

Experiments on direct isolation of *Pythium* spp. from Finnish sugar beet soils

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Abstract. *Pythium* spp. were successfully isolated directly from sugar beet soils by the method of RICCI et al. (1976). The isolates obtained were divided into four groups on the basis of morphological and growth habit characters. The groups showed large differences in *in vitro* pathogenicity. The importance of some factors affecting the estimation of *Pythium* propagule density is studied and discussed.

Index words: *Pythium*, direct isolation, sugar beet soils

Introduction

Scientists have developed a diversity of techniques for the isolation of *Pythium* spp. from soil. Fundamentally these techniques are based on two principles (BOUHOT 1979): 1. Introduction of baits (living or dead plant fragments) into the soil sample, allowing each propagule of the parasite to manifest its presence by the colonization of the baits. 2. Incorporation of soil samples into an artificial selective medium, allowing each propagule of the parasite to develop into a colony.

Baiting with plant materials has often been

used for studying specific soil fungi causing plant diseases. Materials used for detecting *Pythium* include apple (HENDRIX & CAMPBELL 1970), cucumber (BANIHASHEMI 1970), pineapple (KLEMMER & NAKANO 1962), citrus leaf (GRIMM & ALEXANDER 1970) and avocado (ZENTMYER et al. 1960). ROBERTSON (1975) developed a paper disc technique for the recovery of *Pythium* spp. from soil or water.

For the isolation of *Pythium* spp. directly from soil WARCUP'S (1950) soil-plate method, in which soil is dispersed in agar, has proved superior to the dilution-plate method. RICCI and coworkers (1976) developed the soil-plate method further by transferring soil-agar discs from a primary medium to a secondary isolation medium. SCHMITTHENNER (1962) used a

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soil-particle technique for isolating *Pythium ultimum* Trow. and several other *Pythium* spp. from soil particles. Particles of 1, 3 and 7 mg mean weight gave amounts of *Pythium* comparable to the soil plate technique. ANGELL (1954) found that *Pythium* was present in the larger soil particles that sedimented from a suspension and that bacteria were present in the colloidal part. STANGHELLINI and HANCOCK (1970) found that *P. ultimum* grew out from small drops dispersed on the surface of 3-day-old 2 % water agar and that this method could be quantified by making dilutions.

Numerous selective media have been reported for the isolation of *Pythium* spp. from soil. ROSE bengal (SING & MITCHELL 1961, KERR 1963, VAARTAJA & BUMBIERIS 1964, TAKAHASHI & OZAKI 1965, VAARTAJA 1967, FLOWERS & HENDRIX 1969, MARTIN 1970), gallic acid (FLOWERS & HENDRIX 1969) and fungicides like PCNB (VAARTAJA & BUMBIERIS 1964, TAKAHASHI & OZAKI 1965, RICCI et al. 1976) and benomyl (RICCI et al. 1976) were used in these media before the introduction of the polyene antibiotics (TSAO 1970). The polyene antibiotic pimaricin has been widely used to suppress fungi other than *Pythium* spp. in selective media (ECKERT & TSAO 1960, SING & MITCHELL 1961, HINE & LUNA 1963, MERCETICH & FOGLE 1969). Several other antibiotics, eg. penicillin, polymyxin, endomycin, streptomycin, nystatin and vancomycin are also mentioned for this purpose by TSAO (1970) in his review on selective media for isolating pathogenic fungi.

This paper presents some preliminary experiments on direct isolation of *Pythium* spp. from Finnish sugar beet soils contaminated with the damping-off pathogen *Pythium debaryanum* auct. non Hesse.

Materials and method

Soil

The soil used in the various experiments were from sugar beet fields where damping-

off problems had occurred. There were two very finesandy soils (Mietoinen 60°40'N 21°52'E and Laitila 60°56'N 24°40'E), one clay soil from Salo (60°21'N 23°4'E) and two soils were high in organic matter, a loam from Köyliö (61°6'N 22°20'E) and a peaty soil from Janakkala (60°47'N 24°40'E)

Soil samples were stored in semi-moist conditions at room temperature until use.

