Pyrrolnitrin Interferes with Osmotic Signal Transduction in Neurospora crassa

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Pyrrnlitrin, produced by several bacteria that are used in biological control, has an inhibitory effect on the electron transport system of respiration in Neurospora crassa. We have previously described that fludioxonil, a derivative of phenylpyrroles, affects osmotic signal transduction. Both pyrrolnitrin and fludioxonil were highly active against Botrytis cinerea, Fusarium oxysporum, Rhizoctonia solani, and N. crassa. However, a high concentration of pyrrolnitrin (more than 10 μg/ml) inhibited the growth of fludioxonil-insensitive fungi such as Pythium ultimum, Phytophthora capsici, and Saccharomyces cerevisiae. In order to clarify the difference in the antifungal mechanisms between pyrrolnitrin and fludioxonil, we observed cross-resistance in mutants of the osmotic signal transduction pathway, namely, os-1 (histidine kinase), os-4 (MAPKK kinase), os-5 (MAPK kinase), and os-2 (MAP kinase) of N. crassa. All os mutants that were resistant to fludioxonil showed cross-resistance to pyrrolnitrin without exception. The levels of resistance to pyrrolnitrin correlated well with those to fludioxonil in the 10 os-1 mutant alleles with single amino acid substitutions. However, at a concentration of 6.1 μg/ml, pyrrolnitrin inhibited the growth of all strains including the os mutants insensitive to fludioxonil even at 25 μg/ml. When the conidia of the wild-type strain were grown on a medium containing either fungicide at a concentration of 0.1 μg/ml, both fungicides induced the swelling and rupture of conidia without germ-tube formation. At a concentration of 25 μg/ml, pyrrolnitrin inhibited conidia germination without any morphological change in the fludioxonil-insensitive os-5 mutant. Both fungicides at a concentration of 1 μg/ml stimulated glycerol synthesis in the wild-type strain, but the glycerol content was reduced to a considerable extent on treatment with 25 μg/ml pyrrolnitrin. These results suggest that a primary antifungal mechanism of pyrrolnitrin against N. crassa is interference with the osmotic signal transduction pathway rather than inhibition of respiration. © Pesticide Science Society of Japan

Keywords: dicarboximide, glycerol, two-component signal transduction, biocontrol, histidine kinase, respiration.

INTRODUCTION

The antibiotic pyrrolnitrin [3-chloro-4-(3-chloro-2-nitrophenyl) pyrrole] (Fig. 1) is produced by several species of Pseudomonas, and has broad-spectral antifungal activity.1,2) The ability of certain antagonistic bacteria to protect plants from soilborne fungal pathogens, commonly referred to as biocontrol, is strongly correlated with the production of antifungal factors such as antibiotics, hydrolytic enzymes, siderophores. Pyrrolnitrin has been implicated as an important factor in the mechanism of biological control of fungal plant pathogens by several Pseudomonas strains.3–6) Two phenylpyrrole derivatives, fludioxonil and fenpiclonil, are analogues of pyrrolnitrin and used as a fungicide.7) Fludioxonil, when used in seed treatments, is effective against Gibberella fujikuroi, Magnaporthe grisea, and Cochliobolus miyabeanus, which attack rice crops.7) Further, the foliar application of fludioxonil is also effective against Botrytis cinerea, which affects various vegetables.

