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# SCREENING OF POLISH, OTHER EUROPEAN AND TROPICAL ISOLATES OF *VERTICILLIUM CHLAMYDOSPORIUM* TO ASSESS THEIR POTENTIAL AS BIOLOGICAL CONTROL AGENTS OF ROOT-KNOT NEMATODES

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Abstract. Three Polish, four other European and three tropical isolates of the nematophagous fungus *Verticillium chlamydosporium* were screened for their ability to colonise the surface of barley roots, produce chlamydospores and infect eggs of *Meloidogyne incognita* in laboratory tests. PCR-fingerprinting of different Polish isolates and Vc10 was used to detect differences between isolates. Among the European isolates, the Polish isolate which came from a field fertilised with manure produced the greatest number of viable chlamydospores, the largest proportion of infected eggs of *Meloidogyne incognita* and the most prolific root colonisation. Among the tropical isolates, an isolate A produced the largest number of chlamydospores and an isolate B was the most prolific root coloniser and parasitised most of *M. incognita* eggs.

Key words: nematophagous fungus, *Verticillium chlamydosporium*, root-knot nematode, *Meloidogyne incognita*

## I. INTRODUCTION

*Verticillium chlamydosporium* is widespread in soils and is known as a facultative parasite of eggs of cyst and root-knot nematodes (Bourne et al. 1999; De Leij and Kerry 1991; Kerry et al. 1984; Kerry et al. 1993; Stirling 1991). In Poland, this species of fungus plays an important role in population control of the sugar beet nematode (*Heterodera schachtii*) in fields fertilised with straw (Sosnowska 1996; Sosnowska and Banaszak 2000).

There are large differences between isolates of this fungus in terms of virulence and growth (Kerry et al. 1986), and for these reasons the fungus must be carefully screened to select isolates which have the greatest potential for introduction to the soil for the control of nematodes.

Three criteria are considered essential for selection of isolates: colonisation of the rhizosphere, chlamydospore production and egg parasitism. The fungus must be able to colonise the rhizosphere and in screening on the basis of this, it may be possible to eliminate about 50% of the isolates before further screening (Bourne et al. 1994). *V. chlamydosporium* does not cause lesions or affect root growth but appears to proliferate in the rhizosphere (De Leij and Kerry 1991). However, isolates of the fungus differ in their ability to spread on roots and the extent of colonisation differs between plant species (Bourne et al. 1994). Chlamydospores are the preferred type of inoculum and screening for chlamydospore production may eliminate

a further 15% of the test isolates. After screening for egg parasitism, about 10% of the original test isolates remain for further selection in tests in soil. Fungal egg parasites may provide biological control against an important nematode pests such as *Meloidogyne incognita*. In Poland this nematode is a quarantine pest and in glasshouses infects a wide range of vegetable plants, causing root galling and significant yield losses (Brzeski 1998). Control has been based on the use of broad spectrum chemicals such as Vydate (a.i. oxamyl), Temik (a.i. aldikarb), and methyl bromide, which may be hazardous to the environment. Nematophagous fungi that can disrupt nematode egg development are promising biological control agents which are environmentally safe compared to nematicides. Screening of different isolates of *V. chlamydosporium* helps to select these with the greatest potential for control of this pest.

Variations between isolates of the fungus can be detected by rapid and sensitive molecular methods, which have been reported by Arora et al. (1996). PCR-based assays were performed to resolve the genetic variation between different isolates of *V. chlamydosporium* using primers matching enterobacterial repetitive intergenic consensus (ERIC) sequences, which may be considered a rapid tool for the genetic characterisation and detection of different isolates of the fungus.

The purpose of the current investigation was to screen three Polish, four other European and three tropical isolates of *V. chlamydosporium* using the three selection criteria outlined to assess their potential as biological control agents and to use molecular methods to identify differences between Polish isolates of the fungus and Vc10 (The standard isolate used at IACR – Rothamsted).

## II. MATERIALS AND METHODS

Trials were conducted in the Entomology and Nematology Department, IACR-Rothamsted in England. Three Polish isolates of *V. chlamydosporium* were isolated from eggs of *Heterodera schachtii* in Poland, from fields of sugar beet fertilised with cattle manure, straw or mustard (Vc1, Vc2, Vc3). Four other European (C, E, F and D) and three tropical isolates (Vc10, B and A) of the fungus from the Rothamsted collection were included in the trials. The Polish isolates were grown on Potato Dextrose Agar and the other isolates have been stored by freeze-drying. The isolate F used in these experiments was collected from infected eggs of *M. incognita* on outdoor pumpkin, the isolate E was collected from root-knot nematode (*Meloidogyne* sp.) infested soil around orange trees, the isolates C and D were collected from soils. The tropical isolate B was collected from soil and isolate Vc10 was originally collected from infected root-knot nematode eggs (*Meloidogyne* sp.).

