DEPLETION OF PENTACHLOROPHENOL BY DEUTEROMYCETES ISOLATED FROM SOIL


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ABSTRACT

1. A study performed on 100 strains of micromycetes cultivated on solid media with pentachlorophenol (PCP, 0.5 g L\(^{-1}\)) allowed to select strains as a function of the appearance of a light blurring around the inoculation spot. Cultivation in liquid synthetic medium with high concentration of PCP (1 g L\(^{-1}\)) showed that biotic depletion depended on the taxonomic group. The influence of the physiological state of the inoculum and of the amount of glucose have been studied. Cultivation of Phoma glomerata in the dark and with light allowed to evaluate the part played by abiotic factors.

2. A screening was performed on 784 strains of Deuteromycetes cultivated in liquid synthetic medium with PCP (100 mg L\(^{-1}\)). After 5 days, the mean consumption of PCP was 50.7%. The distribution of efficient strains was very heterogeneous depending on the groups and genera. Among the most efficient fungi, 168 reached 70% of depletion and 40 reached 80% (Mucedinaceae, 23; Dematiaceae, 14; Sphaeropsidales, 2; Tuberculariales, 1). Abiotic depletion accounted for 5%.

3. A more detailed study was done on Calcarisporium arbuscula and Oidiodendron echinulatum that both depleted PCP 90% after 5 days. PCP disappeared rapidly from the culture medium of C. arbuscula since, after 24 hours, only 20% of PCP could be detected. The mechanism of PCP depletion is discussed.

Keywords: Pentachlorophenol. Deuteromycetes. Fungi. Degradation. Depletion.
INTRODUCTION

Until recently, pentachlorophenol (PCP) has been used extensively in the wood industry and in agriculture. Numerous reports have shown that PCP was degraded in the environment, but this degradation is slow and the pollution of many terrestrial and aquatic ecosystems persists.

Most studies on the biodegradation of PCP deal with bacteria. Very few concern its metabolism by fungi. Recently, a detailed study was undertaken concerning the biodegradation potential of the white-rot fungus, _Phanerochaete chrysosporium_. We have previously reported on the biotic depletion capabilities of fungi belonging to various taxonomic groups, Ascomycetes, Basidiomycetes, Yeasts and Zygomycetes. The last taxonomic group we investigated concerning fungal depletion was Deuteromycetes. It was by far the least known and only few studies deal with few species and genera. The results obtained are presented in this communication, with focus on the possible role of phenol-oxidases.

MATERIALS AND METHODS

Microorganisms

Seven hundred and eighty four strains of Deuteromycetes from the Mycological Collection of the Laboratory of Cryptogamy (Collection Mycologie Pharmacie Grenoble, CMPG) were used in this study. Most of them were isolated from soil, few from fragments of decayed wood or walnut. Stock cultures were maintained on solid malt extract medium (1.5 %).

Chemicals

Sodium-PCP was purchased from Janssen (Beerse, Belgium). Other products were from Prolabo (Paris, France). The quality of each compound was controlled by high performance liquid chromatography (HPLC) prior to use.

Culture conditions

_Solid media_. Two different solid media (agar 1.5 %) were used for preliminary studies: malt extract medium (ME) (1.5 %) and Galzy and Slonimski synthetic medium (GS) pH 4.5 without glucose. They were autoclaved 20 min at 121°C for sterilization. PCP (100 g L⁻¹) was dissolved in ethanol, sterilized by filtration through a Millipore syringe (pore size, 0.22 μm) and added aseptically to the media before inoculation. First studies were made with a final concentration of PCP varying from 0.1 to 1 g L⁻¹. Then one hundred strains were cultivated with a final concentration of 0.5 g L⁻¹. ME medium was used to point out the toxicity of PCP against
microorganisms. GS medium without glucose was used as a test for consumption or metabolization of PCP. Growth was observed after 15 days at 24°C.

**Preliminary studies in GS liquid medium**: Strains selected after cultivation on solid media were cultivated in GS liquid medium pH 4.5 with glucose (5 and 10 g L\(^{-1}\)). Cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of medium. After sterilization, the media were inoculated with mycelium and spores or only with spores. Cultures were incubated with shaking for 2 days (180 rpm, orbital shaker or magnetic stirrer) in order to allow the biomasses to grow and to reach 4 g L\(^{-1}\)±10% (dry weight). At this stage, no glucose remained in the medium. PCP was added to the 2-day old cultures to a final concentration of 1 g L\(^{-1}\). The depletion of PCP was evaluated after 8 days of cultivation (6 with PCP). Each series of experiment included flasks extracted at time = 0 and photodegradation references. Temperature was 24°C, light was 1200 lux with a photoperiod of 12 h per day. Each experiment was made in triplicate.