Fludioxonil-resistant mutants can be easily obtained in the laboratory in Neurospora crassa and other fungi, and most of them show cross-resistance to iprodione, a dicarboximide, and aromatic hydrocarbons, and sensitivity to osmotic stress. We
have previously demonstrated that fludioxonil interferes with osmotic signal transduction in N. crassa and B. cinerea.9,10 In N. crassa, the osmotic sensitive mutants os-1, os-2, os-4, and os-5 were resistant to fludioxonil and iprodione.9,11 Fludioxonil and iprodione induce glycerol accumulation in the wild-type strain of N. crassa.12 However, glycerol is not accumulated in response to fludioxonil and iprodione in the os mutants highly resistant to these fungicides.10,11 The N. crassa os-1 gene encodes an osmosensor-like histidine kinase13,14 and the genes os-4, os-5, and os-2 encode homologues of Ssk2/Ssk2 MAPKK kinase,15 Pbs2 MAPK kinase,15 and Hog1 MAP kinase,16 respectively, which are components of the MAP kinase cascade in the Saccharomyces cerevisiae HOG pathway.17 Molecular analysis of the os-1 allele mutants revealed that most of the mutations are located in the amino acid repeat region at the N-terminus of the os-1 gene product, which is highly polymorphic with regard to both fungicide resistance and osmotic sensitivity.18,19 A similar result was obtained on the mutation of the os-1 family histidine kinase in plant pathogenic fungi such as B. cinerea and Cochliobolus heterostrophus.20,21 Although most laboratory mutants are highly resistant to both fludioxonil and iprodione and show osmotic sensitivity, some mutations do not confer cross-resistance between fludioxonil and iprodione. In B. cinerea, for example, iprodione-resistant strains isolated from fields show neither osmotic sensitivity nor resistance to fludioxonil.22,23 An iprodione resistance mutation (I365S) in field isolates was also detected within the amino acid repeats of the BcOS1 gene product, an os-1-like histidine kinase, in B. cinerea.24,25

The mode of action of pyrrolnitrin has been extensively studied in N. crassa. The results of these studies indicate that pyrrolnitrin has an inhibitory effect on the electron transport system, as demonstrated in mitochondria isolated from N. crassa.26-29 This inhibition rate was reduced by the addition of N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD), indicating that pyrrolnitrin blocked the electron transfer between the dehydrogenases and the cytochrome components of the respiratory chain. The concentration of fenpiclonil and iprodione required for the uncoupling activity detected in the mitochondria of B. cinerea, was greater than that required to inhibit mycelial growth.22 These results suggest that, unlike pyrrolnitrin, fenpiclonil may not act via a primary effect on respiration. Limited information is available with regard to the effects of pyrrolnitrin on osmotic signal transduction. Thus, in this study, we investigated the cross-resistance between pyrrolnitrin and fludioxonil in the os mutants of N. crassa. We have also described the effect of pyrrolnitrin on the morphology of conidial germination and glycerol synthesis in the wild-type strain and the os mutants.

**MATERIALS AND METHODS**

1. **Strains and Medium**

N. crassa 74-OR31-14a (al-2, pan-2, cot-1) was used as the wild-type strain. The osmotic sensitive mutants os-1 (alleles: M16, M155-1, NM233(t), NM204(t), P668, P6549, P5990, E11200, B135, and Y256M209), os-2 (ALS10), os-4 (Y256M223), and os-5 (NM2160) were obtained from the Fungal Genetic Stock Center (Kansas City, MO, USA). The strains of N. crassa were cultured at 28°C on Vogel's minimal (VM) agar medium that contained 100 µg/ml potassium pantothenate. B. cinerea strains Bc-56 (wild-type), Bc-45 (field isolates, iprodione-resistance), and Bc-DHR3 (laboratory mutant, iprodione-resistance) were cultured on potato dextrose agar (PDA) medium at 18°C. Other fungi Fusarium oxysporum (NBRC 30703), F. solani (LV-1), M. grisea (P2), Rhizoctonia solani (Rs-2-1), Phytophthora capsici (NBRC 9173), and Pythium ultimum (NBRC 32424) were also cultured on PDA medium at 25°C. S. cerevisiae (w303-1A) was cultured on YPD (2% tryptone, 2% yeast extract and 10% glucose) agar medium at 28°C.

