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Correlation of trichome density and length and polyphenol fluorescence with susceptibility of five cucurbits to *Didymella bryoniae*

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CORRELATION OF TRICHOME DENSITY AND LENGTH AND POLYPHENOL FLUORESCENCE WITH SUSCEPTIBILITY OF FIVE CUCURBITS TO *DIDYMELLA BRYONIAE*

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Running title: Trichomes, polyphenols and *D. bryoniae* susceptibility

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SUMMARY

Among species within *Cucurbitaceae,* there are substantial differences in susceptibility to *Didymella bryoniae*, the causal agent of gummy stem blight on cucurbits. The underlying reasons, though, are still unresolved. Susceptibility was characterized with muskmelon (*Cucumis melo*), watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), pumpkin (*Cucurbita pepo*), and zucchini (*C. pepo*). Lesion diameters on leaf disks inoculated with agar plugs were measured 7 days after inoculation, and the necrotized areas of leaf disks inoculated with conidial

suspensions were measured 48 hours after inoculation (hai). For each species, the number of trichomes was counted on 16 leaf pieces using a stereomicroscope. Lengths of ≥21 trichomes per species were measured. Polyphenol autofluorescence was recorded at 48 hai and quantified. Watermelon had the lowest trichome density and the shortest trichomes. Zucchini showed the highest trichome density, and pumpkin had the longest trichomes. Trichome density was negatively correlated with mean necrotized leaf area, and trichome length was highly negatively correlated with lesion diameter. Mean fluorescing area was correlated with lesion diameters and mean necrotized leaf area. This is the first study in which trichome morphology and polyphenol autofluorescence in inoculated cucurbit leaves were correlated with susceptibility to *D. bryoniae*.

KEYWORDS

Trichomes, polyphenols, cucurbits, *Didymella bryoniae*, susceptibility

The ascomycete *Didymella bryoniae* (Auersw.) Rehm (synonym *Stagonospropsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley) is the causal agent of gummy stem blight and black rot on cucurbits. The fungus is distributed worldwide and attacks a broad range of host plants (Keinath, 2011). It is one of the most important pathogens limiting cucurbit production in Brazil (Dos Santos et al., 2009), the United States (Keinath, 2011), Europe (Van Steekelenburg, 1983; Blancard et al., 1994; Grube et al., 2011) and elsewhere (Farr and Rossman, 2014). Within cucurbits there is great variability in susceptibility to *Didymella bryoniae*. *Citrullus lanatus* and *Cucumis melo* are generally considered the most susceptible hosts, whereas *Cucurbita* spp. are among the less susceptible ones (Chiu and Walker, 1949; Dos Santos et al., 2009; Keinath, 2014a; Keinath, 2014b).

47 There is an abundance of trichome morphologies within the *Cucurbitaceae* (Inamdar and
48 Gangadhara, 1975). Trichomes have a number of important functions in plants. They reduce heat
49 load, increase tolerance to freezing, promote seed dispersal and water absorption, protect from
50 UV-B, and repel insects (Adebooye et al., 2012). Trichome densities and lengths have been
51 highly correlated with rust resistance of beans (Mmbaga and Steadman, 1990; Zaiter et al., 1990;
52 Menendez Sevillano et al., 1997). In hops, trichomes are 13 times larger than normal epidermal
53 cells and show an increased susceptibility to powdery mildew, because they have a lower level of
54 defense reactions and physiological activity than other cells (Oberhollenzer et al., 2013). A high
55 polyphenol content in plant tissue has been shown to confer resistance to ascomycete pathogens
56 (Treutter and Feucht, 1990; Gradziel et al., 1998; Mayer, 2006; Giordani et al., 2013). Beckman
57 et al. (1972) observed stored phenolics in bulbous trichomes and later pointed out that phenolic-
58 storing cells play key roles in the defense strategy of plants (Beckman, 2000). However,
59 trichome morphology and polyphenol content has never been linked to the susceptibility of
60 cucurbits to gummy stem blight.
61 The objectives of this study were to i) assess the relative susceptibility of five different
62 cucurbits to *Didymella bryoniae*; ii) measure the trichome density and length as well as the
63 polyphenol autofluorescence in inoculated leaf pieces and iii) correlate these parameters with
64 susceptibility to assess the importance of these factors in the interaction of *Didymella bryoniae*
65 with its major cucurbit hosts.
66 Inoculum of *D. bryoniae* isolates N2 and N3, obtained from two cucumber plants in Lower
67 Bavaria, Germany, was grown on quarter potato dextrose agar (QPDA; 9.75 g/l PDA, 11.25 g/l
68 agar, 100 mg/l aureomycin). Five cucurbit species were grown in the greenhouse for three weeks
69 (Table 1). Four leaf disks with a diameter of 7 cm were cut from mature, fully expanded leaves.

