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Characterisation of energy-dependent efflux of imazalil and fenarimol in isolates of Penicillium italicum with a low, medium and high degree of resistance to DMI-fungicides

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Abstract

Differential accumulation of [^{14}C]imazalil and [^{14}C]fenarimol by germlings of wild-type and DMI-resistant isolates of *Penicillium italicum* was studied at various pH values. At pH 7 and 8 the low-resistant isolate $E_{300\text{-}3}$ accumulated 22% and 35%, respectively, less imazalil than the wild-type isolate W_5. Imazalil accumulation at pH 5 and 6 was similar. Isolate $E_{300\text{-}3}$ also accumulated less fenarimol as compared with the wild-type isolate. This difference was much more obvious than for imazalil and was observed at all pH values tested. Differences in accumulation of both imazalil and fenarimol between low ($E_{300\text{-}3}$), medium (H_{17}) and high resistant (I_{33}) isolates were not observed. These results suggest that decreased accumulation of DMIs is responsible for a low level of resistance only and that additional mechanisms of resistance might operate in isolates with a medium and high degree of resistance. With all isolates fenarimol accumulation was energy-dependent. This was not obvious for imazalil.

The wild-type and DMI-resistant isolates had a similar plasma membrane potential as determined with the probe [^{14}C]tetraphenylphosphonium bromide ([^{14}C]TPP$^+$). Various test compounds, among which ATPase inhibitors, ionophoric antibiotics and calmodulin antagonists, affected the accumulation of [^{14}C]TPP$^+$, [^{14}C]imazalil and [^{14}C]fenarimol. No obvious correlation between the effects of the test compounds on accumulation levels of the fungicides and [^{14}C]TPP$^+$ could be observed. These results indicate that the plasma membrane potential does not mediate the efflux of DMI fungicides by *P. italicum*.

Additional keywords: ATPase inhibitors, calmodulin antagonists, fenarimol, imazalil, ionophoric antibiotics, sterol 14α-demethylation inhibitors, tetraphenylphosphonium bromide.

Introduction

Sterol biosynthesis inhibitors (SBIs) are systemic fungicides used in agriculture for the control of plant diseases. The primary mode of action of most SBIs (e.g. derivatives of imidazoles, triazoles and pyrimidines) is based on inhibition of cytochrome P450-dependent sterol 14α-demethylation (Kato, 1986). Therefore, these SBIs are referred to as demethylation inhibitors (DMIs).

Resistance to DMIs has been reported for a number of fungi in both laboratory and

field (Köller and Scheinpflug, 1987; Brent and Hollomon, 1988). Studies with various fungi suggested that a large number of potential mechanisms of resistance can be involved (Walsh and Sisler, 1982; Taylor et al., 1983; Portillo and Gancedo, 1985; Deas, 1986; Hitchcock et al., 1986; Weete, 1986; Watson et al., 1988; Smith and Köller, 1990; Vanden Bossche et al., 1990). A well documented mechanism is energy-dependent efflux of DMI fungicides from mycelium of *Aspergillus nidulans* and *Penicillium italicum*. Increased efflux from fenarimol-resistant mutants of these fungi leads to secretion of DMIs into the external medium and hence prevents intracellular accumulation. Consequently, the target enzyme becomes less readily inhibited and the mutants less sensitive (De Waard and Van Nistelrooy, 1979, 1984, 1987, 1988). The ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide (DCCD) and the plasma membrane ATPase inhibitor sodium orthovanadate were found to inhibit efflux, causing accumulation of all DMIs tested (De Waard and Van Nistelrooy, 1987). This might be a consequence of dissipation of the electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) maintained by plasma membrane ATPase across plasma membranes (De Waard and Van Nistelrooy, 1987). The $\Delta\tilde{\mu}_{H^+}$ is composed of a proton gradient (Δ_{H^+}) and a plasma membrane potential ($\Delta\Psi$) and is known to drive transport processes of various xenobiotics such as benzoic and sorbic acid (Warth, 1977) and aminoglycoside antibiotics (Eisenberg et al., 1984), respectively . Accumulation of the aminoglycoside antibiotic gentamycin by *Staphylococcus aureus* was also found to be enhanced by DCCD (Gilman and Saunders, 1986). DMI- resistant isolates of *A. nidulans* showed cross-resistance to the aminoglycoside antibiotic neomycin (Van Tuyl, 1977). These results suggest that a similar mechanism may mediate the accumulation of DMIs and aminoglycosides. In addition, resistance to aminoglycoside antibiotics in *Saccharomyces cerevisiae* and *Escherichia coli* was found to correlate with decreased intracellular accumulation and was ascribed to a defect $\Delta\Psi$ (Damper and Epstein,1981; Perlin et al., 1988). Therefore, we investigated the $\Delta\Psi$ of wild-type and DMI-resistant isolates of *P. italicum* using [^{14}C]tetraphenylphosphonium bromide ([^{14}C]TPP$^+$), a lipophilic cation, as a probe.

