EFFECTS OF BENLATE ON HYPHAL MORPHOLOGY OF *PERONOSPORA VICIA*E

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Abstract

To eliminate contamination by other fungi assotiated with *Peronospora viciae*, treating the conidial suspension as an inoculum with fungicide Benlate at a concentration of 0.2 w/v has an effect on hyphal morphology and reduced sporolation. The exposure of *P. viciae* to this fungicide caused stunting and increased branching of hyphae, and also hyphae appeared to be septate and houstoria were not observed.

INTRODUCTION

Oomycetes are a diverse group of organisms, many of which are plant pathogens, including approximately 60 species of the genus *Phytophthora*, numerous genera of the biotrophic downy mildews and more than 100 species of the genus *Pythium* (Kamoun, *et al.*, 1999). These cause devastating disease symptoms on numerous crop and ornamental plant species. Downy mildew diseases alone, account for approximately 17 % of the world fungicide market (Gisi, 2002).

The oomycetes are placed in either Kingdom Straminipila or the Kingdom Chromista, depending on whether heterotrophic or photosynthetic ancestry respectively, is assumed (Dick, 2002a). Within the Straminipila, *Peronospora viciae* (Berk) Caspary (syn *P. pisi* Sydow), which causes downy mildew of pea plants, is placed in the sub-division Peronosporomycotina, class Peronosporomycetes, sub-class Peronosporomycetidae, order Peronosporales, and family Peronosporaceae according to Dick (2002 b). Species of *Peronospora* produce conidia that germinate to give a germ tube and not zoospores.

Downy mildew is an important disease of *Pisum sativum* L. and is caused by the oomycete *P. viciae* (Stegmark, 1994), which is an obligate biotrophic pathogen of

several fabaceous hosts and obtains nutrients from living plant tissues. It causes systemic, local and pod infections, and the disease is widely distributed all over the world, wherever peas are grown for vegetable or seed production (Dixon, 1981; Stegmark, 1994). Systemic infection of seedlings can cause a yield loss up to 30 % in Sweden and 55 % in the UK (Olofsson, 1966; Clark and Spencer-Phillips, 2000). Three different infection types with different symptoms can be recognized during the crop cycle under field conditions (Stegmark, 1994; Clark and Spencer-Phillips, 2000).

P. viciae hyphae grow intercellularly in the tissues of their host and produce haustoria in host cells, which invaginate the cytoplasm of the host cell and may function as absorbing organs. The ultra-structure of *P. viciae* hyphae and haustoria within host tissue were described by Hickey and Coffey (1977). Hyphae of downy mildew fungi are coenocytic, with only the reproductive organs separated from the other hyphae by septa. Septa may also form in response to injury. The cell wall of these fungi contains cellulose instead of chitin as the main fibrillar component.

A variety of methods have been used to isolate the inter- and intracellular infection structures of rust, powdery mildew and downy mildew fungi from infected plant tissues. These include enzymic maceration (Crucefix *et al.*, 1987; Street *et al.*, 1986; Beale *et al.*, 1990; Clark and Spencer-Phillips, 1990; Ashton, 1994; Saito, 1995) or mechanical disruption followed by either density gradient centrifugation (Gil and Gay, 1977; Tiburzy *et al.*, 1992; Cantrill and Deverall, 1993) or lectin affinity chromatography (Hahn and Mendgen, 1992). Cell sorting by density gradient centrifugation may be possible, as in the isolation of rust haustoria from infected wheat leaves (Tiburzy *et al.*, 1992). Enzyme maceration and mechanical disruption have also been used to extract viable *P. viciae* oospores from plant tissues (Van Der Gaag and Frinking, 1996). Workers with these fungi have used cellulase and Macerozyme R10 or R200 to isolate hyphae of *B. lactucae* (Crucefix *et al.*, 1987) and *P. viciae* (Clark 1989; Clark and Spencer-Phillips, 1990; Ashton, 1994).

To study a number of physiological processes such as nutrient uptake, a routine protocol is required to isolate viable hyphae free from other microorganisms. These contaminants need to be avoided by using fungicides treatment to conidial suspension and then surface sterilization of infected leaves, followed by antibiotic treatment

during the enzymic maceration stage (Street *et al.*, 1986; Clark, 1989; Van Der Gaag and Frinking, 1996).