70 For watermelon, twice as many leaf disks were used, since the pinnatifid leaves of this cucurbit
71 only allowed one measurement of lesion diameter per leaf. Disks were rinsed under running tap
72 water for 10 s, washed three times with sterile distilled water and blotted with autoclaved filter
73 paper. Two leaf disks were placed with the upper surface up and two with the lower surface up
74 onto water agar (1.2%) in 10 cm-diameter petri dishes. One 5-mm-diameter agar piece cut from
75 QPDA cultures of isolate N2 was put in the center of each leaf disk, i.e. four leaves per cucurbit
76 were inoculated. The petri dishes with the inoculated leaf disks were then placed in a growth
77 chamber at 20°C and 60% RH for one week. For the initial 24 hours the plates were held in
78 complete darkness. After that period, a light cycle of 12 hours light / 12 hours darkness was
79 applied. After one week of incubation, two perpendicular lesion diameters on leaf disks were
80 measured. The experiment was repeated once.

81 Six leaf disks 15 mm in diameter were cut from leaves of each cucurbit. Leaf disks were
82 rinsed as described above. The leaf disks were inoculated with a suspension of 10^6 conidia/ml of
83 a mix of isolates N2 and N3 in a solution of sucrose (0.1%) and casein (0.05%) using a
84 chromatography sprayer. At 24 and 48 hours after inoculation (hai), non-inoculated and
85 inoculated leaf disks were mounted in water onto microscope slides and analyzed under a
86 fluorescence microscope (Zeiss, Mikroskop Universal, filter setting: G 436, FT 510, LP 520).
87 The percentage of black color in the pictures was measured with ImageJ (Li et al., 2009) to
88 estimate the degree of necrosis in the leaf disks.

89 The number of trichomes on the upper leaf surface of each cucurbit was determined. Three to
90 four pieces 4 mm^2 from at least five leaves of different ages and positions on plants were cut
91 from plants grown in the greenhouse for 3-4 weeks, so that for each cucurbit 16 leaf pieces were
92 examined. All visible trichomes on the upper surface of the leaf pieces were counted using a