### Experiment 1: Screening for chlamydospore production

A sand-barley medium was used, for which 30 ml of milled barley was washed on a 53 μm sieve and then after drying was mixed with an equal volume of coarse sand. The medium was left to partially air dry, put into a 250 ml flask and autoclaved. When cool, the

flasks were shaken to loosen the mixture and were inoculated with 5 x 5 mm plugs of different isolates of fungus growing on Corn Meal Agar. Flasks were incubated at 25°C, and were not shaken at this stage, to allow the fungus to grow off the agar plugs for 2-3 days. The inoculum was then dispersed throughout the medium by vigorous shaking. The flasks were incubated for a total of three weeks. After this period the medium was thoroughly mixed using a spatula, a 1 g subsample was removed and suspended in 9 mls of water agar solution (0.05%). The numbers of chlamydospores were counted in a haemocytometer and chlamydospore production $g^{-1}$ medium calculated. The percentage of germinating chlamydospores was estimated on sorbose agar (12 g technical agar, 2 g sorbose in 1 l distilled water) + antibiotics (streptomycin sulphate, chloramphenicol, chlortetracycline: 50 mg of each per litre of distilled water). There were three replicate flasks for each isolate.

#### Experiment 2: Screening for egg parasitism

Different isolates of the fungus growing on Corn Meal Agar were washed in 2-5 mls of sterile distilled water and 0.2 mls of fungal suspension spread onto a Petri dish (9 cm diameter) of 2% water agar + antibiotics (streptomycin sulphate, chloramphenicol, chlortetracycline: 50 mg of each per 1 litre of distilled water). The Petri dishes were incubated at 25°C for 2 days. Roots of plants infected with root-knot nematode (*M. incognita*) were washed. Several hundred egg masses were hand picked and crushed using a cyst crusher and glass rod. About 200 root-knot nematode eggs were added to each plate and spread around the plate colonised by the fungus. The Petri dishes were incubated at 25°C for 3 days and then the number of parasitised eggs was counted. The number of eggs that hatched during the 3 days of the bioassay was consistent on all plates. Controls were Petri dishes with eggs of root-knot nematode without the fungus.

#### Experiment 3: Rhizosphere colonisation in sterile conditions

Barley seeds were surface sterilised in 7% calcium hypochlorite for 45 minutes on a wrist action shaker and then washed 4-5 times in sterile distilled water and transferred to a seed germinating medium (12 g technical agar, 10 g glucose, 0.1 g peptone and 0.1 g yeast extract in 1 litre distilled water) to detect any contaminants remaining. Seeds were incubated at 25°C for 2 days. Glass tubes (15 x 2.5 cm) were filled to about 2/3 volume with vermiculite, moistened, stoppered with cotton wool and autoclaved. When cool, 4 × 2 mm plugs of fungus on Corn Meal Agar were put below the surface of the vermiculite. Germinated sterile seedlings were placed on top of the plugs so that the roots grew down between the plugs. Tubes were incubated for three weeks at 25°C in a 12 hr light regime. After 3 weeks the roots were cut into 1 cm portions in sterile conditions and placed on water agar. The numbers of colonised root segments were counted after 3 days incubation at 25°C (Bourne et al. 1994). Controls were the roots without fungus plugs.

Analysis of variance was used to determine statistically significant differences.

**Experiment 4: Typing and identification of fungal isolates of *V. chlamydosporium* by PCR**

– fungal growth and DNA extraction

Isolates of *V. chlamydosporium* were grown in Czapek Dox broth medium at room temperature in an orbital shaker at 95 rpm for 2 weeks. The fungal mycelium was separated from the broth using a filter (Whatman 90 mm diameter) and air dried. DNA was extracted from a small amount (approximately 1 g) of fungal material by boiling in 40 µl 0.25 M NaOH for 30 s. Samples were neutralised by addition of 40 µl 0.25 M HCl and 20 µl 0.5 M Tris-HCl, 0.25% (w/v) Igepal (Sigma). Samples were boiled for a further 2 mins, and kept on ice prior to PCR.

