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SHORT COMMUNICATION

Are microconidia infectious principles in *Neonectria ditissima*?

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Abstract
Comparative studies of different isolates of *Neonectria ditissima* obtained from canker lesions and rotten fruit showed that both five-septate macroconidia and aseptate microconidia were capable of germination by germ-tube formation, but that growth commenced earlier and proceeded faster from the former than the latter type of spore. Further, following wound inoculation of apple fruit with different numbers of conidia (50, 500 or 5000 per wound) the resulting rot lesions were always significantly larger with macroconidia than microconidia, and in both conidial types lesion size increased with higher inoculum loads. These data confirm that microconidia are capable of causing infections, but indicate that their contribution to the success of the pathogen in the field is probably negligible.

Keyword Apple · *Cylindrocarpon heteronema* · European canker · Fruit rot · Macroconidia

Introduction

European canker, caused by *Neonectria ditissima* (Tul. & Tul.) Samuels & Rossman, is one of the most important diseases of apple and other pome fruit trees in temperate climatic zones (Swinburne 1975; Saville and Olivieri 2019). Canker lesions are the result of infections through natural or artificial bark wounds giving rise to necrotic regions surrounded by more or less pronounced callus tissue, which is the manifestation of a host defence reaction (Crowdy 1949). In addition, *N. ditissima* may cause two types of fruit rot, viz. blossom-end rot following floral infections and postharvest fruit rot as a result of infections during the weeks before harvest (Xu and Robinson 2010; Holthusen and Weber 2021). Infections by ascospores of the *Neonectria* type and/or by conidia of the *Cylindrocarpon* type may occur throughout the year. There is a growing awareness that conidia play a prominent and previously underestimated role in the disease cycle of *N. ditissima* at least in the mild and wet conditions of Northwestern Europe (Holthusen and Weber 2021; Weber and Børve 2021).

Two types of conidia are, in fact, produced by *N. ditissima*. Elongated cylindrical mostly five-septate macroconidia are abundantly released from sporodochia formed on fresh canker lesions (Zeller 1926). These as well as one- to three-septate macroconidia are also produced by agar cultures. Microconidia, which are aseptate or one-septate, are much smaller than macroconidia (Zeller 1926). In nature, these are produced more commonly by ageing sporodochia (Braun and Riehm 1957), whereas in the laboratory the proportion of macro- to microconidia may vary with the age of the culture (Wesche and Weber, unpublished observations), growth media (Scheper et al. 2014), illumination conditions (Scheper et al. 2014) and temperature (Gelain et al. 2020). In addition, different isolates of *N. ditissima* may vary intrinsically in their ability to produce macro- or microconidia (Scheper et al. 2014; Campos et al. 2017).

Whereas macroconidia are well known as agents of canker and fruit rots (Zeller 1926; Weber 2014), the role of microconidia has remained unclear (Zeller 1926; Saure 1961). Weber (2014) observed that microconidia were able to germinate on standard agar media and suggested that they should be regarded as unicellular macroconidia in functional terms. On the other hand, Xu and Robinson (2010) speculated that the ratio of macro- to microconidia may affect symptom expression in apple fruit. Therefore, the aim of the

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present study was to characterize the pathogenic potential of microconidia by studying their germination and ability to cause fruit rot in direct comparison with macroconidia. For this purpose, we used isolates of *N. ditissima* capable of producing either or both spore types.

Materials and methods

Twelve isolates of *N. ditissima* obtained from different origins and host organs (Table 1) were stored as lyophilised conidial preparations (Smith and Onions 1983) in the Esteburg Centre culture collection. These were revived on potato dextrose agar (PDA; Carl Roth, Karlsruhe, Germany) at the beginning of the study. For conidium production, PDA plates were incubated at room temperature for 10–14 d with a daily 10-min burst of near-UV light ($\lambda_{max} = 365$ nm). The identity of all isolates was confirmed by sequencing of the ITS1—5.8S rRNA—ITS2 region of the ribosomal RNA gene cluster (Weber and Dralle 2013).

In order to examine conidial germination, spores were harvested from 10 to 21 d old PDA plates in sterile deionised water, adjusted to different concentrations (10^6, 10^5 or 10^4 ml^{-1}) and placed as 15 µl drops on fresh PDA or tap-water agar (TWA). Germination rates and germ-tube lengths were determined on both media after different periods of incubation at 20 °C in the dark. Spores were considered to have germinated if the germ-tube was at least 10 µm long. At each time-point for each isolate, 10 germ-tubes emerging from aseptate microconidia and/or 5-septate macroconidia were measured with an AxioScope A.1 light microscope (Carl Zeiss, Germany) fitted with a × 10 objective and an eyepiece graticule (final magnification 100×). All measurements were conducted in triplicate.