Accumulation of fenarimol and other DMIs, and of imazalil by a wild-type and a fenarimol-resistant isolate of *P. italicum* with a low degree of resistance to imazalil, differed in various aspects (De Waard and Van Nistelrooy, 1988). The main difference was that the differential accumulation by low-resistant isolates was obvious for fenarimol but absent for imazalil. Therefore, in the present study fenarimol and imazalil were selected as test compounds. The study compares the accumulation of these fungicides in isolates of *P. italicum* with a low, medium and high degree of resistance at different pH values. In addition, the effect of compounds with known membrane-interfering properties on accumulation of [^{14}C]TPP$^+$ was compared with their effect on accumulation of both fungicides.

Materials and methods

Fungal isolates. *P. italicum* wild-type isolate W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17} and I_{33} with a low, medium and high degree of resistance, respectively, were used. The isolates have been described previously (De Waard et al., 1982; De Waard and Van Nistelrooy, 1990). The fungus was maintained on malt-extract agar medium.

Culture methods and preparation of germling suspensions. Germlings were grown in liquid malt-extract medium (De Waard and Van Nistelrooy, 1984). Flasks (300 ml) with medium (100 ml) were inoculated with 1 ml of spore suspension (10^9 conidia per ml) collected from 7–10-day-old agar plates. The flasks were incubated at 26 °C on an orbital shaker at 200 rpm for 11 h. Germlings were harvested on a sieve (0.21 mm pores) and collected on a second sieve (0.02 mm pores) by intensive washing with tap water. Germlings were washed once again with 25 mM potassium phosphate buffer pH 7.0, containing 0.1 mM calcium chloride and 1% (w/v) glucose. Standard suspensions of germlings with an average dry weight of about 2 mg ml^{-1} were prepared by resuspending 1 g wet weight of germlings in 50 ml of the same buffer.

Fungicides and chemicals. [^{14}C]imazalil and imazalil sulphate (imazalil) were gifts from Janssen Pharmaceutica (Beerse, Belgium); [^{14}C]fenarimol from Lilly Research Center Ltd. (Erl Wood Manor, England). [^{14}C]tetraphenylphosphonium bromide ([^{14}C]TPP^+) was purchased from Amersham (UK), TPP^+ from ICN Pharmaceuticals Inc. (New York, USA); calmidazolium, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), chlorpromazine, diethylstilbestrol (DES), gramicidine-S, nigericine and triflupromazine (TFP) from Sigma (Deisenhofen, Germany). Sodium orthovanadate (vanadate) and valinomycine from Janssen Chimica (Beerse, Belgium). Fungicides and chemicals were prepared as 100-fold concentrated solutions in methanol.