93 stereomicroscope with magnification of 40×. The lengths of trichomes were measured using
94 AxioVision microscope software (Release 4.8.2 (06-2010)). For each cucurbit ≥21 trichomes
95 originating from different positions on at least five different leaves were measured.
96 Leaf disks 15 mm in diameter were prepared and inoculated with a conidial suspension
97 applied with a chromatography sprayer as described above. For each point of time (non-
98 inoculated, 24 hai and 48 hai) at least five different leaf disks from different plants of each
99 cucurbit were examined under a fluorescence microscope and photographed. The presence of
100 phenolic compounds in the upper leaf epidermis and trichomes is indicated by light green
101 fluorescence (filter setting: G 436, FT 510, LP 520) (Kolb et al., 2001). The chlorophyll in the
102 leaf disks is visible through its emission of red fluorescing light (Misra et al., 2012). The
103 percentage of yellow-green area in each picture was analyzed with Adobe Photoshop CS6
104 Extended (Version 13.0.1 x 64) (Luna et al., 2011).
105 The trichome measurements, lesion diameters and values of the necrotized leaf area and
106 polyphenol autofluorescence were analyzed with SAS version 9.4 with PROC GLM.
107 Subsequently a Tukey test was used to separate means. Pearson correlation coefficients between
108 trichome measurements and polyphenol autofluorescence with disease assessments were
109 calculated with SAS PROC CORR.
110 In the leaf disk assay there was no significant effect of leaf side ($P = 0.27$) or repetition ($P =$
111 0.21) on lesion size, and no cultivar-leaf side interaction ($P = 0.50$). After 7 days, lesion
112 diameters on pumpkin leaf disks were the smallest of all tested plants. On average they were 40.6
113 mm in size. Cucumber and zucchini showed significantly larger lesion diameters than pumpkin
114 (Table 1). However, they were significantly smaller than the lesion diameter on watermelon,
115 which was the largest with a mean lesion size of 68.9 mm. This was not statistically different

from muskmelon, cucumber and zucchini but significantly larger than the diameter measured on pumpkin leaf disks. The overall average of lesion diameters on lower leaf surfaces of all tested cucurbits was only 0.77 mm larger than on upper leaf surfaces.

Prior to inoculation, the average percentage of leaf necrosis as measured by autofluorescence showed no significant differences among the five studied plants. The values ranged from 0.00% in muskmelon and watermelon to 0.98% in pumpkin. At 48 hai there was an increase in leaf necrosis in all examined plants (Table 1). Muskmelon and watermelon with extremely severe necrosis of 99.9% and 99.7%, respectively, were clearly the most affected plants. In the second group was cucumber, which showed a mean leaf necrosis of 35.5%. This was significantly lower than muskmelon and watermelon but higher than pumpkin and zucchini. The averages for pumpkin, 4.04%, and for zucchini, only 3.74%, were significantly lower than the necrosis measured on the other three cucurbits.

There were several significant differences in the number of trichomes per unit area among the five cucurbits used for this study. With a mean number of merely 1.11 trichomes / mm^2 watermelon had by far the lowest number of trichomes (Table 1). Consequently, watermelon had significantly ($\alpha = 0.05$) fewer trichomes per square millimeter than the other four cucurbits. Muskmelon showed the second lowest density of trichomes, only 3.89 / mm^2, which was significantly lower than the observed average of zucchini, which had the highest density of trichomes with a value of 8.28 / mm^2 on average (Table 2). On both cucumber and pumpkin a significantly higher number of trichomes compared to watermelon was observed, but they did not differ from muskmelon and zucchini.

With average lengths of 278.48 µm and 317.35 µm, respectively, watermelon and muskmelon had the shortest trichomes among the five examined cucurbits (Table 1). These two hosts had

139 significantly shorter trichomes than cucumber and pumpkin, but they were not statistically
140 different from zucchini. The mean trichome length of pumpkin was the longest with a length of
141 732.73 µm.

142 In non-inoculated leaves of the five cucurbits used for this study, fluorescence of polyphenols
143 was detected exclusively in the trichomes. At 24 and 48 hai bright yellow-green fluorescence of
144 polyphenols also was observed in epidermis cells of inoculated leaf disks. From 0 to 48 hai the
145 mean measured areas of fluorescing upper leaf surfaces of inoculated disks decreased in three
146 cucurbits and increased in muskmelon and watermelon, which showed a clear increase from
147 0.28% to 2.52% and from 0.24% to 3.79%, respectively. Autofluorescence (% area) in
148 watermelon was significantly greater than autofluorescence in cucumber, zucchini and pumpkin
149 (Table 1).