A variety of methods have been used previously to avoid other fungal contamination in cell suspensions (Ashton, 1994).

MATERIALS AND METHODS

Conidia of *P.viciae* isolate prepared, inoculated to the plants and incubated followed by methods as in (El-Gariani 2003 and El-Gariani and Spencer-Phillips 2004). The conidia suspension was prepared with 0.2 % w/v Benlate (Ssynchemicals, UK) to reduce the mycobiotia associated with *P.viciae* befor being applied to pea plants.

Maceration of infected and non-infected leave

The maceration procedure leading to isolation of hyphae as described in (El-Gariani), by using Macerozyme R200 (Yakult, Japan) in phosphate buffered saline based in a method used by (El-Gariani and Spencer-Phillips 2004). Whole leaflets from plants 4d post-inoculation were surface sterilized by immersion in 40 % v/v ethanol, then 1 % v/v sodium hypochlorite, both for 30 sec, and finally washed three times with sterile distilled water (SDW) before infiltration with the maceration medium as in (El-Gariani and Spencer-Phillips 2004).

Microscopic Examinatin

Hypae in suspension

A drop of the suspended cells was placed on a slide and covered with a cover slip, all excess liquid was gently removed with a tissue and the slide was examined by bright field optics (BF).

Hyphae in leaf tissue

This method used to examine hyphae within leaf tissue, leaf discs were cleared for 24 h in solution of equal volumes of chloroform, lactic acid and methanol. Discs were stained for 24 h in a 1 % w/v solution of Aniline Blue in0.07 M tri-potasium phosphate, pH 12. The discs were sensed in water, mounted in Citifluor, and examined with UV10 filter block.

RESULTS

Isolation of hyphae using Macerozyme R200

A large number of preliminary experiments using leaves of *P. sativum* cvs Liviolleta and Krupp Pelushka were undertaken to establish optimal conditions for isolating hyphae from plant leaves. Initial experiments with *P. sativum* cvs Liviolleta and Krupp Pelushka infected by *P. viciae* showed that both cultivars were macerated with no differences found in the extent of maceration. The results of maceration experiments showed that the PBS medium with Macerozyme R200 completely macerated whole leaves after 3 h.

Morphology of isolated intercellular hyphae

Cell suspension prepared as before was examined with bright field (BF) microscopy. Large hyphal fragments containing granular cytoplasm and of variable width had haustoria that were either completely or incompletely detached from plant cells (Fig.1).

Effect of Benlate on hyphal morphology

To eliminate contamination by *C. cladosporioides*, the fungicide Benlate was applied to the *P. viciae* spore suspension because it is supposed to have no effect on oomycetes (Ashton, 1994). The results showed that exposure of *P. viciae* to Benlate caused stunting and increased branching of hyphae (Fig.2). The hyphae also appeared to be septate and haustoria were not observed. Furthermore, sporulation was reduced compared with plants inoculated with sporangia in SDW alone.

Staining methods to examine downy mildew hyphae within leaf tissue

Leaf disks from the leaflets of the Levioleta, 4 d after inoculation, were cleared and stained with aniline blue. Hyphae and haustoria stained a pale white-blue colour (Fig.1&2 b). Intensely fluorescent areas were associated with haustoria at their junction with hyphae arrow.

DISCUSSION

The isolate of *P. viciae* used throughout the experiments reported here was derived from the single plant of cv. Maro obtained from National Institute of Agriculture Botany. In the laboratory at UWE, *P. viciae* often failed to sporulate when maintained for more than few months on a single cultivar (Clark, 1989). The successful maintenance of the *P. viciae* stock for over three years is attributed both to the use of a mixed conidial inoculum collected from several cultivars, and regular harvesting of

conidia. The advantage of the mixed inoculums method was confirmed by (Ashton 1994 and El-Gariani 2003), who showed that sporulation failed after 20 months, equivalent to approximately 44 generations, of maintenance on a single host cultivars (Krupp Pelushka).