Agars and chemicals

Agar media of the following types were used in the experiments:

- Difco corn meal agar
- » potato dextrose agar
- » bacto agar
- Martin's medium (MARTIN 1950) of the following composition: agar 20 g, peptone 5 g, dextrose 10 g, KH₂PO₄ 1 g, MgSO₄ × 7H₂O 0.5 g, Rose bengal 30 mg/l water
- SPB medium (RICCI et al. 1976): agar 25 g, malt 1 g, saccarose 5 g, Ca(NO₃)₂ 1 g, KNO₃ 250 mg, MgSO₄ × 7H₂O 250 mg, KH₂PO₄ 125 mg, citric acid 50 mg, micronutrient solution 1 mg/l water. The micronutrient solution had the following composition: FeSO₄ 1 g/l, ZnSO₄ 1 g/l, CuSO₄ 1 g/l and MnCl₂ 1 g/l water.

Commercial fungicides in the agar media were benomyl in the form of Benlate (Du Pont) as 50 % a.i. and PCNB as Avicol (Kemira) with 50 % a.i.

The antibiotic pimaricin was of Dutch origin, Brocades-Stheeman & Pharmacia.

Suspensions of fungicides and pimaricin were used immediately after preparation or after short storage in a refrigerator at 4°C.

Assessment of *Pythium* spp.

The basis for the isolation of *Pythium* spp. is the WARCUP (1950) soil-plate method as modified by RICCI et al. (1976). In this method small amounts of soil are evenly dispersed in 2.5 % water agar at 40—42°C. Citric acid, 50 mg/l, is added to the agar before auto-

claving. After solidification of the agar, round discs of 1 cm diameter are cut out and transferred to a *Pythium* selective medium. The plates are incubated for 24 h at 15°C in darkness, after which they are exposed to normal day light and darkness. After 4 days the number of plates with mycelia of *Pythium* are recorded and the number of propagules per gram of dry soil is calculated according to the MPN method.

Statistical interpretation, MPN method

In some experiments the number of propagules of *Pythium* was calculated using the most probable number method (MPN) (MALOY & ALEXANDER 1958). This method is based on the appearance or non-appearance of *Pythium* and results are compared to the Poisson distribution. The method involves three successive dilutions for the calculation of the MPN. In the present experiments the dilution coefficient was 10 and the number of replicates also 10.

The MPN estimates of two soil samples differ from one another at the 95 % level of probability when their ratio exceeds 3.3. The MPN estimate differs from a fixed norm at the same probability level when the mutual ratio is at least 2.3.

Patogenicity of Pythium strains in vitro

Strains of *Pythium* were transferred to CMA to which had been added 500 ppm of wheat germ oil for induction of oogonia. After 7 days 10 seeds of Monohill sugar beet were sown in each petri dish (Ø 90 mm) showing fungal growth. As control, plates with no transfers of *Pythium* were used. After 5 days at laboratory temperatures a disease index (DI_{1-10}) was calculated, given by the mean of the index for seed germination (10—1) and seedling death (1—10).

$$DI_{1-10} = \frac{(\text{Seed germination, } 10-1) + (\text{Seedling death, } 1-10)}{2}$$

Results

Testing of methods

Pythium spp. could not be isolated directly from soil using the selective Martin's medium with the dilution plate method, even though inhibitory substances like benomyl, streptomycin or pimaricin were used. The first experiments with the soil-plate method gave positive results in some cases and negative in others, this depending on several factors. Sieving the soil proved to be important and also even dispersal of the soil into the agar. The latter problem was solved by using a hand shaker. The soil was first poured into test tubes with melted agar at 42°C and thence after shaking into petri dishes. The use of sufficiently large soil samples proved important when soils of low propagule densities were tested.