150 The trichome characteristics showed two strong correlations. Trichome density was negatively
151 correlated with the mean necrotized leaf area ($r = -0.89$; $P = 0.0433$), and trichome length was
152 highly negatively correlated with the lesion diameter ($r = -0.98$; $P = 0.0044$) (Fig. 1, Fig. 2).
153 There also was a positive correlation between the mean fluorescing area measured at 48 hai and
154 the lesion diameter measured on leaf disks ($r = 0.88$; $P = 0.0517$), as well as with the mean
155 necrotized leaf area ($r = 0.94$; $P = 0.0162$) (Table 1, Fig. 3).

156 The order of susceptibility among the cucurbits determined in this study is consistent with
157 previous studies. The two *Cucurbita* species showed the lowest susceptibility. *Citrullus lanatus*
158 and *Cucumis melo*, on the other hand, were the most susceptible, and *Cucumis sativus* fell in-
159 between those two groups. Dos Santos et al. (2009) also found that *Citrullus lanatus* and
160 *Cucumis* spp. were most susceptible to *D. bryoniae*, and *Cucurbita* spp. were the most resistant
161 species in their study. Keinath (2014a, 2014b) showed that muskmelon, watermelon and

honeydew melon are the most suitable hosts for the fungus' reproduction and generally more susceptible than *Cucurbita* spp. Grossenbacher (1909) and Chiu and Walker (1949) reported that *Cucurbita* spp. were among the least susceptible cucurbits and even suggested that they are immune to stem cankers under natural conditions.

The role of trichomes in plant-pathogen interactions is a rather ambivalent one. High correlations between trichome densities and lengths with resistance of beans to rust have been reported several times (Mmbaga and Steadman, 1990; Zaiter et al., 1990; Menendez Sevillano et al., 1997). Simple non-glandular trichomes may protect plants, e.g. act as a physical barrier hindering the contact between pathogenic microorganisms, including fungal spores and the leaf surface (Laźniewska et al., 2012). This may explain the negative correlation between trichome density and length with susceptibility to *D. bryoniae*. Trichomes contribute to the spatial organization of the leaf surface. Therefore they have an impact on the infection process, as a physical barrier against pathogenic microorganisms in general, and on plant-pathogen compatibility by altering the leaf topology (Zelinger et al., 2006; Laźniewska et al., 2012). In addition, specialized glandular trichomes that secrete antimicrobial secondary metabolites protect plants from pathogens (Laźniewska et al., 2012). Nonomura et al. (2009) discovered that trichome exudates of *Lycopersicon pennellii* cover the entire leaf surface and act as a chemical barrier that inhibits the germination of *Oidium neolycopersici.* In contrast to this, leaf topology, which is influenced by trichomes, can affect host specificity. The spores of *Stagonospora nodorum* for instance fit the leaf surface of wheat better than that of barley as a result of the distribution of leaf hairs (Zelinger et al., 2006; Laźniewska et al., 2012). Some fungal pathogens use trichomes as preferred sites of penetration e.g. *Colletotrichum acutatum* on strawberry, *Fusarium graminearum* on *Arabidopsis* spp. (Skadsen and Hohn, 2004; Salazar et al., 2007;

185 Laźniewska et al., 2012) and *Podosphaera macularis* ssp. *humuli* on hops (Oberhollenzer et al.,
186 2013).

187 The analysis of leaf pieces in a fluorescence microscope showed that the trichomes are filled
188 with polyphenols, visible through the light green fluorescence they emit, which is in contrast to
189 epidermis cells. This finding is consistent with earlier reports of stored phenolics in bulbous
190 trichomes (Beckman et al., 1972). The high positive correlation of the measured light green
191 fluorescing leaf area with the lesion size implies that the more susceptible a cucurbit is to *D.*
192 *bryoniae*, the more phenolic compounds it produces in reaction to an infection. This is
193 unexpected, as the fungitoxic effect of phenolic compounds is well documented in cucumber
194 (Daayf et al., 1997a; Daayf et al., 1997b; Fawe et al., 1998; Daayf et al., 2000). Moreover, there
195 are several reports of the general finding that plants that are more resistant to fungal pathogens
196 display a substantially higher concentration of phenolic compounds in their tissues (Gradziel et
197 al., 1998; Mayer, 2006; Giordani et al., 2013). In the five cucurbits examined in this study, the
198 defense strategy of the more susceptible species might rely too strongly on the production of
199 polyphenols, or the less susceptible species might produce other, more effective chemical
200 compounds that enable them to defend themselves against *D. bryoniae.*