2. **Chemicals**

The fungicides iprodione and fludioxonil were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Pyrrolnitrin was provided by Fujisawa Pharmaceutical Co. Ltd. (Tokyo, Japan). The chemicals were stored in the dark at 100 mg/ml in dimethyl sulfoxide (DMSO) at 4°C. The test compounds were added to the media from 1000-fold concentrated stock solutions in DMSO.

3. **Antifungal Assays**

In order to determine the minimal inhibitory concentration (MIC) of the fungicides, conidia of N. crassa were inoculated on the VM agar medium (1% sorbose and 0.2% sucrose were used as carbon sources) containing the chemicals and cultured at 28°C and colonial growth was observed after 2 days. In order to determine the concentration required for 50% inhibition of the mycelial growth (EC₅₀), mycelial disks were placed on VM agar medium (1.2% sucrose) containing the chemicals and cultured at 28°C for 20 hr, and mycelial diameters were scored. The EC₅₀ values for each fungicide were calculated using the dosage-response curves. In the case of other fungal species, mycelial disks were placed on PDA medium containing the chemicals, and mycelial diameters were scored when the diameter of the mycelium of the untreated control reached approximately 7 cm. Each assay for fungicide sensitivity was repeated three times.
In order to perform a cytological study, conidia of the wild-type and the os-5 mutant of *N. crassa* were inoculated on the VM agar medium containing fungicides and incubated at 28°C for 20 hr. In *B. cinerea*, conidia of the strains Bc-56 and Bc-DHR3 were cultured on liquid PD medium for 16 hr at 22°C. The resultant hyphae were spotted on the PDA medium containing fungicides and incubated for 12 hr at 22°C. The morphology of the treated *N. crassa* conidia or hyphae of *B. cinerea* was observed using a light microscope (Olympus IX50).

4. Glycerol Accumulation

In order to determine the glycerol content, the conidia of *N. crassa* were cultured in the VM liquid medium for 16 hr, and the mycelial suspension was incubated in the VM liquid medium with chemicals or with 4% NaCl for 4 hr at 25°C. Next, the incubated mycelium was collected by filtration, and extensively sonicated in distilled water, and the glycerol from the mycelia was extracted at 80°C for 5 min. After centrifugation, the glycerol concentration in the supernatant was determined spectrophotometrically at 340 nm by using an UV-glycerol assay kit (Boehringer Mannheim GmbH).12)

### RESULTS

1. Antifungal Activity of Pyrrolnitrin against Fungi

A comparison of the antifungal activity of pyrrolnitrin with that of fludioxonil against several species of fungi is shown in Table 1. Pyrrolnitrin was active against *N. crassa* and *B. cinerea*, and their growth was inhibited by pyrrolnitrin at the concentration of 0.006 μg/ml and 0.025 μg/ml, respectively. *F. oxysporum, F. solani,* and *R. solani* were also highly sensitive to pyrrolnitrin (MIC: 0.1 μg/ml). The antifungal activity of pyrrolnitrin against these fungi was almost at the same level as that of fludioxonil. However, pyrrolnitrin at high dosage showed antifungal activity against oomyceteous fungi; *Py. ultimum* at a MIC of 10 μg/ml and *Ph. capsici* at a MIC of 25 μg/ml, and also an ascomyceteous yeast, *S. cerevisiae* at a MIC of 25 μg/ml. But, fludioxonil did not inhibit their growth even at 25 μg/ml. Thus, the antifungal spectrum of pyrrolnitrin was essentially similar to that of fludioxonil but was broader at a higher dosage.

