Neth. J. Pl. Path. 86 (1980) 251–258

# Mechanism of resistance to pyrazophos in Pyricularia oryzae

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Accepted 7 February 1980

**Abstract**

Pyrazophos-resistant strains of *Pyricularia oryzae* were isolated from sectors in colonies of the fungus on a pyrazophos-containing agar medium. In contrast to the wild-type strain, resistant strains did not metabolize pyrazophos (Afugan, Curamil) into its phosphate analogue and 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine (PP), which is regarded as the fungitoxic principle of pyrazophos. Resistant strains possessed a slightly increased sensitivity to PP. These data indicate that the mechanism of resistance to pyrazophos is not related to a change of the target sites of PP, but rather with inability to convert pyrazophos into PP. The pyrazophos-resistant strains displayed cross-resistance to the organophosphorus fungicides EBP (Kitazin) and edifenphos (Hinosan). The mechanism of cross-resistance involved is not understood.

*Additional keywords:* fungicide-resistance, pyrazophos (Afugan), EBP (Kitazin), edifenphos (Hinosan).

## Introduction

The organophosphorus fungicide pyrazophos[1] (Afugan, Curamil, Hoe 2873) is used for protective and curative control of powdery mildew diseases (Mariouw Smit, 1969). In addition the fungicide is also active against *Pyricularia oryzae* (De Waard, 1974).

*P. oryzae* converts pyrazophos into two fungitoxic breakdown products, PO-pyrazophos and 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)-pyrimidine (PP). The latter compound is believed to be the fungitoxic principle. The ability of a fungus to convert the fungicide into PP seems to determine its selective fungicidal action, since two pyrazophos-insensitive fungi, *Pythium debaryanum* and *Saccharomyces cerevisiae* did not metabolize pyrazophos (De Waard, 1974).

The aim of the present work was to investigate whether resistance to pyrazophos may develop in vitro and if so, to study the mechanism of resistance in relation to the

[1]Chemical names of fungitoxicants: BPA, *O,O*-dibutyl *N*-methyl *N*-phenylphosphoramidate; EBP, *O,O*-diethyl *S*-benzyl phosphorothioate; IBP, *O,O*-diisopropyl *S*-benzyl phosphorothioate; edifenphos, *O*-ethyl *S,S*-diphenyl phosphorodithioate; pyrazophos, *O,O*-diethyl *O*-(5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidin-2-yl) phosphorothioate; PO-pyrazophos, *O,O*-diethyl *O*-(5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidin-2-yl) phosphate; PP, 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine.

metabolic conversion of pyrazophos. In addition, it was investigated whether resistance to pyrazophos was coupled with cross-resistance to the related organophosphorus fungicides EBP (Kitazin) and edifenphos (Hinosan).

## Material and methods

*Fungus. Pyricularia oryzae* strain CBS 433.70 was used and maintained on Gly agar medium containing 2% glucose and 0.5% yeast extract.

*Chemicals.* Pyrazophos, [$^{14}$C]-2-pyrazophos, the phosphate analogue of pyrazophos, and PP were generously supplied by Farbwerke Hoechst A.G., Frankfurt, Germany, EBP and edifenphos were kindly provided by Kumiai Chemical Industry Co., Japan, and Bayer A.G., Leverkusen, Germany, respectively.

*Radial growth test.* Toxicity of fungitoxicants was measured in radial growth tests by adding the chemicals from stock solutions in acetone to the agar; the final solvent concentration in the agar was always 1%. Agar plates were inoculated by placing 5 mm-agar discs with young mycelium upside down on the agar surface. Radial growth was measured in duplicate after incubation for seven days at 25°C.