Determination of plasma membrane potential ($\Delta\Psi$). Standard germling susensions (70 ml) in flasks (300 ml) were incubated on a reciprocal shaker at 26 °C for 30 min [^{14}C]TPP^+ (sp. act. 17.3 MBq $mmol^{-1}$), a commonly used probe for assessing the $\Delta\Psi$ (Kashket, 1985), was added to a final concentration of 10 μM. At time intervals, samples (5 ml) were taken and filtered on a Whatman GF/A glass filter using a Millipore sampling manifold apparatus. Pellets of germlings were washed three times with 1 mM $MgSO_4$ (5 ml) in 10 s. This washing step reduces aspecific binding of [^{14}C]TPP^+ to anionic groups at the cell wall surface of the germlings (Kashket, 1985). A correction of [^{14}C]TPP^+ accumulation for internal, aspecific probe binding was made by subtracting the amount of probe bound under deenergized conditions from the total amount of accumulated probe under energized conditions (Lolkema et al., 1982, 1983; Kashket, 1985). In preliminary experiments, remaining background adsorption of [^{14}C]TPP^+ by deenergized germlings was studied after various treatments: incubation with 0.2 M KCl for 50 min, incubation without carbon source in the presence of 0.2 or 1.0 M KCl for 6 h, heating at 100 °C for 5 min, and incubation with 1% (v/v) toluene at 37 °C or 50 °C for 1 h according to Lolkema et al. (1983). Germling- associated radioactivity was extracted with scintillation liquid (Aqua luma plus) overnight and counted in a liquid scintillation spectrometer. After correction of [^{14}C]TPP^+ accumulation for aspecific probe binding the $\Delta\Psi$ was estimated with the Nernst equation (Kashket, 1985). The intracellular volume of *P. italicum* was taken to be 2.3 μl mg^{-1} dry weight of germlings (De Waard and Van Nistelrooy, 1988). Effects of test compounds on accumulation of [^{14}C]TPP^+ were studied by addition from 500-fold concentrated stock solutions in methanol, 50 min after the addition of [^{14}C]TPP^+. Accumulation of [^{14}C]TPP^+ was determined in samples (5 ml) taken 5 and 15 min after addition of the test chemicals. The correspon-

dent amount of methanol was added to the controls. In these experiments, [^{14}C]TPP$^+$ accumulation in the presence of test compounds was expressed as nmol mg^{-1} dry weight. The corresponding $\Delta\Psi$ values were not calculated, since the effect of these test compounds on aspecific probe binding was not determined.

Accumulation of [^{14}C]imazalil and [^{14}C]fenarimol. Experiments were carried out according to the method previously described (De Waard and Van Nistelrooy, 1984). Standard germling suspensions were shaken on a reciprocal shaker at 26 °C for 30 min. Concentrations of [^{14}C]imazalil (sp. act. 11.1 MBq mmol^{-1}) and [^{14}C]fenarimol (sp. act. 5.6 MBq mmol^{-1}) in the germling suspensions were 10 and 90 μM, respectively. The final methanol concentration in the suspension was below 1%. Accumulation of DMIs was determined in germlings collected from 5-ml samples and washed five times with buffer (5 ml) in 30 s. Further reduction of background adsorption of the DMIs failed since experimental conditions or chemicals tested which led to deenergization of the germlings gave a significant increase in accumulation of the DMIs. Test chemicals were added to the germling suspension after 40 min of incubation with [^{14}C]imazalil and [^{14}C]fenarimol. Effects of the test compounds on fungicide accumulation were determined 10 min after their addition to the suspension.

Results

Determination of plasma membrane potential. Accumulation of [^{14}C]TPP$^+$ by germlings was relatively fast in the initial 10 min and reached a stationary phase in about 50 min. The total amount of [^{14}C]TPP$^+$ accumulated in the stationary phase was similar for isolates W_5, $E_{300\text{-}3}$ and H_{17} (Fig. 1). In preliminary experiments background adsorption of [^{14}C]TPP$^+$ to germlings was determined according to various