The cultivars used in the main experiments described here was Liviolleta, because this was considered as high in susceptibility as Krupp Pelushka, which was the subject of The inoculated plants were maintained in conditions of reduced light, high humidity and a temperature of about 14°C, which were in keeping with conditions optimized by Mence and Pegg (1971) for *P. viciae*. The spore density used in the present study was 5×10^4 ml^{-1}. This has proved optimal for the isolate of *P. viciae* being used here (Michelle Edwards, personal communication), whilst other workers (Mence and Pegg, 1971; Clark, 1989; Ashton, 1994) suggested that 10^4 ml^{-1} spore density produced optimum germination. Mence and Pegg (1971) also found that a period of leaf wetness of 4 hours was essential for germination, while Ashton (1994) found that germination was not initiated until 4 h after inoculation under the conditions used in the present study which kept leaf surfaces constantly wet. It is likely that the cycle of light and dark in the growth cabinet, together with the constant high humidity, were the equivalent of suitable field conditions for infection.

Isolation of *P. viciae* hyphae and staining

The purpose of the maceration of infected tissue was to examine *P. viciae* hyphae growth after application with Benlate.

Crucefix *et al.* (1987) were the first to attempt to isolate endophytic hyphae of a downy mildew pathogen by enzymic maceration working with *Bremia lactuca* infected lettuce leaves. They found that the enzymes affected the hyphal cell wall.

Clark and Spencer-Phillips (1990) and Clark (1989) used Macerozyme R10 in sodium citrate buffer at pH 5.5 and 37°C to macerate *P. viciae* infected leaves, together with a mild treatment with sodium chlorite to render the tissue susceptible to enzymic maceration. Unfortunately plant cells and fungal hyphae were killed by this method. However, it did enable insoluble carbon accumulated by *P. viciae* hyphae to be quantified following treatment of infected leaves with radiolabelled CO_2 and sugars (Clark and Spencer-Phillips, 1993).

Enzymic maceration has also been used to isolate downy mildew hyphae in a viable condition (Ashton and Spencer-Phillips, 1993; Ashton, 1994; El-Gariani 2003). These

experiments used a purer form of Macerozyme, R200, in phosphate buffered saline medium at pH 5.8 and 12°C. This macerated infected leaflets by breaking down the pectic middle lamellae of the host cells, but without appearing to degrade further the walls of either plant cells or *P. viciae* hyphae too extensively.

P. viciae exhibits growth in which the width of hyphae is relatively constant when growing in either palisade or spongy mesophyll tissue. Occasionally, however, the width can vary by about five-fold (Clark, 1989) with the tubular wall expanded to fill the intercellular space (as in Fig. 3). Fraymouth (1956) has recorded this for several downy mildew species, some of which did not appear to form many haustoria. In the present study it was necessary to examine the changes on the hyphal morphology.

Elimination of external and internal contamination

Prior to isolating *P. viciae* hyphae, it was necessary to sterilize the leaves to eliminate other microbial growth associated with these leaves. Sodium hypochlorite, ethanol and sterile distilled water (SDW) were used in a surface sterilization regime to suppress at least epiphytic microorganisms. Other workers (Street *et al.*, 1986; Clark and Spencer-Phillips, 1990) have used similar treatments.

In the experiments with (El-Gariani 2003), treatment of infected leaflets with 40 % ethanol for 30 s, 1 % sodium hypochlorite for 30 s and three times with SDW did not affect *P. viciae* hyphal but strongly suppressed other microorganisms. And also different antibiotics were used to eliminate bacterial growth.

Van Der Gaag and Frinking (1996) found that these antibiotics completely suppressed bacterial growth and did not affect oospore germination of *P. viciae*.

Effect of Benlate on the morphology of *P. viciae* hyphae

As well as bacterial contamination, a fungus often also contaminated samples of macerated leaflets. This study has demonstrated that Benlate applied to pea plants at 0.2 % w/v did not control the contaminant fungus *Cladosporium cladosporiodes*. This result agreed with those of Ashton (1994).

