J. Univ. Kuwait (Sci.) **15**, 1988

Penicillium stipitatum **and** ***Trichoderma harzianum*** **in the biological control of cucumber damping-off disease caused by** ***Pythium aphanidermatum***

FAYADH M. SHARIF, A. M. OKASHA AND K. T. KAZEM

Laboratory of Plant Pathology, Department of Plant Production, Agriculture and Water Resources Research Centre, Council of Scientific Research, P.O. Box 2416, Baghdad, Iraq

ABSTRACT

Biological control of cucumber damping-off seedlings caused by *Pythium aphanidermatum* has been achieved by the fungi *Penicillium stipitatum* (anamorph of *Talaromyces stipitatus*) and *Trichoderma harzianum*. Efficiency of the biological control was equivalent to that obtained by the application of the fungicide ridomil. *T. viride* and *Aspergillus flavus* were ineffective in the biological control of the disease. *In vitro* antagonism of these fungi against the pathogen is discussed. A new nutrient medium, potato–egg yolk–sucrose–agar was formulated which induces intensive oospore production by *Pythium aphanidermatum*.

INTRODUCTION

Pythium species attack a wide range of host plants, causing damping-off of seedlings and rot of storage organs. Being soil inhabitants, they are difficult to control. The use of fungicides for controlling plant pathogens is not always effective and may cause environmental pollution and occasional phytotoxicity (Lifshitz *et al.* 1984). Therefore, alternative control measures become necessary.

Biological control of *Pythium* diseases has been attempted with antagonistic bacteria (Howell & Stipanovic 1980; Becker & Cook 1984; Weller & Graham 1984) and fungi such as *Gliocladium virens* (Howell 1982), *Trichoderma hamatum* (Harman *et al.* 1980; Sivan *et al.* 1984), and *Penicillium oxalicum* (Carol 1981).

The purpose of this study is to evaluate the biocontrol potential of the following fungi: *Penicillium stipitatum* Thom (valid name *Penicillium* anamorph of *Talaromyces stipitatus* C.R. Benjamin), *Aspergillus flavus* Link, new isolates of *Trichoderma harzianum* Rifai and *T. viride* Rifai against damping-off of cucumber seedlings caused by *Pythium aphanidermatum* (Edson) Fitz.

A high yield of oospore concentration is required for successful artificial inoculation with *Pythium* spp., but high yields of oospores are not easily attainable on conventional media. Ayers & Lumsden (1975) obtained high yields of oospores of three *Pythium* spp. by adding cholesterol to V8-broth. Many natural products contain cholesterol, the highest percentage being in egg yolk (Henrietta 1976). Since

egg yolk is a cheap common product, we tested the possibility of substitution of cholesterol by egg yolk in order to formulate a simple nutrient medium that induces oospore production.

MATERIAL AND METHODS

Pythium aphanidermatum was isolated from diseased cucumber seedlings grown in a plastic green house. A stock culture of the fungus was kept in the refrigerator on slants of potato–sucrose–agar (PSA).

Preliminary tests showed that this isolate is pathogenic to cucumber, causing pre- and post-emergence damping-off.

To obtain high yield of oospores, dry egg yolk from boiled eggs was added to PSA in proportions of 0, 0·5, 1, 1·5, 2, 2·5, 3, 3·5 and 4% (w/v). In another experiment sucrose was added to potato–egg yolk–agar (PEYA: 20, 2, 2%) in concentrations of 0, 0·5, 1, 1·5, 2, 2·5, 3, 3·5 and 4% (w/v). One hundred ml portions of each medium were autoclaved at 121°C for 20 min. Penicillin and streptomycin were added to reach a final concentration of 50 ppm each before solidification of media. Inoculation of media was made by transferring a mycelial disc (5 mm diameter) from a 3-day-old colony of *P. aphanidermatum*. Incubation was made at 30°C for 14 days. The mycelial mat with the underlying surface medium was homogenized in 100 ml water with the aid of a blender at a high speed for 1 min. Oospores concentration was estimated using a haemocytometer and yield per Petri dish calculated. Five replicates per treatment were used.