2. Resistance to Pyrrolnitrin in Neurospora os Mutants

In *N. crassa*, the osmotic sensitive mutants os-1 (NM233t, histidine kinase), os-4 (Y256M223, MAPKK kinase), os-5 (NM216o, MAPK kinase), and os-2 (ALS10, MAP kinase) were highly resistant to fludioxonil and iprodione as described previously.10) These *os* mutants grew on the medium containing 1.6 μg/ml of pyrrolnitrin, while the wild-type strain was sensitive to 0.006 μg/ml of pyrrolnitrin (Table 2). Thus, an obvious cross-resistance between pyrrolnitrin and fludioxonil was observed in all these *os* mutants of *N. crassa*. However, pyrrolnitrin was still active against these *os* mutants at a concentration of 6.1 μg/ml, while fludioxonil and iprodione did not show any antifungal activity against them even at a concentration of 25 μg/ml. This indicates that the cross-resistance pattern of pyrrolnitrin was similar to that of fludioxonil and iprodione, although pyrrolnitrin inhibited the growth of all the strains at a higher dosage.

### Table 1. Antifungal activity of pyrrolnitrin and fludioxonil in various fungal species

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Pyrrolnitrin (MIC (μg/ml))</th>
<th>Fludioxonil (MIC (μg/ml))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Neurospora crassa</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.006</td>
<td>0.013</td>
</tr>
<tr>
<td><em>Botrytis cinerea</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Fusarium solani</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Rhizoctonia solani</em>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Magnaporthe grisea</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td><em>Pythium ultimum</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10</td>
<td>&gt;25</td>
</tr>
<tr>
<td><em>Phytophthora capsici</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25</td>
<td>&gt;25</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25</td>
<td>&gt;25</td>
</tr>
</tbody>
</table>

<sup>a</sup>Minimum inhibitory concentration. <sup>b</sup>Ascomycetes. <sup>c</sup>Basidiomycetes. <sup>d</sup>Oomycetes, chromista. Tests were repeated three times and average values are shown.

### Table 2. Cross-resistance between pyrrolnitrin and fludioxonil in the *os* mutants in *N. crassa*

<table>
<thead>
<tr>
<th>Strains (allele)</th>
<th>Corresponding protein</th>
<th>Pyrrolnitrin (MIC (μg/ml))&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fludioxonil (MIC (μg/ml))&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Iprodione (MIC (μg/ml))&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td></td>
<td>0.006</td>
<td>0.013</td>
<td>3.1</td>
</tr>
<tr>
<td>os-1 (NM233t)</td>
<td>Histidine kinase</td>
<td>6.1</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>os-4 (Y256M223)</td>
<td>MAPKK kinase</td>
<td>25–6.1</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>os-5 (NM216o)</td>
<td>MAPK kinase</td>
<td>6.1</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>os-2 (ALS10)</td>
<td>MAP kinase</td>
<td>6.1</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
</tbody>
</table>

<sup>a</sup>Minimum inhibitory concentration. Tests were repeated three times and average values are shown.
3. Effect of Pyrrolnitrin on Conidial Germination of N. crassa and on Hyphal Elongation in B. cinerea

Cross-resistance between pyrrolnitrin, fludioxonil, and iprodione was observed in N. crassa os mutants; however, pyrrolnitrin inhibited the growth of all the strains at a high dosage. In order to investigate the antifungal mechanism of pyrrolnitrin at a high dosage and compare it with that at a low dosage, cytological observation of conidial germination was carried out (Fig. 2a). At a concentration of 0.1 µg/ml, both fungicides showed similar modes of toxicity against the wild-type strain; Most conidia of the wild-type strain swelled and ruptured without germ tube formation. Fludioxonil induced abnormal morphology even at a concentration of 25 µg/ml. However, pyrrolnitrin at a high dosage (25 µg/ml) caused the rupture of conidia without swelling. When the os-5 conidia were grown in a medium containing fludioxonil, they germinated and elongated normally even at a high dosage (25 µg/ml). In contrast, pyrrolnitrin at a concentration of 25 µg/ml completely inhibited the germination of conidia without any rupture or swelling.