The direct isolation of *Pythium* using the modified soil-plate method of RICCI et al. (1976) proved very successful, irrespective of the kind of agar media used (Table 1). In subsequent experiments, therefore, this method was used as the basis for estimating *Pythium* propagule densities of soils.

Some factors affecting the isolation method

Fungal growth inhibitors

The commonly used polyene antibiotic pimaricin inhibited the growth of six *P. debaryanum* isolates out of eight (Table 2). No growth whatsoever was observed when pimaricin was used at concentrations of 50 and 100 ppm. Reduced growth was noticed at 10 ppm. The fungicide PCNB together with benomyl inhibited completely the growth of one isolate and caused reduced growth in some others, especially when PCNB was used at 100 ppm. Two isolates from the Janakkala site showed

Table 1. Success in direct isolation of *Pythium* spp. from soil by different methods.

| Method | Origin of soil | Isolation medium | Success in isolating <i>Pythium</i> |
|---|----------------------|---|-------------------------------------|
| Dilution-plate | Janakkala Laitila | — Martin + 50 ppm benomyl + 20 ppm pimaricin — CMA — PDA | — |
| Dilution-plate | Laitila | — Martin + 10 ppm benomyl + 100 ppm pimaricin + 100 ppm streptomycin sulphate | — |
| Soil-plate | Laitila | — Martin + 50 ppm PCNB | + |
| Soil-plate | Laitila | — Water agar | — |
| » » | Salo | — Water agar + 50 ppm citric acid + 15 ppm benomyl + 50 ppm PCNB | — |
| » » | Salo | — CMA + 50 ppm citric acid + 15 ppm benomyl + 50 ppm PCNB | — |
| » » | Mietoinen | — Martin | — |
| Soil-plate as modif. by RICCI et al. (1976) | Köyliö | — Martin + 15 ppm benomyl + 15 ppm PCNB | + |
| » | » | — Martin + 15 ppm PCNB + 5 ppm pimaricin | + |
| » | » | — Martin + 15 ppm benomyl + 15 ppm PCNB + 100 ppm streptomycin sulphate | + |
| » | » | — SPB + 100 ppm streptomycin sulphate | + |

Table 2. Growth rate of 8 isolates of *Pythium debaryanum* on 7 agar media. Room temperature.

| Medium | Daily growth rate (mm) of <i>Pythium debaryanum</i> | | | | | | | | |
|--------|---|-----|------|-----------------|------|------|--------------------|------|------|
| | Mietoinen isolates | | | Köyliö isolates | | | Janakkala isolates | | Mean |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | |
| 1 | 7.5 | 7.5 | 10.0 | 11.3 | 11.3 | 11.3 | 10.0 | 10.0 | 9.9 |
| 2 | 5.0 | 9.3 | 7.5 | 3.8 | 3.0 | 3.5 | 6.0 | 7.5 | 5.7 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 4.3 | 4.0 | 1.0 |
| 4 | 3.0 | 6.8 | 0 | 3.0 | 3.8 | 2.8 | 4.5 | 4.0 | 4.0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 4.3 | 5.0 | 1.2 |
| 6 | 2.5 | 6.8 | 3.8 | 2.5 | 0 | 1.8 | 5.0 | 6.3 | 3.6 |
| 7 | 2.3 | 5.0 | 3.0 | 1.3 | 0 | 2.8 | 4.0 | 5.0 | 2.9 |

Media:

- 1 CMA + streptomycin sulphate 100 ppm
- 2 Martin's medium
- 3 » » + pimaricin 50 ppm
- 4 » » + » 10 » + Benlate 10 ppm
- 5 » » + » 100 » + » » »
- 6 » » + Brassicol 10 » + » » »
- 7 » » + » 100 » + » » »

Table 3. The influence of storage on the content of *Pythium* in soil from Köyliö with sugar beet and oats as preceding crops. Propagule densities estimated by the method of Ricci et al. (1976). Semi-moist storage of soil at room temperature.