For infection of apple fruit (cv. Elstar), seven representative isolates were chosen (see Table 1). Conidia were harvested in water as above. Where both micro- and macroconidia were present, the conidial density was adjusted on the basis of macroconidia. The viability of all spore preparations was determined as the percentage of germinated conidia after incubating drops of suspension on PDA for 24 h at 20 °C. Following surface-sterilization by swabbing the fruit surface with 70% (w/v) ethanol, four wounds (2 mm diam., 2 mm depth) were created on each fruit using a blunt sterile nail, and inoculated with 20 µl conidial suspension adjusted to give 50, 500 or 5000 spores per wound, or with 20 µl sterile water as a control. For each isolate, 25 fruit were inoculated and incubated at room temperature in a randomized block design of moist chambers, each containing five fruit per isolate. The radius of each fruit rot lesion was measured after 14, 21 and 28 d incubation as the average of the smallest and largest distance from the margin of the wound to the advancing margin of the rot lesion. After 28 d, squares of tissue (2–3 mm side length) were removed from representative lesions with a sterile scalpel and placed on fresh PDA augmented with antibiotics (200 mg penicillin G and 200 mg streptomycin sulfate l^{-1}; both from Carl Roth) in order check for the viability of inoculum in cases where no spreading rot lesion had been observed, and to re-examine the properties of all *N. ditissima* isolates after their passage through the fruit.

Data of germ-tube length after 24 h were $\log_{10}$ transformed to improve variance homogeneity and subjected to an analysis of variance (ANOVA) with block design. The Tukey test ($P = 0.05$) was used as a multiple means comparison between isolates and conidial types. For the infection test on apple fruit, the mean value of rot lesion radius after 28 d was calculated for each replicate and the measured values were subsampled over the individual replicates. Statistically

Table 1 Details of *Neonectria ditissima* isolates used in this study

Isolate	Region (production)	Cultivar (disease)	Year	Types of conidia	
				Macro	Micro
OVB12-104[a]	Lower Elbe (IPM)	unknown (fruit rot)	2012	X	X
OVB13-062[a,b]	Lower Elbe (IPM)	Evelina (fruit rot)	2013	X	X
OVB21-001[a]	Lower Elbe (IPM)	Jonagold (canker)	2021	X	X
NeoDit21-011[a,b]	Lower Elbe (IPM)	Nicoter (canker)	2021		X
NeoDit21-013[a,b]	Lower Elbe (IPM)	Nicoter (canker)	2021		X
NeoDit21-019[a]	South Tyrol (IPM)	Nicoter (canker)	2021	X	X
NeoDit21-020[a]	South Tyrol (IPM)	Nicoter (canker)	2021	X	X
NeoDit21-026[a,b]	Lower Elbe (organic)	Fresco (canker)	2021		X
NeoDit21-027[a,b]	Lower Elbe (organic)	Fresco (canker)	2021		X
NeoDit22-003[a,b]	Lower Elbe (abandoned)	Gloster (canker)	2022	X	X
NeoDit22-006[a]	Lower Elbe (IPM)	Rockit (fruit rot)	2022	X	X
NeoDit22-008[a,b]	South Tyrol (IPM)	Cosmic Crisp (canker)	2022	X	

[a]Isolates used for germ-tube measurements; [b]isolates used for the fruit inoculation assay

significant differences between isolates and conidial concentration, and possible interactions between these variables, were examined using a two-factorial ANOVA with block design. To ensure variance homogeneity, data were $\log_{10}$ transformed. The Tukey test ($P = 0.05$) was used as a post hoc test. In addition, the infestation incidence was determined for each replicate, arcsin transformed and compared with the ANOVA and Tukey tests ($P = 0.05$). Variance homogeneity was ensured for all analyses by the Levene test. All analyses were performed with the SPSS 27.0 software (IBM, USA).