Similar results were obtained in B. cinerea (Fig. 2b). When hyphae in the liquid culture were treated with fungicides, swelling and rupturing were induced by fludioxonil at a concentration of 1.0 µg/ml and 25 µg/ml, and also by pyrrolnitrin at a concentration of 1.0 µg/ml in the wild-type strain. The iprodione-resistant isolate Bc-DHR3 was highly resistant to fludioxonil. However, the treatment with pyrrolnitrin at 25 µg/ml immediately stopped the elongation of the hyphae and induced vacuolation in both the wild-type and the Bc-DHR3 strains. This suggests that the mode of toxicity of pyrrolnitrin at high dosage differs from that at low dosage.

4. Effect of Pyrrolnitrin on Glycerol Synthesis in N. crassa

Fludioxonil (1.0 and 25 µg/ml) increased glycerol levels in the hyphae of the wild-type strain of N. crassa, and the stimulation of glycerol synthesis was eliminated in the os-1 mutant (NM233t), as described previously.11) Pyrrolnitrin also stimulated glycerol biosynthesis in the wild-type strain at 1.0 µg/ml but not in the os-1 mutant (NM233t). However, this pyrrolnitrin-induced glycerol synthesis was decreased at a concentration of 10 µg/ml and almost absent at 25 µg/ml in the wild-type strain. In the os-1 mutant (NM233t), neither fungicide stimulated glycerol synthesis, while a higher dosage of pyrrolnitrin caused a decrease in basal glycerol contents (Fig. 3).

5. Pyrrolnitrin Resistance in the Histidine Kinase os-1 Mutant Alleles

Molecular analysis of iprodione-resistant plant pathogens and other fungi reveals that most mutations were in the sensor region of the os-1 family histidine kinase genes.18–21,24,25,31) Therefore, os-1 histidine kinase is considered to be a potential target for phenylpyrroles and iprodione. Furthermore, the iprodione-resistant Bc-45 strain of B. cinerea, in which a mutation (I365S) occurred in the amino acid region of histidine kinase BcOS1p, did not show cross-resistance to both fludioxonil and pyrrolnitrin (data not shown). We compared the level of resistance (EC50) of the os-1 mutant alleles with a single...
a) The site of mutation and amino acid change in each os-1 mutant. The mutation of each os-1 allele is indicated in the schematic structure of the putative os-1 histidine kinase. The six amino acid repeats, the histidine kinase domain, and the response regulatory domains are indicated by gray boxes, a black box, and a hatched box, respectively.
b) Comparison of EC₅₀ values of fludioxonil with those of pyrrolnitrin in each os-1 mutant. EC₅₀ values of fungicides are plotted on the graph as squares. When the EC₅₀ was more than 25 μg/ml, the mutant was plotted as a black box, and a hatched box, respectively.
c) Comparison of EC₅₀ values of fludioxonil with those of pyrrolnitrin with those of iprodione in each os-1 mutant. EC₅₀ values of fungicides are plotted on the graph as squares. When the EC₅₀ was more than 25 μg/ml, the mutant was plotted as a black box, and a hatched box, respectively.