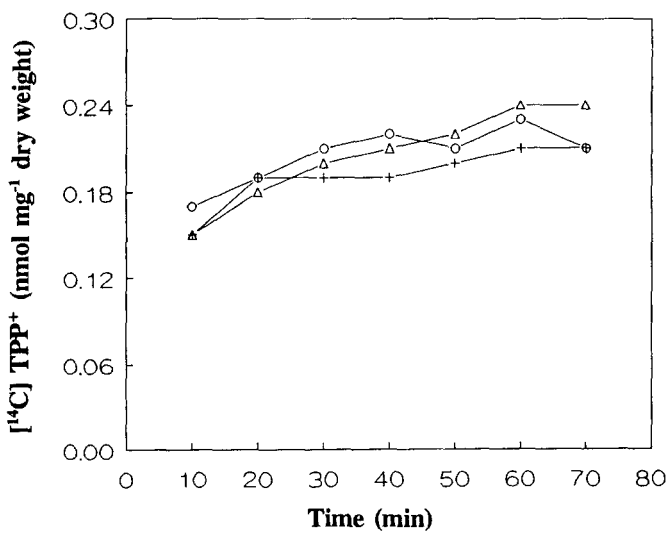

Fig. 1. Accumulation of [^{14}C]TPP$^+$ (10 μM) by wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (Δ) and H_{17} ($\bigcirc$) of *Penicillium italicum* in 23.4 mM potassium phosphate buffer pH 7, containing 0.1 mM calcium chloride and 1% (w/v) glucose.

Table 1. Relative effect of various treatments of germlings on accumulation of [^{14}C]TPP$^+$ (10 μM) by *Penicillium italicum* isolates W_5, $E_{300\text{-}3}$ and H_{17}

Treatment	Relative effect of treatment on accumulation level[a,b]		
	W_5	$E_{300\text{-}3}$	H_{17}
Heating (100 °C, 5 min)	1.0	1.3	1.3
Toluene (1% (v/v), 37 °C, 1 h)	4.4	4.2	–[c]
Toluene (1% (v/v), 50 °C, 1 h)	0.7	0.7	0.8
KCl (0.2 M, 50 min)	0.8	0.8	0.8
Starvation[d] and KCl (0.2 M)	0.6	0.6	0.6
Starvation and KCl (1.0 M)	0.3	0.3	0.3

[a] Average accumulation levels of [^{14}]TPP$^+$ in untreated germlings of isolates W_5, $E_{300\text{-}3}$ and H_{17} under equilibrium conditions were 0.25 ± 0.04, 0.27 ± 0.04 and 0.29 ± 0.06 nmol mg^{-1} dry weight, respectively (n = 6).

[b] Ratio between acumulation level in treated and untreated germlings.

[c] Not tested.

[d] Incubation without glucose in orbital shaker at 180 rpm for 6 h.

methods. Heat treatment of germlings slightly increased [^{14}C]TPP$^+$ accumulation (Table 1). Germlings treated with toluene at 37 °C accumulated approximately 4-fold more [^{14}C]TPP$^+$ than untreated germlings. In contrast, treatment of germlings with toluene at 50 °C slightly reduced accumulation of the probe, as did the incubation of germlings with 0.2 M KCl for 50 min (Table 1). Germlings which were deenergized by starvation for 6 h in the presence of 1.0 M KCl showed the lowest level of [^{14}C]TPP$^+$ accumulation, suggesting a background adsorption of about 30% (Table 1). This percentage was used to correct $\Delta\Psi$ for internal, aspecific probe binding. The $\Delta\Psi$ values under equilibrium conditions were calculated to be 55.9 ± 4.3, 58.1 ± 4.3 and 59.8 ± 6.7 mV (n = 6) for isolates W_5, $E_{300\text{-}3}$ and H_{17}, respectively.

Accumulation of [^{14}C]imazalil and [^{14}C]fenarimol. Accumulation of [^{14}C]imazalil by germlings of the wild-type isolate W_5 was pH-dependent, gradually reached a stable level in about 30 min and increased with pH, except for pH 8 (Fig. 2). At pH 5 and 6 [^{14}C]imazalil accumulations by W_5 and the low-resistant isolate $E_{300\text{-}3}$ were similar, but at pH 7 and 8 accumulation by isolate $E_{300\text{-}3}$ was 22 and 35% less compared to the wild-type isolate (Fig. 2). No differential accumulation of [^{14}C]imazalil among isolates $E_{300\text{-}3}$, H_{17} and I_{33} was found (Fig. 3). The accumulation pattern of [^{14}C]fenarimol by wild-type isolate W_5 at pH 7 (Fig. 4) was similar to the typical transient accumulation curve of [^{14}C]fenarimol demonstrated previously by De Waard and Van Nistelrooy (1984), who used 16-h-old mycelium. A minor difference was that in the present experiments equilibrium in accumulation was obtained less readily. Accumulation of [^{14}C]fenarimol was not significantly affected by changes of extracellular pH (results not shown). The accumulation of [^{14}C]fenarimol by resistant isolate $E_{300\text{-}3}$ was much lower than for wild-type isolate W_5 (Fig. 3). No differential accumulation of [^{14}C]fenarimol among isolates $E_{300\text{-}3}$, H_{17} and I_{33} was perceived. For all following experiments pH 7 was selected as a standard pH.