Results

All 12 isolates of *N. ditissima* possessed an identical ITS1−5.8S—ITS2 sequence which was, furthermore, identical to sequences previously obtained for Northern German *N. ditissima* isolates (Weber and Dralle 2013). Even at first glance, there were obvious differences between the rapid growth of germ-tubes emerging from macroconidia as compared to the much slower growth of those emitted from microconidia (Fig. 1). When measured after 24 h incubation at 20 °C, these differences in germ-tube length were significant ($P < 0.05$) not only between macroconidia and microconidia of the same isolate, but also between different isolates, i.e., germ-tubes from microconidia of all 11 isolates producing them were significantly shorter than germ-tubes from macroconidia of all 8 isolates producing them in our experiment. This was observed on PDA (Fig. 2) as well as on TWA (not shown), whereby germ-tube growth after 24 h was significantly higher on the former than the latter medium, irrespective of isolate and conidium type. There were no significant interactions between the different isolates and culture media. The longest germ-tubes after 24 h were measured for NeoDit22-008, an isolate which produced exclusively macroconidia of the 5-septate type. Repeated measurements at 1, 2, 3, 4, 6, 8, 10, 14, 20 and 24 h of the length of germ-tubes revealed that germ-tube growth commenced earlier from macroconidia than microconidia, and that the subsequent growth rate was also higher. This held true on both growth media. An illustration is made here for isolate NeoDit22-003 (Fig. 3); the graphs for the other six isolates producing both types of conidium were almost superimposable (not shown).

Spore preparations of all seven isolates used for apple fruit inoculation possessed a high viability exceeding 90% germination on PDA after 24 h for both macro- and microconidia. A comparison of these isolates for their ability to cause wound infections on apple fruit after 28 d incubation at room temperature showed that those three isolates inoculated as macroconidia (with or without accompanying microconidia) gave rise to significantly larger fruit rot lesions than those four which had been inoculated exclusively as microconidia (Figs. 4, 5a, b). There were also statistically significant increases in lesion size associated with increasing concentrations of conidia per wound. The disease incidence (percent inoculated wounds giving rise to a visible rot lesion) correlated in a similar way with inoculum dose

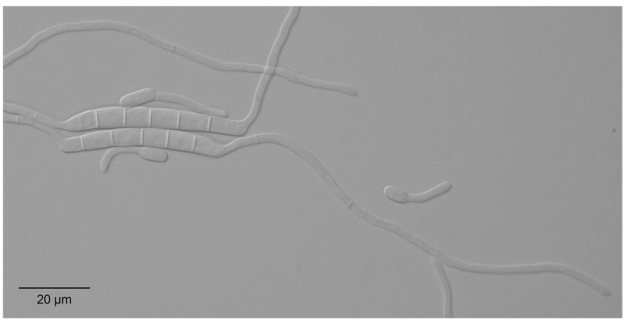

Fig. 1 Appearance of germ-tubes emitted by macroconidia and microconidia of *N. ditissima* isolate NeoDit22-003 on PDA after 6 h at 20 °C

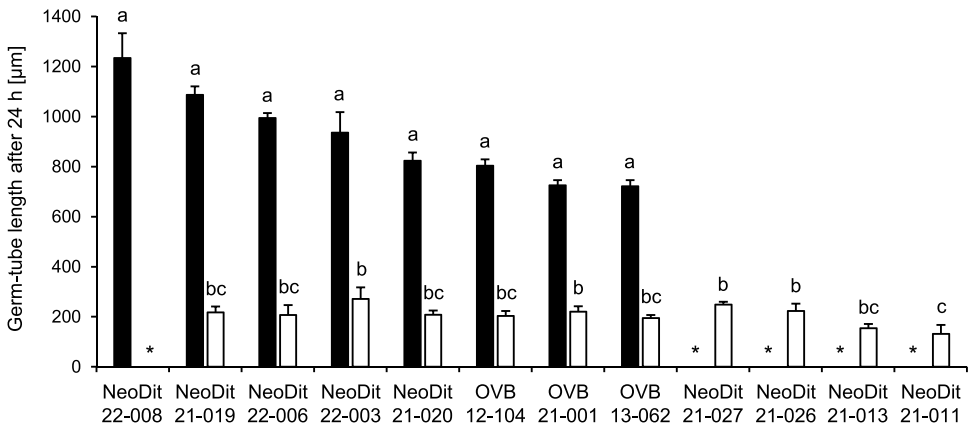

Fig. 2 Length of germ-tubes emitted from macroconidia (black bars) and/or microconidia (white bars) of 12 isolates of *N. ditissima* after 24 h incubation on PDA at 20 °C, shown as average + standard error ($n = 3$). Different letters indicate significant differences at $P < 0.05$. Missing spore stages are indicated (*)