Phenylpyrrole fungicides, such as fludioxonil and fenpiclonil, are derivatives of pyrrolnitrin, which are produced by various bacteria that exhibit biocontrol activity. Therefore, it is easy to assume that these compounds have the same modes of antifungal activity. The mode of action of pyrrolnitrin has been extensively studied in a highly sensitive model fungus, N. crassa, and it has been reported that pyrrolnitrin has an inhibitory effect on the mitochondrial electron transport system. However, we had previously revealed that fludioxonil affected osmotic signal transduction in N. crassa and B. cinerea. Thus, we compared pyrrolnitrin with fludioxonil and iprodione with regard to their antifungal activity. Pyrrolnitrin and fludioxonil, exhibited a similar antifungal spectrum; they are highly active against N. crassa, B. cinerea, Furarium spp., and R. solani. The antifungal spectrum of pyrrolnitrin essentially resembles that of iprodione, a dicarboximide fungicide, because neither fludioxonil nor iprodione is active against oomyceteous fungi and ascomycetous yeast. However, a high dosage of pyrrolnitrin inhibited the growth of fludioxonil-resistant fungi such as Py. ultimum, Ph. capsici, and S. cerevisiae (Table 1). Fludioxonil and iprodione interfere with the osmotic signal transduction pathway consisting of os-1 histidine kinase, os-4 (MAPKK kinase), os-5 (MAPK kinase), and os-2 (MAP kinase) in N. crassa, and their mutants are resistant to both fungicides. Kojima et al. recently reported that fludioxonil treatment activated the phosphorylation of MAP kinases related to Neurospora OS2p in plant pathogenic fungi, including B. cinerea. An obvious pattern of cross-resistance in these os mutants was also observed in pyrrolnitrin; the MIC values of pyrrolnitrin to os mutants were approximately 1000 times higher than that of pyrrolnitrin to the wild-type strain. However, pyrrolnitrin was active against all os mutants at concentrations ranging from 6.1 to 25 μg/ml, whereas fludioxonil and iprodione did not inhibit the growth of the os mutants even at 25 μg/ml (Table 2). Furthermore, pyrrolnitrin induced abnormal morphology in the germination of conidia of N. crassa and in hyphal elongation of B. cinerea, and also stimulates glycerol synthesis in the wild-type strain of N. crassa. These results indicate that pyrrolnitrin, similar to fludioxonil and iprodione, interferes with the osmotic signal transduction pathway. However, both the abnormal morphology and stimulation of glycerol synthesis disappeared at a higher dosage of pyrrolnitrin. For example, the conidial germination of the os-5 mutant strain was completely inhibited by pyrrolnitrin (25 μg/ml) and no morphological change occurred. The production of glycerol induced by pyrrolnitrin (1 μg/ml) decreased at a concentration of 10 μg/ml and was almost eliminated at a concentration of 25 μg/ml. These results suggest that pyrrolnitrin has another antifungal property at a high dosage, which could be the inhibition of respiration described previously in N. crassa.

The targets of phenylpyroles and iprodione have not yet been identified. However, it has been proposed that these fungicides target a component of the osmosensing histidine kinase pathway. Recently, Motoyama et al. revealed that the os-1 family gene from M. grisea can confer sensitivity to fungicides on S. cerevisiae, although fungicides generally do not have a fungicidal effect on yeast. Resistance to these fungicides is often the result of mutations in the histidine kinases regulating osmotic signal transmission. The os-1 gene mutations confer pleiotropic phenotypes in fungi-
cide resistance and osmotic sensitivity. Mutations of the 10 os-1 alleles have been determined by Ochiai et al. and Miller et al. Miller et al. reported that the os-1 mutants E11200, M155-1, and P5990 have two sites of mutation within the os-1 gene; however, we found only one mutation in each os-1 mutant. Therefore, in this study, we described the mutations of the os-1 alleles, as shown in Fig. 4a. Most mutations are located in the N-terminal amino acid repeat region of os-1 histidine kinase. The levels of resistance to fludioxonil in these os-1 mutants correlated well with those to pyrrolnitrin. Interestingly, the resistance levels of fludioxonil also essentially correlated with those to iprodione, despite the difference in chemical structure between phenylpyrrole and iprodione. Therefore, most mutations of the os-1 gene might affect the function of histidine kinase rather than the binding site of fungicides and cause resistance. However, os-1 E11200 with the mutation M639V was highly resistant to iprodione but moderately resistant to phenylpyrroles. Botrytis filiformis isolates with the mutation I365S and resistant to iprodione did not show cross-resistance to fludioxonil nor pyrrolnitrin. The function of the N-terminal region of os-1 histidine kinase and its correlation with fungicide resistance remain unclear.

These results indicate that the primary antifungal mechanism of pyrrolnitrin is the interference of the osmotic signal transduction pathway, and the secondary mechanism is probably the inhibition of respiration, which is active at a high dosage in N. crassa and other fungi.

REFERENCES