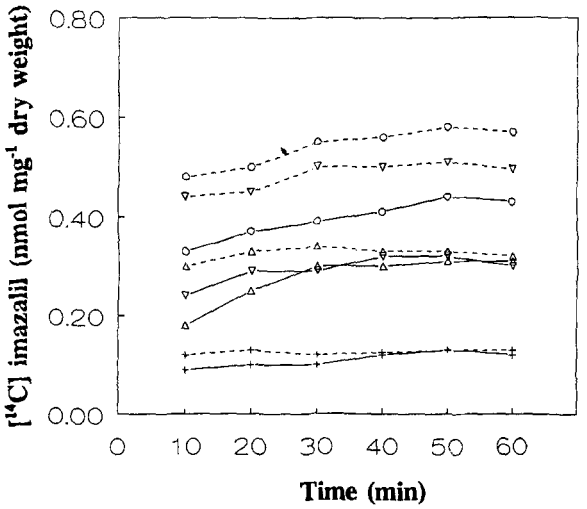

Fig. 2. Effect of external pH on accumulation level of [^{14}C]imazalil (10 μM) by wild-type isolate W_5 (----) and DMI-resistant isolate $E_{300\text{-}3}$ (——) of *Penicillium italicum* in 23.4 mM potassium phosphate, containing 0.1 mM calcium chloride and 1% (w/v) glucose at pH 5 (+), 6 (Δ), 7 ($\bigcirc$) and 8 (∇).

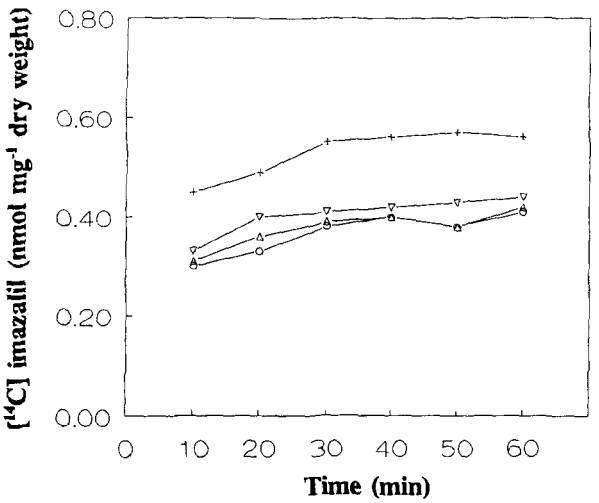

Fig. 3. Accumulation of [^{14}C]imazalil (10 μM) by wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (Δ), H_{17} ($\bigcirc$) and I_{33} (∇) of *Penicillium italicum* in 23.4 mM potassium phosphate buffer pH 7, containing 0.1 mM calcium chloride and 1% (w/v) glucose.

Effects of chemicals on accumulation of [^{14}C]TPP$^+$, [^{14}C]imazalil and [^{14}C]fenarimol. Differential accumulation of [^{14}C]imazalil and [^{14}C]fenarimol was observed between wild-type W_5 and low-resistant isolate $E_{300\text{-}3}$ only. Therefore, these two isolates were selected for experiments in which the effects of chemicals with known membrane-interfering properties on accumulation of [^{14}C]TPP$^+$ and both