201 There is an abundant range of trichome morphology among cucurbits. The cucurbits used in
202 this study comprise only a fraction of the trichome morphologies that occur in this plant family
203 (Inamdar and Ganggadhara, 1975). Including species or varieties with other trichome
204 morphologies in a future study might further the understanding of the role of trichomes in the
205 susceptibility or resistance of cucurbits to *D. bryoniae*. Differences in trichome morphology and
206 polyphenol fluorescence can likely be attributed to differences between cultivars within one
207 particular species rather than differences between species.

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306

Table 1. Mean values of susceptibility parameters, trichome characteristics and fluorescing leaf area.

Species	Ø[1]	NECLA[2]	Trich. No.[3]	Trich. length[4]	% fluorescence[5]
Cucumber (cv. Platina)	54.58^{b}	35.45^{c}	5.59^{bc}	689.85^{bc}	1.09^{a}
Muskmelon (cv. Charentais)	62.63^{bc}	99.94^{b}	3.89^{b}	317.35^{a}	2.52^{ab}
Pumpkin (cv. Aspen)	40.63^{a}	4.04^{a}	6.20^{cd}	732.73^{c}	0.54^{a}
Watermelon (cv. Red Star)	68.94^{c}	99.72^{b}	1.11^{a}	378.48^{a}	3.79^{b}
Zucchini (cv. Diamant)	56.07^{b}	3.74^{a}	8.28^{d}	468.32^{ab}	0.56^{a}

[1] Lesion diameter in mm measured 7 days after inoculation

[2] Necrotized leaf area in percent 48 hours after inoculation (hai)

[3] Trichome number per mm^2

[4] Trichome length in µm

[5] Percentage of leaf area with polyphenol fluorescence 48 hai

[a-d] Letters indicate significant differences for ANOVA with Tukey test ($\alpha = 0.05$)

307

Table 2. Correlations of trichome density and length and polyphenol autofluorescence with disease parameters.

	Trichome density		Trichome length		PA[a] at 48 hai	
	PCC[b]	P value	PCC[b]	P value	PCC[b]	P value
Lesion diameter	- 0.6864	0.2006	- 0.9761	0.0044	0.8756	0.0517
Necrotized leaf area	- 0.8896	0.0433	- 0.7953	0.1077	0.9429	0.0162