| Amount of soil mg/ petridish | Number of positive petri-dishes/10 | | | |
|---------------------------------|------------------------------------|----------------------|-------------------------|----------------------|
| | Sugar beet field | | Oat field | |
| | Soil tested immediately | Soil stored 154 days | Soil tested immediately | Soil stored 154 days |
| 14.0 | 10 | 10 | 9 | 10 |
| 2.5 | 10 | 10 | 1 | 3 |
| 0.8 | 8 | 9 | 1 | 1 |
| 0.2 | 8 | 6 | 0 | 2 |

no or only minor influence by the inhibitory agents used in this experiment.

Soil properties

Storage of the soil samples under semi-moist conditions at laboratory temperatures for 154 days did not affect their contents of *Pythium* propagules (Table 3). Drying the soil sample for 6 days before introduction into the agar medium gave fewer propagules as compared to drying overnight, especially when potato-dextrose agar was used as the isolation medium (Table 3). On the other hand, keeping the soil in the first isolation medium for 6 days gave a higher number of propagules as compared with keeping it in this medium overnight.

Evaluation of the isolation method

The method of Ricci et al. (1976) for the direct isolation of *Pythium* species from soil gave somewhat differing amounts of *Pythium* propagules depending on the type of isolation agar used (Table 4). Consistent trends were also noticed. The amount of propagules was the highest in the Köyliö soil on each of the four isolation media. On average, the number of propagules was highest on CMA and lowest on PDA.

The direct isolation of *Pythium* from soil yielded several species and strains. From the sugar beet soils of Köyliö, Salo, Laitila and

Janakkala four different types of *Pythium* could be recovered on corn meal agar:

1. Oogonia with cell wall projections
2. » » smooth cell walls of the type *P. debaryanum* — *P. ultimum*. Mycelial growth irregular growth on CMA
3. Like type 2, but with far more abundant mycelial growth on CMA
4. No oogonia observed. Mycelial growth radial on CMA.

Type 1 had the lowest DI in the pathogenicity test. Most of the transfers of this group showed a DI of 1, which is the same as in the control (Fig. 1). Transfers of *Pythium* type 2 showed a wide diversity of pathogenicity, while the third group consisted of highly pathogenic transfers with a DI peak of 8–9. The fourth group of *Pythium* showed low to moderate pathogenicity.

When *Pythium* spp. were isolated directly from soil, several slow-growing fungi oc-

Table 4. *Pythium* densities in four soils on four media. Estimation according to the MPN method.

| Origin of soil | No of <i>Pythium</i> propagules/g dry soil as estimated by the MPN method | | | |
|----------------|---|------------|------|------|
| | Martin's medium | SPB medium | CMA | PDA |
| Köyliö | 205a | 75a | 205a | 40b |
| Salo | 7a | 2b | 60c | 13ad |
| Laitila | 23a | 14a | 105b | 12a |
| Janakkala | 20a | 18a | 50a | 16a |
| Mean | 64ab | 27a | 105b | 20a |

Row values marked with different letters differ significantly ($P < 0.05$)

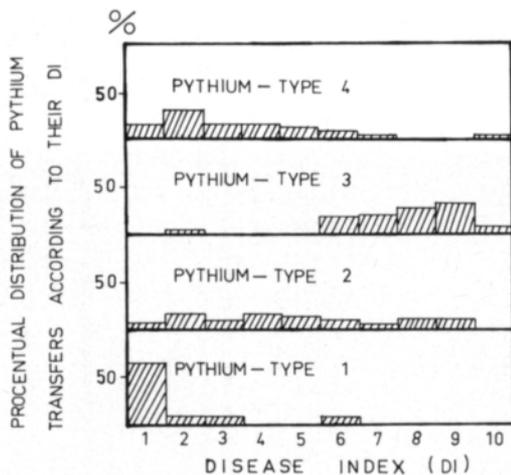


Fig. 1. Percentage distribution of four types of *Pythium* transfers according to their disease indices as estimated *in vitro*.

