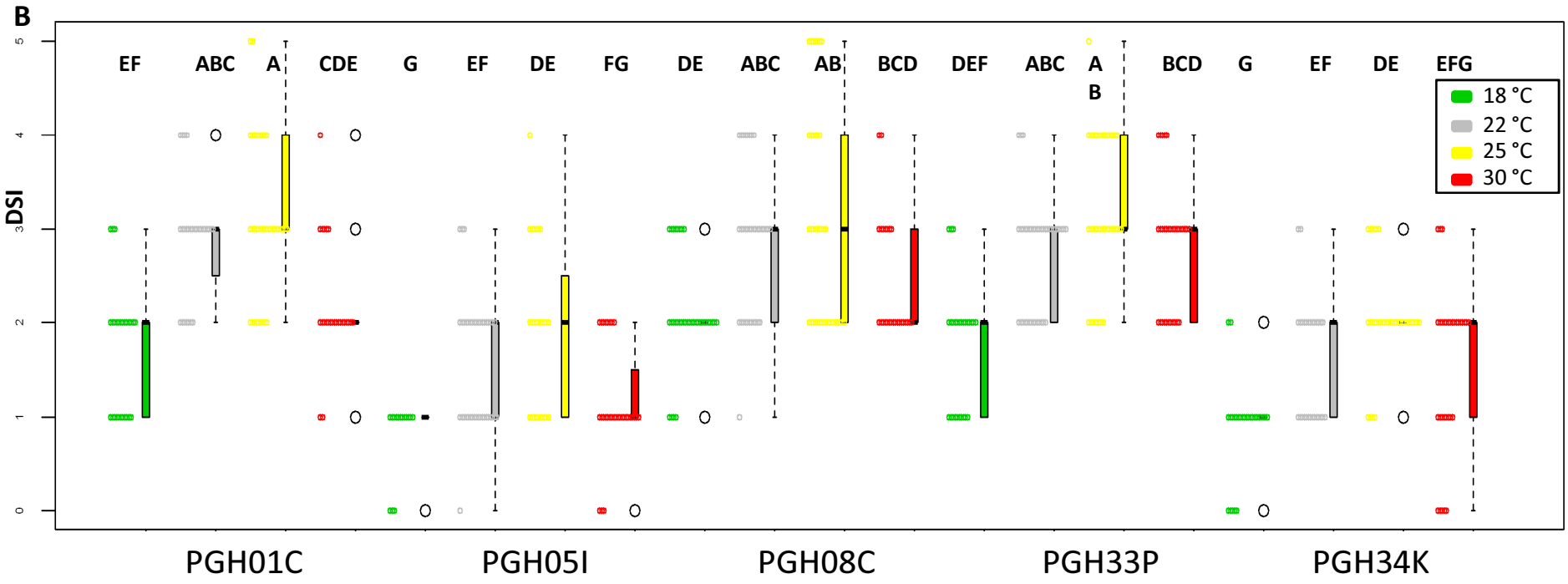


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Figure 4(on next page)

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

Dually stained samples visualize the infection process (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). 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 it did not succeed to grow through the papilla of the resistant cultigen PGH05I. C: 3D rendering of a papilla representative for the susceptible cultigen PGH12P. D: 3D rendering of a papilla representative for the resistant cultigen PGH05I. A smaller number of stacked pictures generated a shorter papilla. E: Visualization of a successful infection of the susceptible cultigen PGH12P at 4dpi. Arrows from left to right denote: Germinating conidium; 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 at 4dpi. G: Typical actin networks crossing cells of the uninfected leaves (presented: susceptible cultigen PGH12P at 0dpi). H: Rapid polarization of the actin networks, tightly surrounding the papilla deposited beneath the perceived pathogen attack (presented: resistant cultigen PGH05I at 2dpi).