The putative biocontrol agents used were: *Penicillium stipitatum*, *Aspergillus flavus*, *Trichoderma harzianum* (isolates Th-1, Th-2, Th-p) and *T. viride* (isolates Tv-1, Tv-2). All of them had been isolated from soil except isolate Th-p of *T. harzianum* which had been isolated from a culture of the pathogen. The inoculum of these biocontrol agents was prepared by inoculating 200 g portions of moistened barley grains (1:1 grains:water w/v) in 1 l Erlenmyer flasks with a dense conidial suspension of the biocontrol fungus from 10-day-old cultures on PSA slants. Inoculated flasks were incubated at 25°C for 10 days, then cultures were air-dried for 24 h, milled and kept in the refrigerator for use.

Fifty ml of oospore suspension containing 7×10^5 oospore/ml of the pathogen were added to 500 g of unsterilized sandy loam soil in plastic pots (9 cm diameter). Equivalent quantities of water were added to pots to serve as un-inoculated controls. Pots were covered with nylon bags and kept for 7 days before adding the biocontrol inoculum. Five grams inoculum of the biocontrol agent per pot were mixed thoroughly with the soil and returned to the pot. Equal quantities of autoclaved un-inoculated barley grains were added to the controls. A set of pathogen-infested pots were treated with 50 ml of ridomil (DL-methyl N-(2,6-dimethylphenyl)-N-(2-methoxy-acetyl) alaninate) suspension (1500 ppm) and these served as the fungicide treatment. Soil moisture was brought to about half its field capacity for all pots. Pots were then covered with nylon bags. Twenty-five seeds of cucumber (*Cucumis sativus* L. cv Beit Alpha) were sown 14 days after treatment with the biocontrol agents. Four replicates per treatment were used and the experiment was repeated twice. Pre- and post-emergence damping-off of cucumber seedlings was recorded and the pathogen aseptically isolated from ungerminated seeds and diseased seedlings on PSA plates.

Antagonism (*in vitro*) of the biocontrol fungi against the pathogen was tested by

inoculating PSA plates with a mycelial disc (5 mm diameter) of the antagonist and of the pathogen, 3 cm apart. Since the pathogen is a very fast growing fungus, its mycelial disc was placed 24 h after that of the antagonist. After 5 or 10 days of incubation at 28°C the class of antagonism in each combination was evaluated according to a 5-point scale described by Bell *et al.* (1982) (class 1, the antagonist completely overgrows the pathogen and covers the entire medium surface; class 2, the antagonist overgrows at least two-thirds of the medium surface; class 3, the antagonist and the pathogen each colonizes approximately one-half of the medium surface and neither organism appears to dominate the other; class 4, the pathogen colonizes at least two-thirds of the medium surface; and class 5, the pathogen completely overgrows the antagonist and occupies the entire medium surface). The fungus is considered highly antagonistic to the pathogen if it gives a mean score of 2 or less. Five replicates per treatment were used.

In order to test for growth-suppressing metabolites, each of the test fungi was grown on 50 ml of potato–sucrose broth in 250 ml Erlenmyer flasks at 28°C for 14 days. The culture filtrates obtained from these were divided into two portions; one portion was sterilized by autoclaving at 121°C for 15 min, the other was sterilized by passing through a bacterial proof Sartorius membrane filter with a pore size of 0·15 μm. Culture filtrates were added separately to autoclaved, cooled (50°C) PSA in a concentration of 10% (v/v). The media were dispensed in Petri dishes each of which was inoculated with a mycelial disc of the pathogen and incubated at 30°C. The growth pattern was compared after 2 days of incubation. Five replicates per treatment were used. The complete randomized block design was adopted and the results of disease control were subjected to statistical analysis using Duncan's Multiple Range Test.

RESULTS

Fig. 1A shows that egg yolk added to PSA induced intensive production of oospores by the fungus *Pythium aphanidermatum*. Induction of oospore production was directly related to egg yolk concentration up to 2% where the number of oospores per plate reached $67{\cdot}22 \times 10^6$ within an incubation period of 14 days compared to zero oospore on PSA alone.