– polymerase chain reaction

PCR was performed with two sets of primers. Firstly, ERIC primers which are useful for the typing of fungal isolates were used as described by Arora et al. (1996). Secondly, primers for the positive identification of isolates as *V. chlamydosporium*, which are based on its beta-tubulin gene, were used as previously described by Hirsch et al. (2000). PCR products were separated on 3% agarose gels (Helena Bioscienes) in 0.5% TBE buffer, stained with ethidium bromide (0.5 µg $ml^{-1}$ in $H_2O$) and digitised using an EagleEye® II sytem (Stratagene). The 123 bp DNA ladder (Life Technologies Ltd, U.K.) was used as a size marker.

## III. RESULTS AND DISCUSSION

**Experiment 1: Chlamydospore production**

Significantly greater numbers of chlamydospores were produced by the tropical isolate A (Tab. 1), 1.7 times more chlamydospores than the isolate D and 1.7 times more then Vc10. Among the three Polish isolates, the isolate from the field fertilised with manure (Vc1) produced the greatest number of chlamydospores (2 x $10^6$ $g^{-1}$ medium), second was the isolate

Table 1

**Production and viability of chlamydospores of different isolates of *V. chlamydosporium***

| Isolates | Chlamydospores produced × $10^6$ $g^{-1}$ medium | Chlamydospore germination (%) | Number of viable chlamydospores × $10^6$ $g^{-1}$ medium |
|---|---|---|---|
| A | 5 | 88 | 4 |
| Vc1 | 2 | 87 | 2 |
| Vc2 | 1 | 25 | 0.3 |
| Vc3 | 0.8 | 8 | 0.06 |
| Vc 10 | 3 | 10 | 0.3 |
| B | 0.07 | – | – |
| C | 1 | 77 | 1 |
| D | 3 | 6 | 0.2 |
| E | 0 | 0 | 0 |
| F | 0 | 0 | 0 |
| | P<0.001 SED=0.728 | P<0.001 SED=3.26 | P<0.001 SED=0.261 |

from the field fertilised with straw (Vc2) (1 x $10^6$ $g^{-1}$ medium) and the smallest amount of spores was produced by the isolate from the field fertilised with mustard (Vc3) (0.8 x $10^6$ $g^{-1}$ medium) (Tab. 1). Isolates E and F did not produce any chlamydospores on this medium, and the isolate B produced only 7 x $10^4$.

Although the greater numbers of chlamydospores were produced by isolates A, D and Vc10 the greatest chlamydospore viability observed was with isolate A, the Polish isolate from the manure field (Vc1) and isolate C. There were significant differences in the numbers of viable chlamydospores between these three isolates.

Isolates that produce the greatest number of viable chlamydospores are more suitable for application, because a larger area of soil may be treated for a given amount of medium. Chlamydospores are the most convenient form of inoculum and have provided consistent control of nematode multiplication in a range of laboratory tests and small plot trials (De Leij et al. 1993). If the fungus is to be applied without a nutrient source it must be applied as a chlamydospore inoculum to ensure establishment in the soil and rhizosphere (Kerry et al. 1993). For this reason, production of viable chlamydospores is important in the use of this fungus for biological control of nematodes.

The three isolates that produced the greatest number of viable chlamydospores (A, Vc1 and C) were selected, and the other *V. chlamydosporium* isolates were rejected on this criterion alone.

#### Experiment 2: Egg parasitism

All isolates of *V. chlamydosporium* tested were able to infect eggs of *M. incognita*. There were no significant differences in the numbers of eggs parasitised by the seven isolates: A, Vc1, Vc2 and Vc3, Vc10, B and C. All these isolates infected more then 60% of eggs of *M. incognita* (Tab. 2). Isolates D, E and F parasitised significantly fewer eggs and they could be rejected on the basis of this criterion in the screening.

Among the Polish isolates, all three were able to infect *M. incognita* eggs, which is promising, because they were isolated from eggs of *H. schachtii* (Sosnowska 1996). The Polish isolate occurring in the field fertilised with manure (Vc1) was selected for control of *M. incognita* in glasshouses in Poland as it produced relatively large numbers of viable chlamydospores and effectively parasitised eggs.