curred on the isolation media. In Table 5 are summarized the fungi of four sugar beet soils and their occurrence on four agar media. There were differences between the occurrence of fungi on different media. On the whole, CMA showed only a few species, this being due to the abundant growth of bacteria on this

medium. *Chrysosporium pannorum* (Link) Hughes and *Penicillium* spp. were the most common fungi on Martin's medium, PDA and SPB, while for instance *Acremonium* spp. could be found especially on Martin's medium and SPB and *Zygorhynchus* spp. on Martin's medium and PDA (Table 5).

Discussion

Pythium spp. were readily isolated directly from sugar beet soils by the soil-plate method (WARCUP 1950) as modified by RICCI et al. (1976). Even common media like CMA and PDA could be used as isolation media in this method. However, cultures of *Pythium* obtained on MARTIN'S medium (1950) and the SPB medium were more easily recognizable than cultures on CMA or PDA.

In the present investigation *Pythium* species were not identified, but they were grouped on the basis of morphological and growth habit characters. There were four groups of differing pathogenicity *in vitro*. These four groups certainly do not represent the whole *Pythium* flora of the soil samples studied, be-

Table 5. Occurrence of fungi other than *Pythium* spp. on agar media used for direct quantitative isolation of *Pythium* in soil. 114 petri-dishes with Martin's agar, 118 dishes with PDA, 82 dishes with CMA and 125 petri-dishes with SPB.

| Fungus | Number of petri-dishes showing fungal growth on different media | | | |
|---|---|-----|-----|-----|
| | Martin's agar | PDA | CMA | SPB |
| <i>Absidia</i> sp. | 0 | 2 | 0 | 0 |
| <i>Acremonium</i> spp. | 12 | 0 | 1 | 13 |
| <i>Alternaria alternata</i> (Fr.) Keissl. | 2 | 1 | 0 | 0 |
| <i>Chrysosporium pannorum</i> (Link) Hughes | 5 | 23 | 0 | 32 |
| <i>Doratomyces stemonitis</i> (Pers. ex Steud.) Morton & G. Sm. | 0 | 1 | 0 | 4 |
| <i>Fusarium</i> spp. | 0 | 0 | 0 | 2 |
| <i>Geotrichum candidum</i> Link ex Leman | 3 | 0 | 0 | 0 |
| <i>Graphium</i> sp. | 0 | 0 | 1 | 0 |
| <i>Moniliella</i> sp. | 1 | 7 | 2 | 0 |
| <i>Monodictys levis</i> (Wiltsh.) Hughes | 0 | 0 | 0 | 1 |
| <i>Mortierella</i> sp. | 1 | 0 | 0 | 0 |
| <i>Mucor</i> spp. | 1 | 2 | 0 | 0 |
| <i>Paecilomyces</i> sp. | 2 | 0 | 0 | 0 |
| <i>Penicillium</i> spp. | 20 | 10 | 0 | 7 |
| <i>Ulocladium botrytis</i> Preuss | 7 | 3 | 0 | 1 |
| <i>Zygorhynchus</i> sp. | 15 | 15 | 0 | 1 |
| Unidentified fungi | 2 | 3 | 0 | 4 |

cause only one isolation method was used. In fact, it is well known that the application of different isolation methods well yield a range of *Pythium* species and that no single method yields the whole *Pythium* flora of the soil (HENDRIX & CAMPBELL 1973). For example, the Flowers & Hendrix gallic acid medium is good for isolating *P. acanthicum* Drechs. — *P. oligandrum* Drechs. group, *P. ultimum* Trow and *P. vexans* de Bary, while the Kerr medium is best for *P. afertile* Kanouse & Humphrey, *P. aphanidermatum* (Edson) Fizz. and the *P. dissotocum* Drechs. — *P. perniciosum* Serbinow group (HENDRIX & CAMPBELL 1970). To approach a total qualitative estimate of the *Pythium* flora, several different techniques should therefore be used on each soil sample.