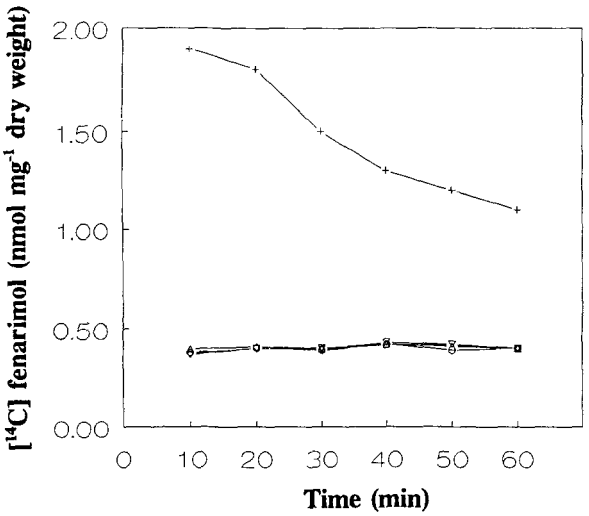

Fig. 4. Accumulation of [^{14}C]fenarimol (90 μM) by wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (Δ), H_{17} (○) and I_{33} (∇) of *Penicillium italicum* in 23.4 mM potassium phosphate buffer pH 7, containing 0.1 mM calcium chloride and 1% (w/v) glucose.

[^{14}C]DMIs were studied. The chemicals selected were the protonophoric uncoupler CCCP, the membrane ATPase inhibitors DES and vanadate, the calmodulin anatagonists chlorpromazine and TFP, and the ionophoric antibiotics gramicidine-S, nigericine and valinomycine. CCCP, DES, gramicidine- S and vanadate caused a slight increase in accumulation of [^{14}C]TPP$^+$ in both isolates (Table 2). The effect of TFP was more pronounced, but became weaker in time. Chlorpromazine slightly inhibited accumulation of [^{14}C]TPP$^+$, while nigericine and valinomycine hardly showed any effect. On the other hand, all test compounds used strongly enhanced accumulation of [^{14}C]fenarimol in both isolates (Table 2). The relative effect of most compounds on accumulation by isolate $E_{300\text{-}3}$ was higher than by isolate W_5. The effect of the test compounds on accumulation of [^{14}C]imazalil was generally much weaker and not comparable with that of [^{14}C]fenarimol. Only CCCP and DES caused a small increase in accumulation, whereas all other compounds tested led to a slightly lower accumulation level (Table 2).

Discussion

Energy-dependent efflux of a number of DMIs from DMI-resistant isolates of *A. nidulans* and *P. italicum* has been well documented (De Waard and Van Nistelrooy, 1979, 1984, 1987, 1988). The increased efflux of DMIs in resistant isolate $E_{300\text{-}3}$, which is high-resistant to fenarimol and low-resistant to imazalil, is probably responsible for a lower level of accumulation at which the DMIs do not readily saturate the target enzyme in sterol biosynthesis. Imazalil was the only exception among DMIs, since no differential accumulation between 16-h-old mycelium of sensitive and resistant isolates could be observed (De Waard and Van Nistelrooy, 1988). However, the present study indicates that a significant difference in accumulation could be

Table 2. Effect of various test compounds on accumulation of [^{14}C]imazalil (10 μM), [^{14}C]fenarimol (90 μM) and [^{14}C]TPP^{+} (10 μM) by germlings of *Penicillium italicum* isolates W_5 and $E_{300\text{-}3}$ at pH 7.0.