*Metabolic conversion of pyrazophos.* Standard mycelial suspensions of *P. oryzae* were prepared from 3-day-old vigorously growing cultures in a liquid 2% glucose, 0.5% yeast extract medium (De Waard, 1974). Suspensions (10 ml) were incubated with $10^{-3}$ M [$^{14}$C] pyrazophos (spec. act. 7 μCi/mmol) and 50 μg/ml terramycin on an orbital shaker at 180 rpm and 25°C for 12, 24, and 48 h. Then, cultures were acidified with perchloric acid (PCA) to a final concentration of 0.5% and after 30 min centrifuged at 3000 rpm for 5 min. The supernatants (culture media) were decanted and subsequently extracted four times with equal volumes of toluene. The toluene fractions were combined, concentrated to a small volume and spotted on silicagel F254 thin-layer plates (Merck). Plates were developed using benzene/acetone 3:1 as a solvent. Radioactivity in the chromatograms was scanned using an Actigraph III. Plates were bioassayed for fungitoxic chemicals by spraying them with spore suspensions of *Cladosporium cucumerinum* in a nutrient medium (Homans and Fuchs, 1970). Radioactivity in fungitoxic spots was measured quantitatively again by transferring non-overgrown silicagel to vials with scintillation liquid and by counting in a Nuclear Chicago Mark I liquid scintillation spectrometer (De Waard, 1974).

## Results

*Isolation of pyrazophos-resistant strains.* Discs with mycelium of *P. oryzae* were transferred to Gly agar containing $10^{-3}$ M pyrazophos, which is slightly less than the minimal inhibitory concentration. After one week of incubation small colonies were formed, which all developed relatively fast growing sectors in the next two to three weeks. Mycelium of different sectors (strains I, II, and III) was subcultured first three times on agar with $10^{-3}$ M pyrazophos and subsequently at least 10 times on fungicide-free agar for more than one year. During this period radial growth of the strains on fungicide-free medium always proved to be slower than that of the wild-type strain (Table 1). Sporulation of all mutated strains was lower compared to the wild-type

Table 1. Radial growth of the wild-type W and pyrazophos-resistant strains I, II, III of *Pyricularia oryzae* on Gly agar with $10^{-3}$ M pyrazophos.

| | W | | I | | II | | III | |
|---|---|---|---|---|---|---|---|---|
| | mm | % | mm | % | mm | % | mm | % |
| Gly – pyrazophos | 34.5 ± 3.6[1] | 100[3] | 27.1 ± 2.7 | 78.6[3] | 23.1 ± 1.6 | 67.2[3] | 24.3 ± 2.9 | 70.4[3] |
| Gly + pyrazophos | 3.7 ± 0.3[2] | 10.7[4] | 15.3 ± 0.4 | 56.4[4] | 14.7 ± 4.5 | 63.4[4] | 17.9 ± 0.1 | 73.7[4] |

[1] Average of four experiments.
[2] Average of two experiments.
[3] Percentage of growth of wild-type strain.
[4] Percentage of growth of non-treated control.

*Tabel 1. Radiale groei van het wild-type W en pyrazofos-resistente stammen I, II, III van Pyricularia oryzae op Gly agar met $10^{-3}$ M pyrazofos.*

strain. No quantitative assessment was made. All strains isolated from sectors showed a decreased sensitivity to the fungicide (Table 1) and may, therefore, be regarded as pyrazophos-resistant strains.

*Toxicity of pyrazophos and PP.* The wild-type and pyrazophos-resistant strains I, II, and III were tested in radial growth tests for their sensitivity to pyrazophos. $ED_{50}$ values, calculated from dosage-response curves (Fig. 1) are given in Table 2. Radial

Fig. 1. Dosage-response curves of pyrazophos, 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo-(1,5-a) pyrimidine (PP), EBP, and edifenphos for radial growth of the wild type W (0) and pyrazophos-resistant strains I (●), II (□), III ( ■) of *Pyricularia oryzae* on Gly agar.