**Biotic and abiotic depletion of PCP by Phoma glomerata**: A more detailed study was done with *Phoma glomerata*. 125 ml flasks containing 25 ml of GS medium, pH 4.5 with glucose (5 g L\(^{-1}\)) were prepared. After sterilization, one series was kept for evaluation of abiotic degradation and the other inoculated with mycelium and spores and grown under a 4800 lux illumination, with a photoperiod of 12 h per day. Cultures were incubated with shaking (180 rpm, orbital shaker) and the temperature was 28°C. After 2 days, PCP was added (100 mg L\(^{-1}\) final concentration) as well in the inoculated than in the uninoculated flasks. One series was shaken in the dark, the other under light (under the same conditions as for incubation). Each day from day 0 to day 8, and on day 12, two flasks from each of the 4 series (abiotic dark, abiotic light, *Ph. glomerata* dark, *Ph. glomerata* light) were analyzed for remaining PCP.

**Screening in GS liquid medium**: 784 strains of Deuteromycetes were cultivated in GS liquid synthetic medium pH 4.5 with glucose (5 g L\(^{-1}\)). Cultures were grown in 125 ml flasks containing 25 ml of medium. After sterilization by autoclaving for 20 min at 121°C, cultures were inoculated with mycelium and spores and incubated at 24°C with shaking (180 rpm, orbital shaker). PCP was added as previously described to a final concentration of 100 mg L\(^{-1}\). The consumption of PCP was evaluated after 7 days of incubation (5 days after PCP addition). Each series of experiment included cell free flasks; some were extracted at time = 0 after PCP addition and others were abiotic degradation references incubated 7 days (5 days after PCP addition). Light was 1200 lux with a photoperiod of 12 h per day. Each series of experiment was made in triplicate.

**Evaluation of ethyl acetate extractible PCP**.

Fungal mycelia and culture media were acidified to pH 2 by HCl 6N and extracted with ethyl acetate (3 times with one volume). After the third extraction, it was checked that no PCP could be extracted either from the medium or from the mycelium. The combined crude extracts were dried over anhydrous sodium sulfate and evaporated with a stream of nitrogen. The residues were dissolved in 5 ml of methanol for HPLC analysis performed with a liquid chromatograph Shimadzu equipped with a pump LC 6A, an automatic injector Shimadzu SIL-9A and a UV detector SPD 6A. The separation column was 4.0 mm inside diameter x 300 mm long filled with μ-Bondapak C\(_{18}\). The mobile phase was methanol, water (90:10 v/v) pH 5 (phosphoric acid). The flow rate was 1 ml min\(^{-1}\)
and analyses were performed at 230 nm. Each sample was injected at least 3 times and the mean taken. Results were within a ±10% range.

**Phenol-oxidases production**

Phenol-oxidases were produced by growth of the strains (12 days) on solid malt extract medium (1.5 %) following the methods of Kii~rik, Harkin and Obst, Harkin et al., Ander and Eriksson, and Dagron. Stock solutions 0.1 M of reagents (benzidine, guaiacol, o-anisidine, pyrogallol, α-naphtol, p-cresol, tyrosine, gallic acid) were prepared in 95 % ethanol. Syringaldazine was 0.1 % (w:v) solution in 95 % ethanol to which could be added a 0.03 % hydrogen peroxide aqueous solution for the detection of peroxidases. R56 reagent was amidopyrine (11.5 %), N-N-diethylaniline (2.5 %) in 95 % ethanol. Reactions were terminated and read after 24 h except for syringaldazine which was incubated for 20 min. Each experiment was made in triplicate.

Intensity of the colour reactions has been evaluated and expressed from 0 to 4. The values given by the 11 reagents have been summed up to establish a global POx index from 0 to 44. Over 10 a strain was considered as high producer, medium producer between 6 to 10, low under 6 and no producer with 0.

**RESULTS AND DISCUSSION**

**Selection of fungi on solid media**

The aim of this work was to find strains of fungi that were able to consume PCP. Preliminary studies were carried out on solid media with 0.5 g L⁻¹ of PCP which was added at the same time when the media were inoculated. Toxicity assay on ME medium showed that PCP was very toxic because all of the strains were inhibited except *Fusarium moniliforme*. Consumption assay with PCP as the only carbon source on solid GS medium confirmed these results. However, at low pH, PCP precipitated in the culture medium which looked opaque; with some strains it was observed the appearance of a light blurring around the inoculum; owing to the very poor solubility of PCP at acidic pHs and as the pH remained low, it can be assumed that it was not simply dissolved but had disappeared from this zone.