Most of the numerous *Pythium*-media available contain one or several antimicrobial agents. The polyene antibiotic pimaricin inhibits the growth of almost all fungi, except a small group including the pythiaceae fungi (TSAO 1970). However, reports of inhibition of some *Pythiaceae* fungi by pimaricin also exists (HINE 1962). In the present study, the growths of several isolates of the pathogenic *P. debaryanum* isolated from sugar beet seedlings were strongly inhibited by pimaricin. Therefore, pimaricin will not be used in further investigations. PCNB and benomyl, on

the other hand, did not inhibit, or only slightly inhibited the growth of *P. debaryanum*.

The estimation of *Pythium* was quantified by making dilutions of test soil into water agar according to the method of RICCI et al. (1976). Although the method proved successful, it has some practical shortcomings. It is laborious and *in vitro* and *in vivo* tests are needed to separate pathogenic and nonpathogenic species or strains from each other. The method proved reliable with *Pythium* inoculum densities of 10 or more propagules per gram of soil. At very low densities, ie. 1 propagule or less per gram of soil, the method is not feasible because the amount of soil to be incorporated into the water agar is too high.

The number of *Pythium* propagules per unit of soil is only one of several factors expressing the likelihood of plant disease. However, this knowledge might be valuable, for example, when establishing a certain minimum inoculum level above which possibilities for disease development exists.

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SELOSTUS

Pythium-sienen suora eristäminen suomalaisista sokerijuurikasmaista

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Helsingin yliopiston kasvipatologian laitos

Vuodesta 1978 alkaen on Helsingin yliopiston kasvipatologian laitoksen ja Sokerijuurikkaan Tutkimuskeskuksen välisenä yhteistyönä tutkittu sokerijuurikkaan taimipoltetta. Tämä kirjoitus käsittelee taimipolteen tärkeimmän aiheuttajan, *Pythium*-sienen eristämistä maasta. *Pythium*-sientä pystyttiin eristämään maasta ranskalaisella menetelmällä, jossa pieniä määriä kuivaa ja seulotua maata (3, 30, 300 ja 3000 mg) sekoitettiin 42°C:een vesiagariin. Jähmettyneestä agarista siirrettiin n. 1 cm kokoisia agarkiekkokoja multineen petrimaljoihin Martinin alustalle, johon oli lisätty benomyyliä ja PCNB:tä muiden paitsi *Pythium*-sienen kasvun hidastamiseksi. Petrimaljat pidettiin ensin 1 vrk pimeässä +15°C:een lämpötilassa ja tämän jälkeen 5 vrk normaaleissa laboratorioolosuhteissa. Tämän jälkeen tehtiin havainnot mahdollisista *Pythium*-kasvustoista. Saatujen lukujen perusteella

laskettiin näytteille todennäköisin itiötiheys/g ilmakeivä maata.

Käytetyllä menetelmällä saatiin tutkituista juurikasmaista esiin sekä tautia aiheuttavia että saprofyttisiä lajeja ja kantoja. Nämä jaettiin morfologisten ja kasvutapaominaisuuksiensa perusteella neljään ryhmään. Ryhmien välillä todettiin patogeenisuuseroja.

Yllä selostetulla menetelmällä saatu tieto on käyttökel-poista esim. verrattaessa käsittelyjä keskenään tai määrittäessä sitä alinta *Pythium*-sienen määrää, jonka yläpuolella taimipolteen puhkeamisvaara kasvaa. Tällä menetelmällä on myös haittapuolensa. Hyvin alhaisia *Pythium*-sienen itiötiheyksiä ei voida määrittää luotettavasti. Patogeenisten lajien ja kantojen erottaminen saprofyttisistä vaatii laajoja laboratorio- ja kasvihuonekokeita.