[a] Polyphenol autofluorescence

[b] Pearson Correlation Coefficient

308

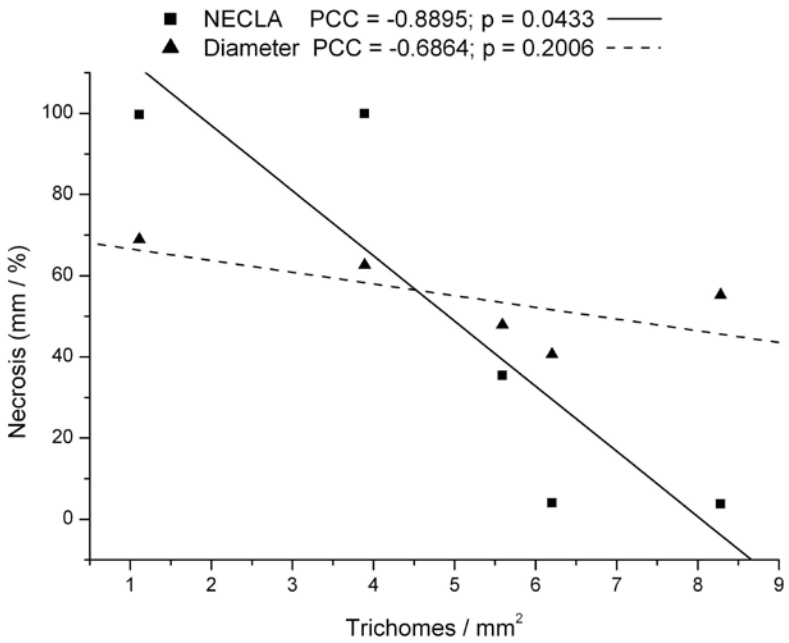

Fig. 1 Correlation of necrotized leaf area (%) and lesion diameter (mm) with trichome density.

309

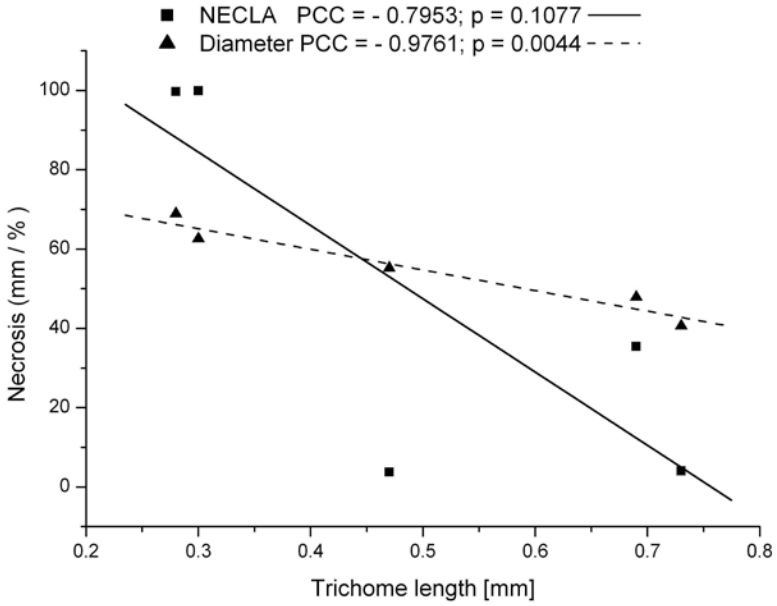

Fig. 2 Correlation of necrotized leaf area (%) and lesion diameter (mm) with trichome length.

310

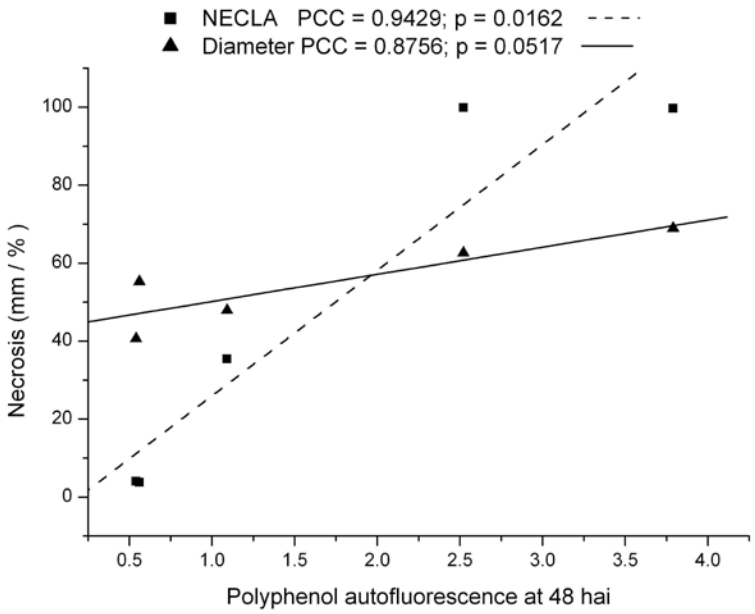

Fig. 3 Correlation of necrotized leaf area (%) and lesion diameter (mm) leaf area showing autofluorescence of polyphenols.