Benlate at this concentration also affected hyphae of *P. viciae* by causing a change in hyphal morphology. These effects were observed in *P. viciae* even at 10 d after inoculation of plants with conidia treated with Benlate. The development of septa was associated with an altered growth form and segmentation of the hyphae into thick-

walled chlamydospore-like structures. Other workers found that fungicides applied to *Phytophthora* spp. induced similar phenotypic and morphological changes but did not suppress fungal growth (Kuhn *et al.,* 1991; Groves and Ristaino, 2000; Staples, 2001b). However, they did report that the form of development induced by Benlate included enhanced or altered patterns of branching at the tip. The lack of haustoria following Benlate treatment further supports the view that downy mildew fungi do not need to produce haustoria for successful colonization of plant tissues (Spencer-Phillips, 1997).

These septate hyphae may be more robust and have higher viability than normal hyphae, and merit further investigation for use in other experiments.

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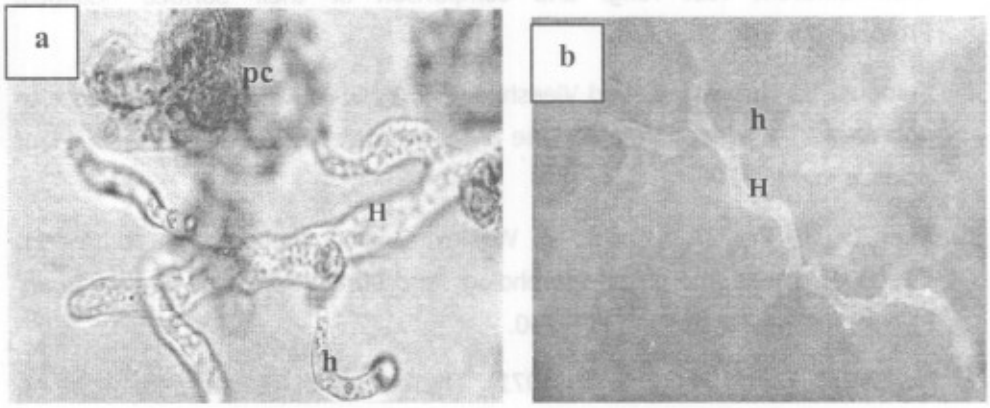

Figure 1. Non fungicide treatment, hyphae of *P. viciae* isolated by maceration method as in (a) and within spongy mesophyll of *P. sativum* 4 d post-inoculation as in (b). Hypha (H), haustoria (h) and plant cell (pc). Hypha in normal branching following inoculation with conidia suspended in SDW.

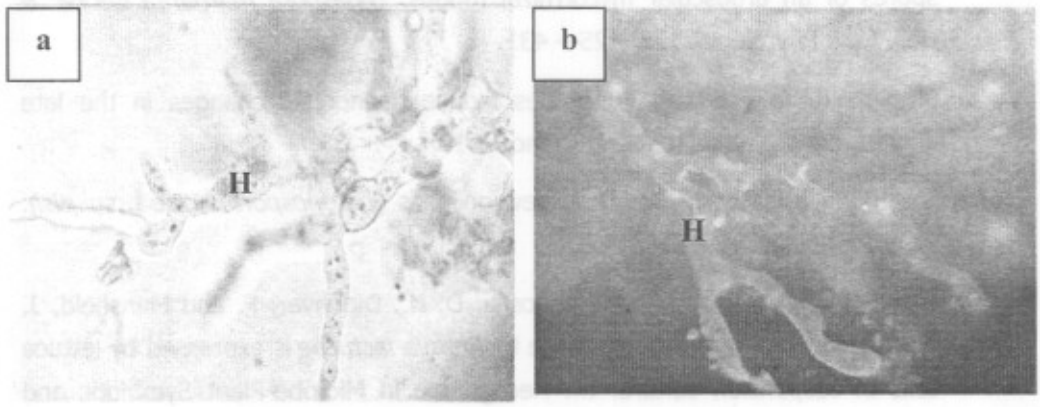

Figure 2. Hyphae of *P. viciae* isolated by maceration method as in (a) and within spongy mesophyll of *P. sativum* 4 d post-inoculation as in (b). Hypha (H),. Hyphae resulting from conidial suspension amended with 0.2 % w/v of Benlate before inoculation of plants. in normal branching following inoculation with conidia suspended in SDW.