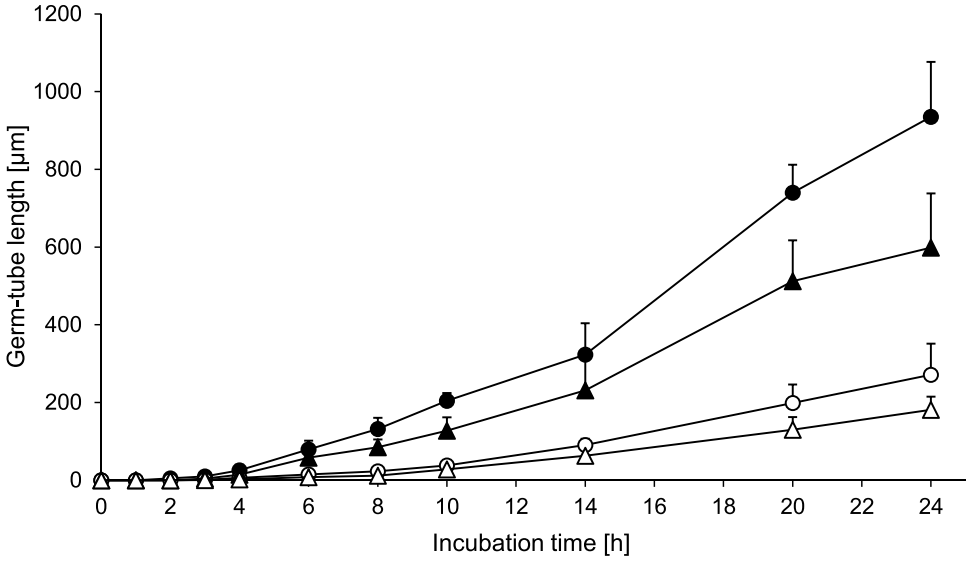

Fig. 3 Elongation of germ-tubes emitted from macroconidia (black symbols) and microconidia (empty symbols) on PDA (circles) and TWA (triangles) for *N. ditissima* isolate NeoDit22-003, shown as average + standard error ($n = 3$)

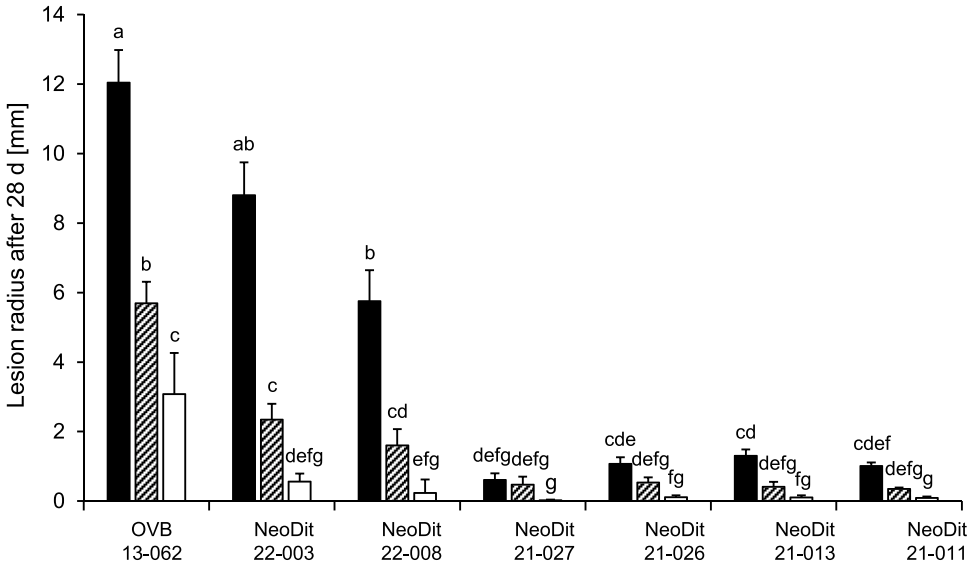

Fig. 4 Lesion radius for seven isolates of *N. ditissima* after 28 d incubation of artificially wound-inoculated apple fruit at room temperature, shown as average + standard error ($n = 5$). Different letters indicate significant differences at $P < 0.05$. Wounds were inoculated with 50 (white bars), 500 (hatched bars) or 5000 (black bars) conidia