Fungal isolate	Compound	Concentration (μm)	Relative effect of chemicals on accumulation[a]			
			Imazalil	Fenarimol	TPP[b]	
					t = 5	t = 15
W_5	Calmidazolium	10	0.8[c]	2.7[c]	–[d]	–
		25	0.8	3.2	–	–
	CCCP	50	1.8	4.5	1.2	1.5
		100	1.2	5.2	1.6	1.7
	Chlorpromazine	10	0.5	1.4	–	–
		50	0.7	3.9	–	–
		100	0.6	2.9	0.9	0.9
	DES	10	0.9	2.2	–	–
		50	1.3	4.7	1.0	1.3
		100	1.4	3.0	1.3	1.7
	TFP	10	–	1.8	–	–
		50	0.7	5.0	1.7	1.4
		100	0.8	2.9	2.2	1.9
	Gramicidine – S	50	–	–	1.5	1.2
		100	–	1.3	1.6	1.5
	Nigericine	100	–	2.7	–	1.0
	Valinomycine	100	–	2.0	–	1.0
	Vanadate	100	–	2.0	1.1	1.2
E_{300-3}	Calmidazolium	10	0.8	4.6	–	–
		25	0.8	6.5	–	–
	CCCP	50	1.7	7.9	1.2	1.5
		100	1.5	–	1.5	1.7
	Chlorpromazine	10	0.7	3.0	–	–
		50	0.9	7.8	–	–
		100	0.5	1.1	0.6	0.6
	DES	10	1.0	1.1	–	–
		50	1.2	7.7	1.2	1.5
		100	1.3	7.1	1.3	1.8
	TFP	10	–	4.8	–	–
		50	0.7	12.7	1.0	0.8
		100	0.6	9.2	1.5	1.0
	Gramicidine-S	50	–	–	1.3	1.3
		100	–	3.8	1.4	1.3
	Nigericine	100	–	14.6	–	0.9
	Valinomycine	100	–	5.7	–	0.9
	Vanadate	100	–	1.6	1.2	1.2

[a] Average accumulation levels of [^{14}C]imazalil, [^{14}C]fenarimol and [^{14}C]TPP^{+} in untreated mycelium 50 min after adding the radiolabeled compounds were 0.58 ± 0.05, 1.10 ± 0.07 and 0.26 ± 0.04 nmol mg^{-1} dry weight mycelium for isolate W_5 and 0.40 ± 0.05, 0.42 ± 0.04 and 0.28 ± 0.05 nmol mg^{-1} dry weight mycelium for isolate $E_{300\text{-}3}$, respectively.

observed if 11-h-old germlings were used and the pH of the external medium was 7.0 or higher (Fig. 1). Mycelium (16-h-old) probably accumulates more aspecifically bound imazalil than germlings and may, therefore, mask small differences in accumulation. In this respect, it is noted that the toxicity of imazalil to 11-h-old germlings of *P. italicum* in liquid medium is relatively high as compared to its toxicity to 16-h-old mycelium in liquid cultures, but comparable to its fungicidal activity determined in radial growth experiments. (Guan et al., 1989). In addition, imazalil has a pK_a value of 6.5. Hence, at pH 7.0 it is mainly in a neutral form and accumulation may not be affected by the presence of its protonated form as compared with pH 6.0 used in previous studies (De Waard and Van Nistelrooy, 1988). Fenarimol is in a neutral form at all pH values tested. This probably explains why the external pH does not affect accumulation of this fungicide.

Increase in [^{14}C]fenarimol accumulation by the protonophoric uncoupler CCCP, the membrane ATPase inhibitors DES and vanadate, the calmodulin antagonists chlorpromazine and TFP, and the ionophoric antibiotics gramicidine- S, nigericine and valinomycine (Table 2), confirms previous results that a mechanism of energy-dependent efflux is probably involved in accumulation of fenarimol and other DMIs (De Waard and Van Nistelrooy, 1987, 1988). In contrast to fenarimol, accumulation of imazalil only slightly increased upon incubation with CCCP and DES while all other test chemicals decreased imazalil uptake (Table 2). *P. italicum* wild-type W_5 also did not exhibit transient accumulation of imazalil in time as demonstrated for fenarimol and other DMIs (Fig. 2; De Waard and Van Nistelrooy, 1988; Kapteyn et al., 1992). These results suggest that imazalil accumulation is not or only slightly mediated by a mechanism of energy-dependent efflux. It might be that the energy-dependent efflux of imazalil is hampered by intracellular protonation of the fungicide.

Isolates $E_{300\text{-}3}$, H_{17} and I_{33} with a low, medium and high degree of resistance to DMIs, respectively, did not show differential accumulation of fenarimol and imazalil (Figs. 2 and 3). An explanation is that increased resistance in isolates H_{17} and I_{33} is based on other mechanisms, such as decreased affinity of the target site for the toxicant. Alternatively, it might also be that relatively high background adsorption of the fungicides masks any further decrease in accumulation in medium- and high-resistant isolates.