Table 2

**Infection of eggs of *M. incognita* by different isolates of *V. chlamydosporium***

| Isolates | % of parasitised eggs |
|---|---|
| A | 74 |
| Vc1 | 77 |
| Vc2 | 68 |
| Vc3 | 76 |
| Vc10 | 64 |
| B | 78 |
| C | 67 |
| D | 50 |
| E | 52 |
| F | 55 |
| Control | 5 |
| | P<0.001; SED=6.52 |

#### Experiment 3: Rhizosphere colonisation

This test detected only the presence or absence of the fungus on the root surface. All isolates colonised the barley rhizosphere. The most prolific root colo-

Table 3

**Extent of rhizosphere colonisation of barley roots by different isolates of *V. chlamydosporiuum***

| Isolates | Colonisation of root segments (%) |
|---|---|
| A | 82 |
| Vc1 | 79 |
| Vc2 | 81 |
| Vc3 | 56 |
| Vc10 | 49 |
| B | 84 |
| C | 55 |
| D | 72 |
| E | 68 |
| F | 78 |
| Control | 0 |
| (excluding control) | n.s. |

nisers were the isolates B, A and Vc2, which colonised more than 80% of the root segments. The Polish isolate Vc1 colonised 79% of the root segments. Isolates D and F colonised more then 70% of root segments; the isolate E more then 68%; isolates C and Vc3 colonised more then 55% of root segments, and isolate Vc10 colonised only 49% of root segments. There were no statistically significant differences in the results (Tab. 3).

In practice, isolates that colonised <80% of the root segment are generally not effective colonisers of the rhizosphere in tests in field soil (Kerry, unpubl.). From the literature it is known that root colonisation by *V. chlamydosporium* increases after infection by root-knot nematodes possibly due to changes in the release of root exudates (Bourne et al. 1996). In addition, root colonisation by *V. chlamydosporium* has been shown to vary among different plant species (Bourne et al. 1994). Isolates selected by laboratory screening may perform less well in field experiments, but screening helps to select the isolate which has potential as a biological control agent. Isolates that proliferate on the root surface without causing lesions or affecting root growth are important because they are likely to be more able to parasitise sufficient numbers of nematode eggs, and so limit the multiplication of root-knot populations, than those that are poor colonisers of the rhizosphere (De Leij and Kerry 1991).

### Experiment 4: Typing and identification of fungal isolates as *V. chlamydosporium* by PCR

The β tubulin PCR analysis of Polish isolates confirmed that they are all *V. chlamydosporium* as each gave a single PCR product of the appropriate size. However, these isolates had different fingerprint profiles with ERIC-PCR indicating genetic differences. ERIC-PCR fingerprinting may be useful for discriminating or identifying these isolates in future experiments.

## IV. CONCLUSIONS

1. The Polish strain of *V. chlamydosporium* isolated from eggs of *H. schachtii* in a field fertilised with manure showed potential as a biological control agent for the control of the root-knot nematode *M. incognita*. Compared with other European isolates this isolate produced the greatest number of viable chlamydospores on sand-barley medium, infected a greater number eggs of *M. incognita* and was a more prolific root coloniser than the other isolates.

2. Among the tropical isolates, the tropical isolate A showed greatest potential as a biological control agent for control of root-knot nematodes.
3. Substantial polymorphism between Polish *V. chlamydosporium* isolates was detected using ERIC PCR.

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# TESTOWANIE POLSKICH, INNYCH EUROPEJSKICH I TROPIKALNYCH IZOLATÓW *VERTICILLIUM CHLAMYDOSPORIUM* W CELU OKREŚLENIA ICH POTENCJAŁU JAKO CZYNNIKÓW BIOLOGICZNEGO ZWALCZANIA GUZAKÓW (*MELOIDOGYNE* SPP.)

STRESZCZENIE

Testy przeprowadzono w Zakładzie Entomologii i Nematologii IACR-Rothamsted w Anglii. Badano trzy polskie szczepy nicieniobójczego grzyba *Verticillium chlamydosporium* izolowanego z jaj mątwika burakowego *Heterodera schachtii* z pól z różnym nawożeniem organicznym. Porównywano je z innymi europejskimi i tropikalnymi szczepami grzyba pod względem produkcji chlamydospor, infekowania jaj guzaka *Meloidogyne incognita* oraz kolonizacji korzeni jęczmienia. Te trzy kryteria selekcji szczepów pozwoliły wybrać najlepszy szczep do zwalczania nicieni w praktyce.

Wśród badanych 10 izolatów grzyba najlepszym był szczep tropikalny A. Wśród izolatów europejskich jedynie polski szczep pochodzący z populacji mątwika burakowego z pola nawożonego obornikiem może być brany pod uwagę w praktyce w biologicznym zwalczaniu guzaków. Testowane trzy kryteria selekcji pozwoliły wyeliminować ponad 60% izolatów grzyba *V. chlamydosporium*. Zastosowana metoda oceny polskich izolatów na poziomie molekularnym stosując test PCR wykazała wyraźne różnice pomiędzy badanymi izolatami grzyba.