Lack of sucrose or its presence in concentrations up to 1·5% in potato–egg yolk–agar still permitted comparatively high yield of oospores ($13{\cdot}23 - 17{\cdot}02 \times 10^6$ oospore/plate). However, 2–4% sucrose gave the maximum yield (Fig. 1B).

Results show that *Penicillium stipitatum* significantly reduced the pre-emergence damping-off of cucumber seedlings (Fig. 2). Disease incidence was statistically equivalent to that shown by the ridomil treatment and by non-infested soil. *Trichoderma harzianum* isolates Th-p, Th-1 and Th-2 also reduced disease incidence to a level statistically comparable to that induced by *Penicillium stipitatum*. However, *Aspergillus flavus* and both isolates, Tv-1 and Tv-2 of *Trichoderma viride* caused no significant reduction in disease incidence. Only *T. harzianum* isolate Th-p and ridomil showed significant reduction in post-emergence damping-off (Fig. 2). The overall effect of the biocontrol fungi revealed that treatment with inoculum of *Penicillium stipitatum* and all isolates of *Trichoderma harzianum* resulted in a significant reduction of the disease (pre- and post-emergence) which is equivalent to the fungicide treatment or non-infested soil. On the other hand, *Aspergillus flavus* and *Trichoderma viride*

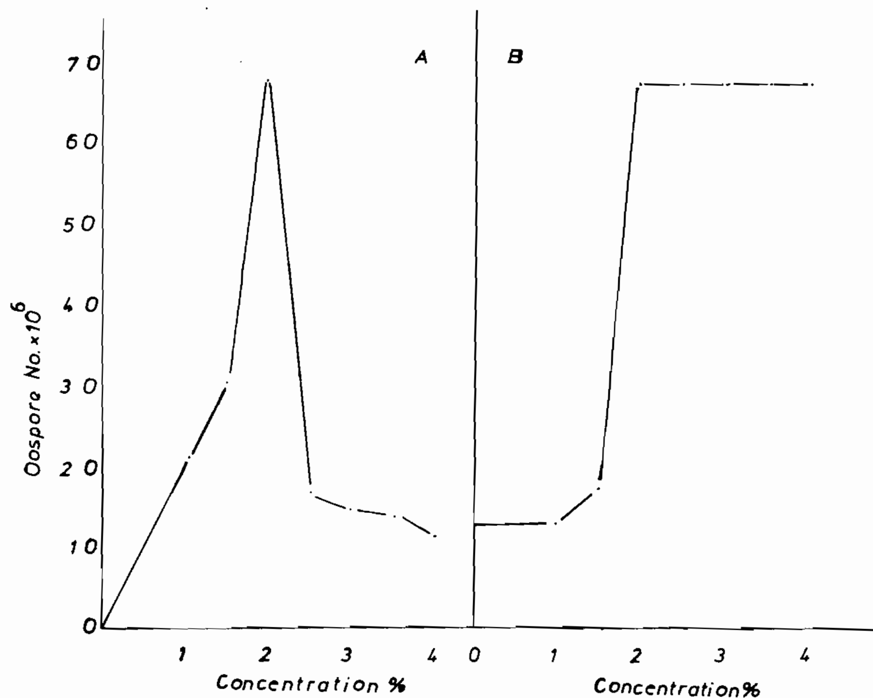

Fig. 1. Effect of egg yolk in potato–sucrose–agar medium (A) and sucrose in potato–egg yolk–agar medium (B) on oospore production by *Pythium aphanidermatum*.