**Preliminary studies in liquid synthetic medium**

As some strains were not completely inhibited, even at the high concentrations of PCP used on solid medium, it was chosen to look if under less drastic conditions, they were able to deplete high concentrations of PCP in liquid medium, as the strains were allowed to grow on glucose for 2 days before PCP addition, they should be more competitive. Fifty micromycetes selected on solid media were cultivated in liquid GS medium, glucose (10 g L⁻¹), PCP (1 g L⁻¹). Photodegradation occurred for 25 % after 6 days. In order to evaluate the amount of depletion for which the fungi were solely responsible, the biotic depletion was calculated upon PCP remaining after photodegradation had been taken into account. On the whole, the percentages of biotic depletion were not very high, but it must be emphasized that PCP concentration (1 g L⁻¹) was certainly responsible of a toxicity pointed out by Mileski et al. These authors showed that biodegradation of PCP by *Phanerochaete chrysosporium* was 50 % for a concentration of 1 mg L⁻¹ while it was only 7 % for a concentration of 500 mg L⁻¹. We obtained the best results with Zygomyctes (mean depletion, 25%), *Cunninghamella bainieri* being the
lowest (13%). PCP was not depleted by Yeasts (0.5%). Among Deuteromycetes, Tuberculariales depleted little (7%). Among Mucedinaceae some strains were very sensitive to PCP, others depleted 10 to 15% of PCP. The same observation was made for Dematiaceae. On first look heterogeneous results were obtained for Ascomycetes. The only Basidiomycete we have cultivated, *Dichomitus squalens*, depleted 8% of PCP.

**Preliminary studies : variation of parameters**

Based on these results, twelve strains were selected in order to study three parameters: glucose concentration (5 to 10 g L\(^{-1}\)), type of agitation (magnetic stirring or orbital shaking) and type of inoculum (spores or mycelium plus spores). Biotic depletion was calculated upon PCP remaining after photodegradation had been taken into account. It can be noticed that the increase of glucose concentration repressed the depletion of PCP, as mentioned by others\(^2\). The best depletion was obtained with glucose concentration of 5 g L\(^{-1}\) (Figure 1).

![Figure 1. Depletion of PCP (1 g L\(^{-1}\)) with 2 concentrations of glucose.](image)

The second observation concerns the agitation of the cultures. An orbital type agitation favoured the biotic depletion of PCP by one half of the strains (Figure 2). The last observation deals with the inoculum (Figure 2). Spores of half of the strains were very sensitive to PCP and a 48 h cultivation before the addition of PCP seemed to be not long enough for developing a mycelial mat and to circumvent the toxic effect of PCP. This observation had already been mentioned by Mileski *et al.*\(^4\). Since PCP concentration of 4 mg L\(^{-1}\) or higher prevented any growth when cultures of *Phanerochaete chrysosporium* were inoculated with spores. It was necessary to establish a mycelial mat before the addition of PCP. The difference of sensitivity between spores of strains belonging to the same taxonomic group is unclear. The 4 strains belonging to Zygomyces developed a mycelial mat, however, only *Syncephalastrum racemosum* was inhibited when cultures were initiated with spores. The same observations were made for Deuteromycetes. So when cultures are inoculated with spores, the development of mycelial mat seems to be necessary but another factor must be elucidated to explain the observed differences of sensitivity of the spores.
Biotic and abiotic depletion of PCP by *Phoma glomerata*

Depletion study of PCP (100 mg L\(^{-1}\)) in the dark by *Ph. glomerata* gave 14% of depletion 24 hours after the addition of PCP (Figure 3). Later, the depletion increased slowly with a maximum at 31% on day 12. We also determined the degree of the abiotic degradation i.e. the oxidation by air enhanced by stirring of the medium. This degradation was low but not negligible and reached 8% on day 12. Then, when the abiotic degradation is subtracted from the global results, it appears that fungal depletion occurred only during the first 2 days. Abiotic degradation alone can explain the increase observed during the following days.