The lipophilic cation TPP^+ is a commonly used probe for measuring $\Delta\Psi$ (Kashket, 1985). Preliminary experiments showed that various methods to determine the background adsorption of the probe by deenergized germlings did not provide comparable results (Table 1). This finding is in agreement with data from Lolkema et al. (1983). It is noted that toluene treatment of the germlings at 37 °C, a standard method used by them to determine the background adsorption of [^{14}C]TPP^+ to bacterial cells, was not useful (Table 1). Apparently, different methods have to be used

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Footnotes Table 2 (contd)

[b] Relative effect measured 5 and 15 min after addition of the test compounds.

[c] Ratio between accumulation level in treatments and controls, determined 10 min after addition of the test compounds.

[d] Not tested.

Abbreviations used: DES (diethylstilbestrol), CCCP (carbonyl cyanide 3-chlorophenylhydrazone), TFP (trifluprom azine), TPP^+ (tetraphenylphosphonium bromide).

for different organisms. In our tests starvation of germlings in the presence of 1 M KCl for 6 h led to the lowest accumulation levels of the probe. With this method about 30% of the accumulated probe remained associated with the deenergized germlings (Table 1). This value was arbitrarily chosen to correct the amount of [^{14}C]TPP$^+$ accumulated for aspecific probe binding. The $\Delta\Psi$ values were calculated to be 55.9 ± 4.3, 58.1 ± 4.3 and 59.8 ± 6.7 mV for isolates W_5, $E_{300\text{-}3}$ and H_{17}, respectively. These results indicate that the isolates do not differ in $\Delta\Psi$.

The relative effects of various compounds on the accumulation of [^{14}C]TPP$^+$ by *P. italicum* isolates W_5 and $E_{300\text{-}3}$ were also studied. The effects observed were marginal (Table 2). For example, CCCP, DES, gramicidine-S and vanadate only enhanced accumulation of [^{14}C]TPP$^+$ with a factor below 2.0, while nigericine and valinomycine hardly had any effect (Table 2). There is no obvious explanation for the fact that addition of CCCP and DES resulted in an increased accumulation of [^{14}C]TPP$^+$, which was unexpected from their known effects on membrane processes. No correlation between the effects of compounds on accumulation of [^{14}C]TPP$^+$ and both fungicides could be observed. For example, accumulation of [^{14}C]fenarimol could be significantly enhanced by test compounds, such as nigericine and valinomycine, which hardly influenced accumulation of [^{14}C]TPP$^+$ (Table 2). Therefore, the results suggest that the $\Delta\Psi$ is not involved in accumulation of fenarimol and imazalil by germlings of wild- type and DMI-resistant isolates of *P. italicum*.

Energy-dependent efflux of DMI fungicides (except for imazalil) in fungi resembles in several aspects multi-drug resistance (MDR) in mammalian cancer cells (Bradley et al., 1988). Firstly, these mammalian MDR cells are cross-resistant to structurally non-related drugs. DMI-resistant isolates of *A. nidulans*, *Botrytis cinerea* and *Ustilago avenae* also show cross resistance to unrelated fungitoxicants and antibiotics such as cycloheximide, acriflavine, neomycin, 8-azaguanidine, thiourea and nikkomycin (Leroux et al., 1976; De Waard and Gieskes, 1977; Van Tuyl, 1977; Krämer et al., 1987). In this respect, it is worthwile to investigate whether the DMI-resistant isolates of *P. italicum* are also cross resistant to unrelated fungitoxic xenobiotics. Secondly, in both types of organism resistance is based on reduced accumulation of compounds mediated by energy-dependent efflux. Thirdly, the accumulation of both anticancer drugs and DMI fungicides can be reversed by calmodulin antagonists such as TFP, chlorpromazine and calmidazolium (Table 2; Endicott and Ling, 1989). In addition, cycloheximide which reversed drug accumulation in MDR cells (Gottesman and Pastan, 1988) also increased accumulation of fenarimol in *A. nidulans* (De Waard, unpublished results). These lines of similarity suggest that DMI resistance in fungi may be based on a similar mechanism as present in MDR mammalian cancer cells. This topic will be the subject of future studies.

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