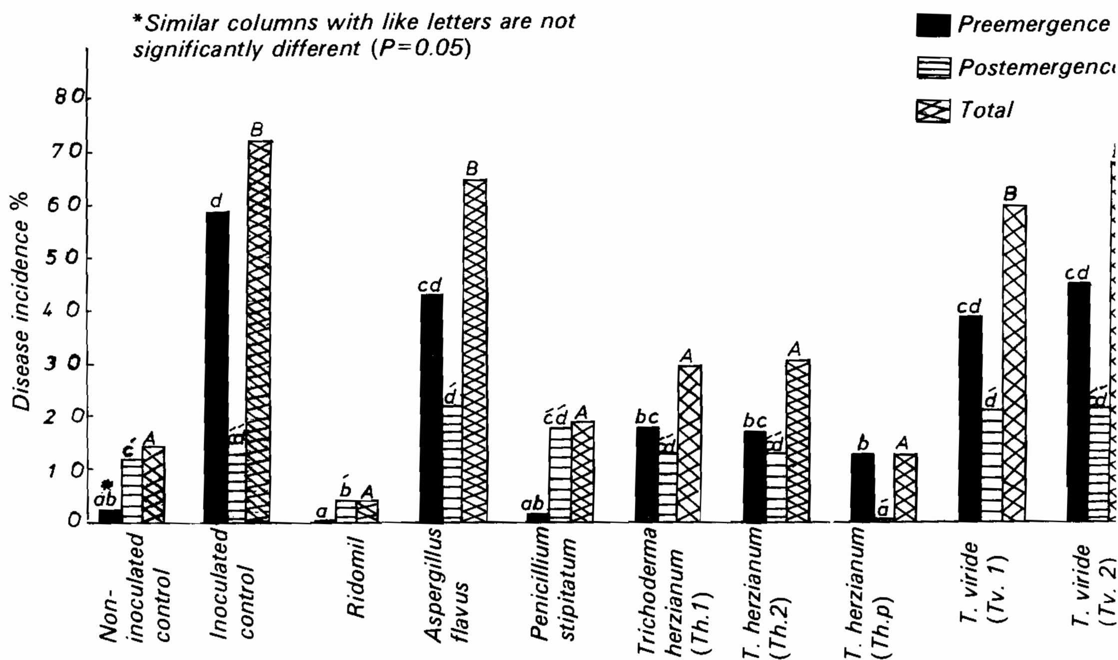

Fig. 2. Effect of biocontrol agents and ridomil on damping-off of cucumber seedlings induced by *Pythium aphanidermatum* 14 days after sowing.

Table 1. Class of antagonism (*in vitro*) of the biocontrol agents against *Pythium aphanidermatum* on potato–sucrose–agar at 28°C

Biocontrol agent	Class of antagonism (1–5)*	
	After 5 days	After 10 days
Aspergillus flavus	2	1
Penicillium stipitatum	4	2
Trichoderma harzianum (Th-1)	2·2	1
T. harzianum (Th-2)	1	1
T. harzianum (Th-p)	1·6	1·4
T. viride (Tv-1)	2·4	1
T. viride (Tv-2)	3·8	3·6

* The antagonist growing over the whole plate, 1; over >67% of the plate, 2; over 50% of the plate, 3; the pathogen growing over >67% of the plate, 4; over the whole plate, 5.

Table 2. Fungitoxicity of the culture filtrates of the biocontrol agents against the growth of *Pythium aphanidermatum* two days after inoculation

Biocontrol agent	Fungitoxicity*	
	Autoclaved culture filtrate	Filter-sterilized culture filtrate
Aspergillus flavus	0	+
Penicillium stipitatum	0	+ + +
Trichoderma harzianum (Th-1)	0	+ +
T. harzianum (Th-2)	0	+ + +
T. harzianum (Th-p)	0	+ + +
T. viride (Tv-1)	0	+ +
T. viride (Tv-2)	0	+ + +
Control	0	0

* Growth strongly affected, + + +; moderately affected, + +; weakly affected, +; growth not affected, 0.

proved to be ineffective in disease control. The pathogen *Pythium aphanidermatum* has been isolated from almost all non-germinated seeds or diseased seedlings.