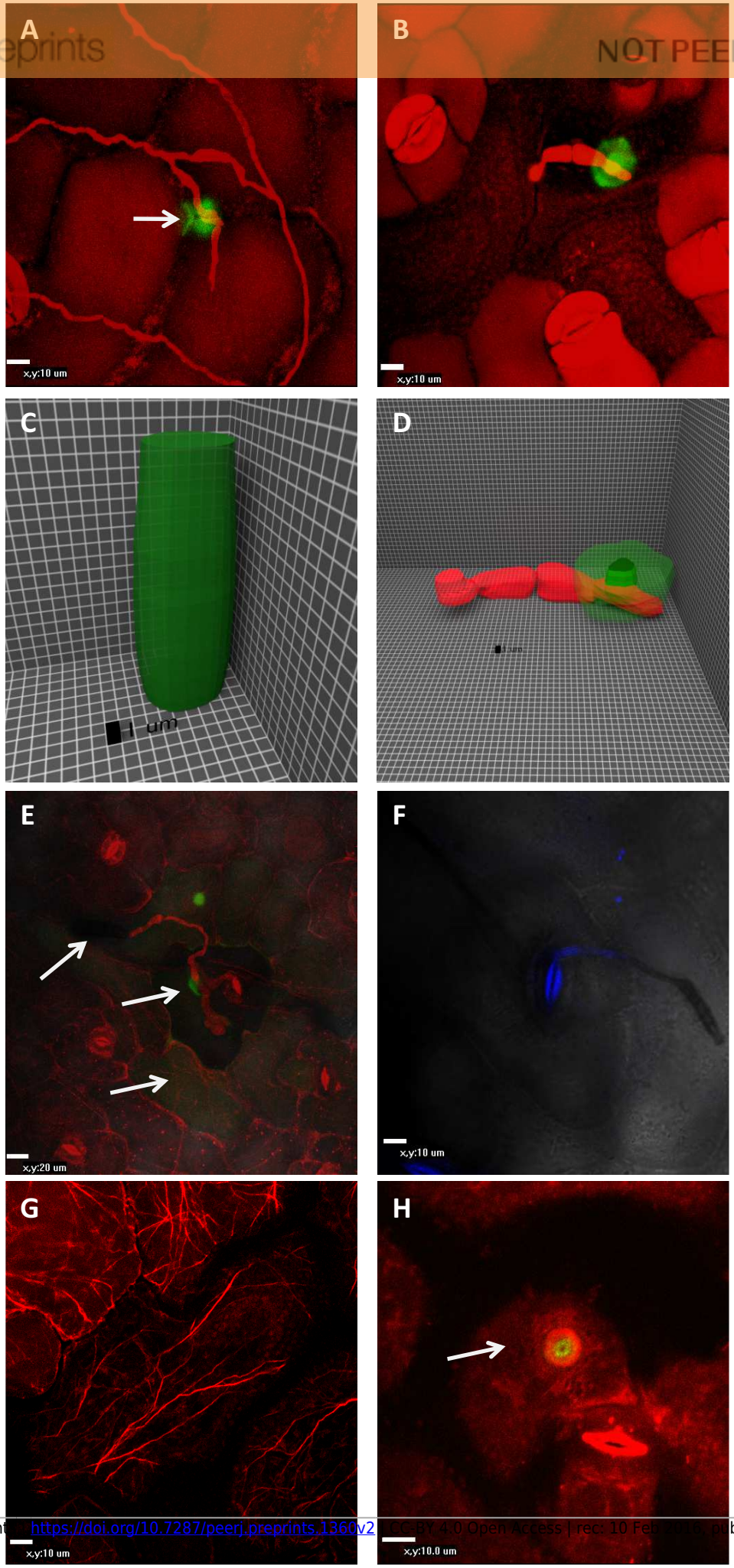

Table 2 (on next page)

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

[a]Cultigens were arranged according to their seedling bio-assay performance (increasing DSI; see Table 1). [b]Density on stomata per cm^2 (mean±SD) on the abaxial (upper) leaf side; data from at least 5 microphotograph counts (670 µm×670 µm each). Upper letter indexes represent results of the Tukey's HSD testing after one-way ANOVA of the stomata counts. [c]Direct (epidermal) or stomatal penetration counts, based on the analyzed dual stained samples. [d]Results of statistical analyses (*P* from the χ^2 test) of the pathogen's non-preference hypothesis.

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
Stomata density [cm^{-2}][b]	17362±2435^{A}	15691±1083^{B}	13688±3768^{C}	11392±3314^{D}
DIRECT[c]	2	12	32	15
STOMATAL[c]	13	6	11	15
χ^2 against 50%-50%[d]	**0.005**	0.157	**0.001**	1

[a] Cultigens were arranged according to their seedling bio-assay performance (increasing DSI; see Table 1).
[b] Density on stomata per cm^2 (mean±SD) on the abaxial (upper) leaf side; data from at least 5 microphotograph counts (670 µm × 670 µm each). Upper letter indexes represent results of the Tukey's HSD testing after one-way ANOVA of the stomata counts.
[c] Direct (epidermal) or stomatal penetration counts, based on the analyzed dual stained samples.
[d] Results of statistical analyses (*P* from the χ^2 test**)** of the pathogen's non-preference hypothesis.