Novel antifungal compounds isolated from plant-growth promoting rhizospheric Pseudomonas fluorescens strains

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Introduction

The application of chemical pesticides in agriculture has produced harmful effects on the environment. This situation has led to an enhanced interest in biological control and the use of microbial products to suppress plant diseases. Bacteria of the genus Pseudomonas have been regarded as promising biocontrol agents because of their rhizospheric abundance, catabolic versatility, root-colonizing abilities, and their capacity to produce a diverse array of antifungal compounds. Antibiotics are commonly reported as responsible for biocontrol activity of fluorescent Pseudomonas. The elucidation of the mechanisms involved in biological control is a key feature for the consistent application of this technology.

Pseudomonas fluorescens CFBP2392 (syn A6) and UP148 were isolated from the rhizosphere of healthy plants growing in soils with damping-off incidence. Both strains are able to reduce the infection of Solanum lycopersicum (tomato) or Lotus corniculatus plants, caused by the phytopathogens Rhizoctonia solani or Pythium ultimum.

Objective: To purify and characterise the antibiotic compounds produced by Pseudomonas fluorescens A6 and UP148.

Isolation of antifungal compounds

The strains were grown in a modified PSF liquid medium and organic extractions of the supernatants were made. The antifungal activity of the extracts was evaluated by the agar disc diffusion test (Fig. 1).

The compounds extracted were fractionated by preparative chromatographic methods and the antifungal activity of the fractions was evaluated by the agar dilution method. A thin layer chromatography (TLC) band containing antibiotic activity was identified from culture extracts of UP148 and A6 (Fig. 2).

The active compounds were further purified by high performance liquid chromatography (HPLC) (Fig. 4) in order to characterise them by spectroscopic analysis.

Cytological effects of the A6 antibiotic on R. solani

The effects of the antibiotic produced by A6 on the R. solani ultrastructure were observed by Transmission Electron Microscopy (TEM). The compound induced cytoplasmic alterations such as higher vacuolization, hypertrophied mitochondria and appearance of membranous structures with granular contents (Fig. 7).

Structural characterisation of the antibiotics

Spectroscopic techniques were used to determine the chemical nature of the antibiotics produced by the strains A6 and UP148.

A6: UV absorption spectrum, 1H- NMR (COSY, TOCSY, HMBC y HMBC) and Electrospray Ionization Mass Spectrometry studies indicated that the compound produced by A6 is a cyclic tetrapeptide composed of Val, Leu, Ile and Glu.

UP148: UV absorption spectrum, 1H- NMR (COSY) and Electron-Mass Spectrometry results (Fig. 4 & 5) showed that UP148 produces a phenazine derivative with a methanecarboxylic and a hydroxyl substituents (Fig. 6).

Role of antifungal compounds of UP148 in biocontrol activity

Table 1. Phenotypic characteristics of P. fluorescens UP148 and its mutants obtained by Tn 5::lux insertion and selected for its reduced inhibition of P. ultimum in vitro.

<table>
<thead>
<tr>
<th>Strain</th>
<th>P. ultimum inhibitiona</th>
<th>Production of metabolites</th>
<th>Protocatechuate</th>
<th>Phe N-acylated aminopeptidase</th>
<th>Phe acetylated aminopeptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>UP148 wt</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>100 %</td>
<td>+</td>
</tr>
<tr>
<td>mB</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>20 %</td>
<td>+</td>
</tr>
<tr>
<td>mK</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>24 %</td>
<td>-</td>
</tr>
<tr>
<td>mK6</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>12 %</td>
<td>-</td>
</tr>
</tbody>
</table>

A combination of several metabolites seem to be necessary for complete plant protection by UP148. The A6 cyclopeptide seems to affect the metabolic activity of the fungus, acting mainly on mitochondrial membranes.

Conclusions

Two non-previously described antifungal metabolites were purified and partially identified. It remains to determine the sequence of amino acids in the A6 cyclic tetrapeptide and the positions of the substituents in the UP148 phenazine ring.

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Figure 1. Antagonistic activity of the organic extract of A6 culture, assessed by the agar disc diffusion test (left). Right plate shows normal growth of Rhizoctonia solani.

Figure 2. (a) Separation of A6 extract by TLC. (b) Evaluation of antifungal activity of the fractions obtained by TLC. N: normal growth of the fungus; T: total extract; A-B: TLC fractions.

Figure 3. HPLC elution profiles of UP148 (a) and A6 (c) extracts. The active compound isolated from A6 is indicated with an arrow. The compound g is the active metabolite produced by UP148 as tested by the agar diffusion test in the right plate. (b) Left plate shows normal growth of Pythium ultimum.

Figure 4. Electron impact mass spectrum (EI-MS) of the antibiotic produced by UP148. Assignment of some fragments is shown.

Figure 5. Protection of Lotus corniculatus infected by P. ultimum achieved by UP148, mB and mK. G.C: Germination Control, I.C.: Infection Control. Different letters represent significant differences by ANOVA and Fisher’s protected LSD test (p<0.05).

Figure 6. One of the proposed structures of the antibiotic produced by UP148. Possible position of substituents: 1-3 or 2-4.

Figure 7. TE micrographs of R. solani growing without (a) or with the peptide (b and c). Sections were stained by double contrast. m: mitochondria; mc: membranous vesicles.