Results of the evaluation of the class of antagonism shown by the test fungi against the pathogen are presented in Table 1. It appears that after 5 days of incubation, *Trichoderma harzianum* isolates Th-2 and Th-p and *Aspergillus flavus* showed a class of antagonism of 2 or less which is considered to be a highly antagonistic reaction. After 10 days, however, all test fungi except *Trichoderma viride* isolate Tv-2 exhibited similar antagonistic reactions. There was no evidence of inhibition (antibiosis). However, hyphae of the antagonists were progressively growing over those of the pathogen.

All the fungi tested produced fungitoxic metabolite(s) in liquid medium that variably affected growth of the pathogen (Table 2). The inhibitory metabolite(s) were destroyed by autoclaving. *Penicillium stipitatum*, *Trichoderma harzianum* isolates Th-2, Th-p and isolate Tv-1 of *T. viride* produced metabolites that caused thin, restricted growth of the pathogen. However, this effect declined with time.

DISCUSSION

Results presented in this study revealed that the addition of egg yolk to PSA induced abundant production of oospores by *Pythium aphanidermatum*. The yield of oospores recovered from potato–egg yolk–sucrose–agar was about seven times the maximum yield obtained by Ayers & Lumsden (1975) on V8-broth to which 30 mg/l cholesterol were added. It seems that the high percentage of cholesterol (225 mg/egg) present in egg yolk (Henrietta 1976) meets the sterol requirement of the fungus for oospore production.

Despite the lack of exogenous sugar in potato–egg yolk–agar, a high yield of oospores was obtained, but the addition of 2–4% sucrose gave a peak yield. So a new nutrient medium, potato–egg yolk–sucrose–agar, composed of 200 g potato, 20 g egg yolk, 20 g sucrose, 20 g agar, 50 mg penicillin, 50 mg streptomycin and 1000 ml distilled water, proved to be adequate for maximum production of oospores by *P. aphanidermatum*. Further tests may show a similar response in other *Pythium* spp.

Results show for the first time that the fungus *Penicillium stipitatum* is an effective biocontrol agent equivalent to ridomil application or to isolates of *Trichoderma harzianum* (Th-p, Th-1 and Th-2), in controlling damping-off of cucumber seedlings caused by *Pythium aphanidermatum*. However, *Penicillium stipitatum* and *Trichoderma harzianum* (isolates Th-1 and Th-2) failed to control post-emergence damping-off. Liu & Vaughan (1965) reported that *T. viride* or *Penicillium frequentans* reduced pre- but not post-emergence damping-off of table beet caused by *Pythium* infection. Similarly, *Gliocladium virens* reduced only pre-emergence damping-off induced by *Pythium ultimum* (Howell 1982).

Failure to control post-emergence damping-off might be due to inoculum decline of the biocontrol agents in the soil. This could be overcome by manipulating media and inoculation technique (Lewis & Papavizas 1984). On the other hand, *Aspergillus flavus* and *Trichoderma viride* (Tv-1 and Tv-2) were shown to be ineffective in disease control, although the first two organisms showed a powerful *in vitro* antagonistic activity against the pathogen.

Results of *in vitro* antagonism reveal that *P. stipitatum* and *T. harzianum* (Th-1) attained the second and the first class respectively, i.e. highly antagonistic activity, according to Bell *et al.* (1982) scale after 10 days rather than 5 days of incubation. However, they showed efficient antagonism *in vivo*. This suggests that evaluation of antagonism interaction over a period of incubation of 10 days could be considered.

Although *P. stipitatum* and *T. harzianum* produced antifungal metabolite(s) in culture media (Table 2), this was not sufficient to explain the mode of antagonism towards the pathogen, since that activity declined with time and no antibiosis was observed on solid media.

Biotrophism seems to be involved in the antagonistic activity of these biocontrol fungi, since their hyphae were growing over those of the pathogen leading to restriction of their growth. Previous workers reported that *Trichoderma* spp. could affect *Pythium* spp. through mycoparasitism (Harman *et al.* 1980; Elad *et al.* 1982; Lifshitz *et al.* 1986). Accordingly, results of the biological control of *Pythium aphanidermatum* obtained in this work could be explained on the basis of mycoparasitic action of the biocontrol agents on the mycelium of the pathogen.