When *Ph. glomerata* was cultivated with light (4800 lux, 12 h per day), depletion after 24 hours was higher (22%) than in the dark and increased until the second day to 54% (Figure 3). On day 12, it was 50%. Like i
the dark, we have determined the part played by abiotic degradation i.e. photodegradation. It was quite high and reached 25% after 12 days. We can then hypothesize that *Ph. glomerata* is able to deplete PCP efficiently and fast but that, nevertheless, it is sensitive to the toxicity of PCP and is greatly inhibited after 48 hours, as well in the dark as with light. Biotic depletion by *Ph. glomerata* after 24 hours was lower in the dark than with light, but the difference was slight (17 vs 14%). The difference increased on the second day (27 vs 19%) and remained constant during the following days, which is consistent with the inhibition of the activity of *Ph. glomerata* after 48 hours.

**Screening (100 mg L⁻¹)**

We have later explored systematically the biotic depletion capacity of the different taxonomic groups of our collection by cultivating them with glucose (5 g L⁻¹) and PCP (100 mg L⁻¹). The first study was done with Zygomycetes², then we explored Ascomycetes, Basidiomycetes and Yeasts¹⁰. This last study concerns Deuteromycetes. No variation of the biomasses (4 g L⁻¹±10%) was observed: when PCP was added, glucose was entirely consumed and the amount of PCP (100 mg L⁻¹) was insufficient to give a significant growth increase.

Results after 5 days in liquid GS medium are given by taxonomic groups. Five classes of activity were constituted: 0-20, 20-40, 40-60, 60-80 and more than 80% depletion of PCP (Figure 4).

**Aspergillus and Penicillium** were the most active. They have been considered separately from other Mucedinaceae as we had previously shown that they produced much higher levels of POx than the others. Towards PCP too, they behave differently since the other Mucedinaceae were among the least active, together with Tuberculariales.
The results obtained concerning as well PCP depletion than POx production by Deuteromycetes were very scattered, with the exception of some genera from the Mucedinaceae like Aspergillus and Penicillium, homogeneous in regard to POx production (Figure 5).

These results must be considered, keeping in mind the taxonomic heterogeneity of the fungi constituting this artificial class, based only on their look. We have particularly to focus on Aspergillus niger which is classified with Dematiaceae but gave us results that do not fit with that group (POx production). A better homogeneity would be reached if the genus Aspergillus was considered as a whole, notwithstanding the fact that some of them are considered as Dematiaceae and other as Mucedinaceae. The same observation could be done for Ascomycetes, another confusing taxonomic group, since PCP depletion occurred with strains producing or not POx, while better defined groups gave much more homogeneous results: Yeasts did not deplete PCP and did not produce POx. Basidiomycetes were high POx producers and depleted moderately PCP. The behaviour of Zygomycetes, another well defined group was also homogeneous since most of the strains were able to deplete extensively PCP and were devoid of any POx activity. On a whole, correlation between POx production and PCP disappearance was low or null. However, though there were several exceptions, better correlations were obtained when POx production and PCP disappearance were positively correlated while the correlations were lower when they tended to be inversely correlated.

The mechanism of disappearance of PCP from a medium is not yet understood. If POx are possibly concerned for some taxonomic groups like Basidiomycetes, they do not seem to play any part for others, like Zygomycetes. Other enzymatic systems or other phenomena must be implicated. Detailed investigations on selected strains are under progress concerning this problem. Beside this aspect, this work has allowed the selection of the most efficient strains able to deplete PCP. Their ability to tolerate high concentrations of this xenobiotic should make them competitive for the bioremediation of highly contaminated soils. Another important result is given by a detailed examination of the data (Figure 6), showing that when several strains of the same species are considered, from different origins, the results can be quite scattered. We had previously made the same observation in another research. This justifies to use several strains of the same species when conducting a screening for biotic depletion.

Two strains among the best were submitted to kinetic studies both on short period (0-24h) and longer (0-5 days) (Figure 7). After 8 hours, with Calcarisporium arbuscula, PCP depletion reached 40%, and 74% after 24 hours. With Oidiodendron echinulatum, 15% after 8 hours and 40% after 24 hours. Abiotic degradation was negligible after 24 hours (2%). On longer periods, with Oidiodendron echinulatum, 47% of PCP had disappeared after
48 hours. After 5 days, depletion had not much increased and reached only 64%. With *Calcarisporium arbuscula*, a high 83% disappeared after 48 hours, and again after 5 days not much more (88%). Photodegradation was about 10% after 5 days and had been withdrawn from the biotic depletion.

Such a rapid disappearance of PCP, extracted from the culture medium by ethyle acetate at pH 2 draws questions about the mechanism involved. The hypothesis of a strong adsorption on the mycelium as a first step can be made. We are now looking after that mechanism.

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REFERENCES