(not shown). We found no significant interaction between these two parameters of isolate and conidium concentration. Some of the inoculated wounds did not give rise to a spreading rot lesion that could be quantified, but nonetheless showed a distinct browning of the wound margin (Fig. 5b). When these fruit were sliced open, a corking of the subepidermal tissue around the wound margin became apparent (Fig. 5c). This type of discoloration was stronger and more deeply seated than the phenolic browning associated with the physical wound reaction in the immediate vicinity of uninoculated wounds (Fig. 5d, e). Attempts to isolate *N. ditissima* from stalled lesions of inoculated wounds onto PDA with antibiotics were successful in 19 of 41 cases, whereas *N. ditissima* was not isolated from any of 35 uninoculated control wounds.

We re-isolated all seven *N. ditissima* isolates from growing wounds in order to re-examine their properties in pure culture. Isolates NeoDit21-027 and NeoDit22-008, which had previously produced only microconidia or only macroconidia, respectively, were able to produce both spore types after apple infection. The other five isolates did not change in their production of conidia as a result of passing through a fruit infection cycle.

Discussion

The results obtained in this study demonstrate that microconidia and macroconidia of *N. ditissima*, produced on agar culture under identical conditions, differed significantly in

Fig. 5 Fruit infections by *N. ditissima* using 50, 500 or 5000 conidia per wound, photographed after 28 d incubation at room temperature. **a** Aggressive rot caused by macroconidia of isolate OVB13-062. **b** Stalled infections by microconidia of *N. ditissima* NeoDit21-011. **c** Section through a stalled infection revealing limited tissue necrosis in deeper regions of the apple tissue. **d** Uninoculated control wound. **e** Section through an uninoculated control showing the wound reaction as a faint browning at the wound surface

their germ-tube growth rate as well as in their ability to cause fruit rot on artificially inoculated apple fruit, despite possessing an equivalent ability to germinate (always > 90%). There is good evidence to assume a direct causal relationship between germ-tube growth vigor and extent of fruit rot because *N. ditissima* is known as a relatively crude pathogen that overwhelms the host's defense system by mass hyphal growth (Crowdy 1949). This, in turn, is reflected in several studies which revealed that successful infection requires a minimum number of spores to be present, typically in the order of 50–100 or more per wound in the case of leaf scars and pruning cuts (Dubin and English 1974; Xu et al. 1998), or above 100 per wound in fruit rots (Swinburne 1971). Infections caused by a lower number of spores often lead to arrested or quiescent fruit or bark infections from which the pathogen may or may not be re-isolated (Swinburne 1971; Walter et al. 2016). This association of stalled infections with low spore numbers and a correlation of lesion size with the number of infecting conidia was also observed in our current study, both with microconidia and macroconidia as inoculum.

The temperature optimum for germination has been reported to be 20 °C for ascospores (Latorre et al. 2002) and 20–25 °C for macroconidia (Latorre et al. 2002; Gelain et al. 2020). Our comparative measurements have shown identical temperature curves for macroconidia and microconidia (Wesche, unpublished observations). Therefore, differential temperature effects can be ruled out as a factor to account for the differences in lesion size associated with these two conidial types. Rather, the low intrinsic growth vigor of microconidial germ-tubes is the most plausible explanation. Therefore, longer periods of surface wetness and/or higher numbers of spores per wound at a given temperature are likely to be required for successful infections by microconidia in nature, as compared to macroconidia or ascospores. Our conclusion is that although microconidia are principally able to cause infections, as we have shown in the present work, in practice their importance to the fungus is likely to be minor or even negligible as compared to ascospores and macroconidia. Further work is under way to test this hypothesis on bark infections leading to cankers.

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Authors' contributions JW performed the experiments and evaluated the results. Both authors designed the study. The first draft of the manuscript was written by RW. Both authors read and approved the final manuscript.

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Data availability All isolates used in this study have been deposited at the culture collection of the Esteburg Centre and will be made available for scientific study by the corresponding author.

Declarations

Conflict of interest The authors have no conflict of interest to declare that are relevant to the contents of this article.

Ethics approval The experiments did not involve human participants and/or animals.

Consent to participate Both authors agreed to participate.

Consent for publication Both authors agreed to publish the manuscript as a Short Communication in the Journal of Plant Diseases and Protection.

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