REFERENCES

Ayers, A.W. & Lumsden, R.D. 1975. Factors affecting production and germination of oospores of three *Pythium* species. Phytopathology **65**: 1094–1100.

Becker, O.J. & Cook, J.R. 1984. *Pythium* control by siderophore-producing bacteria on roots of wheat (Abstr.). Phytopathology **74**: 805.

Bell, D.K., Wells, H.D. & Markham, C.R. 1982. The *in vitro* antagonism of *Trichoderma* species against six fungal plant pathogens. Phytopathology **72**: 379–82.

Carol, E.W. 1981. Growth of *Penicillium oxalicum* as a biological seed treatment on pea seed and soil. Phytopathology **71**: 929–33.

Elad, Y., Chet, I. & Henis, Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Canadian Journal of Microbiology **28**: 719–25.

Harman, G.E., Chet, I. & Baker, R. 1980. *Trichoderma hamatum* effects on seedling disease induced in radish and pea by *Pythium* spp. or *Rhizoctonia solani*. Phytopathology **70**: 1167–72.

Henrietta, F. 1976. Introduction to nutrition. Macmillan, New York, 552 pp.

Howell, C.R. 1982. Effect of *Gliocladium virens* on *Pythium*, *Rhizoctonia solani* and damping-off of cotton seedlings. Phytopathology **72**: 496–98.

Howell, C.R. & Stipanovic, R.D. 1980. Suppression of *Pythium ultimum* induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. Phytopathology **70**: 712–15.

Lewis, J.A. & Papavizas, G.C. 1984. A new approach to stimulate population proliferation of *Trichoderma* species and other potential biocontrol fungi introduced into natural soil. Phytopathology **74**: 1240–44.

Lifshitz, R., Sneh, B. & Baker, R. 1984. Soil suppression to a plant pathogenic *Pythium* species. Phytopathology **74**: 1054–61.

Lifshitz, R., Windham, M.T. & Baker, R. 1986. Mechanism of biological control of pre-emergence damping-off of pea by seed treatment with *Trichoderma* spp. Phytopathology **76**: 720–25.

Liu, S. & Vaughan, E.K. 1965. Control of *Pythium* infection in table beet seedlings by antagonistic microorganisms. Phytopathology **55**: 986–89.

Sivan, A., Elad, Y. & Chet, I. 1984. Biological control effects of a new isolate of *Trichoderma harzianum* on *Pythium aphanidermatum*. Phytopathology **74**: 498–501.

Weller, D.H. & Graham, M.C. 1984. Application of fluorescent pseudomonads to improve the growth of wheat (Abstr.). Phytopathology **74**: 806.

(Received 2 November 1986, revised 24 March 1987)

استخدام فطر پنسليم ستيپيتاتم وفطر ترايكودرما هارزيانم في المكافحة الحيوية لمرض تسقيط بادرات الخيار الذي يحدثه فطر پيثيم أفانيدرماتم

فياض محمد شريف و أحمد محمد عكاشة و قسمة طالب كاظم
مختبر أمراض النبات ، قسم الانتاج النباتي ، مركز البحوث
الزراعية والموارد المائية ، مجلس البحث العلمي ،
ص . ب . ٢٤١٦ بغداد ، العراق

خلاصة

تم احداث المكافحة الحيوية لمرض تسقيط بادرات الخيار الذي يسببه فطر پيثيم أفانيدرماتم ، وذلك باستخدام كل من فطر پنسليم ستيپيتاتم وعزلات جديدة من فطر ترايكودرما هارزيانم ، وكانت كفاءة المكافحة الحيوية مساوية لكفاءة المبيد ريدوميل . أما الفطران ترايكودرما ڤيريدي وأسپرجلس فلاڤس فقد فشلا في مكافحة المرض .

وقد تمت مناقشة ظاهرة التضاد بين هذه الفطريات والفطر الممرض . كما أمكن التوصل إلى وسط غذائي جديد مكون من البطاطا وصفار البيض والسكروز والأجار يحفز الفطر الممرض على انتاج الأبواغ البيضية بغزارة .