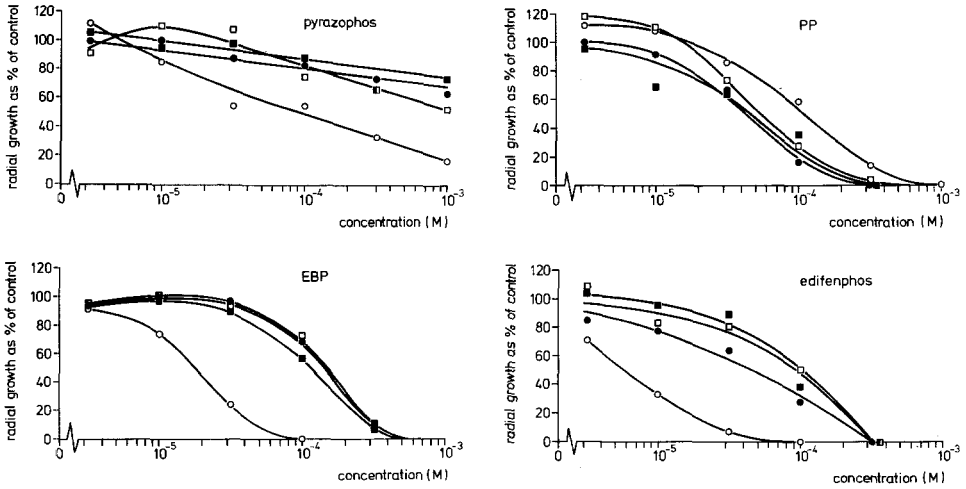


*Fig. 1. Dosis-respons curven van pyrazofos, 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo-(1,5-a)-pyrimidine (PP), EBP, en edifenfos voor radiale groei van het wild-type W (0) en pyrazofos-resistente stammen I (●), II (□), III ( ■) van Pyricularia oryzae op Gly agar.*

Table 2. $ED_{50}$ values of pyrazophos, 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)-pyrimidine (PP), EBP, and edifenphos to radial growth of the wild-type W and pyrazophos-resistant strains I, II, III of *Pyricularia oryzae* on Gly agar.

| Strain | $ED_{50}$ (μM) | | | |
|---|---|---|---|---|
| | pyrazophos | PP[1] | EBP | edifenphos |
| W | 90 | 100 | 6 | 18 |
| I | > 1000 | 43 | 45 | 108 |
| II | ⩾1000 | 54 | 94 | 110 |
| III | > 1000 | 46 | 105 | 103 |

[1] Gly agar contained 0.05 McIlvaine buffer, pH 4.5.

*Tabel 2. $ED_{50}$ waarden van pyrazofos, 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)-pyrimidine (PP), EBP en edifenfos voor radiale groei van het wild-type W en pyrazofos-resistente stammen I, II, III van Pyricularia oryzae op Gly agar.*

growth tests with PP were performed in a similar way using Gly agar with 0.05 M McIlvaine buffer, pH 4.5, since fungitoxicity of PP can only be demonstrated at low pH values (De Waard, 1974). $ED_{50}$ values of PP calculated from dosage-response curves (Fig. 1) are given in Table 2.

*Cross-resistance to EBP and edifenphos.* EBP and edifenphos are organophosphorus fungicides in practical use against *P. oryzae* on rice. It seemed of interest to test the toxicity of these chemicals to the pyrazophos-resistant strains isolated. Radial growth tests (Fig. 1; Table 2) showed that resistance to pyrazophos was accompanied with decreased sensitivity to both fungicides.

*Metabolic conversion of pyrazophos.* Mycelial suspensions of wild-type and pyrazophos-resistant strain III were incubated with $10^{-3}$ M [$^{14}$C] pyrazophos. After 12, 24, and 48 h of incubation the culture media were analyzed for the presence of pyrazophos and metabolic conversion products by thin-layer chromatography (TLC). UV-absorbing spots in the chromatograms were detected by UV-light. TLC-radioscans showed that radioactivity in chromatograms of the wild-type strain was present in spots which co-chromatographed with pyrazophos, PO-pyrazophos, and PP. In addition radioactivity was also detected at the origin in chromatograms of samples taken after 24 and 48 h of incubation (Fig. 2). In chromatograms of pyrazophos-resistant strain III UV-light absorbing or radioactive spots were only detected which co-chromatographed with pyrazophos itself (Fig. 2). In order to demonstrate fungitoxic chemicals in the chromatograms, plates were bio-assayed with *Cladosporium cucumerinum*. This test-fungus is sensitive to PO-pyrazophos and PP, but insensitive to pyrazophos, probably because of inability to convert pyrazophos into toxic breakdown products. Results (Fig. 3) confirmed the formation of fungitoxic amounts of PO-pyrazophos and PP only by the wild-type strain. Experiments with strains I and II carried out with unlabelled pyrazophos gave similar results. The fungitoxic spot present in chromatograms of resistant strain III (Fig. 3) was not visible

Fig. 2. TLC-radioscans of culture media of the wild-type and pyrazophos-resistant strain III of *Pyricularia oryzae*, incubated for 12, 24 and 48 h with $10^{-3}$ M [$^{14}$C]pyrazophos in Gly medium. Control: $10^{-3}$ M [$^{14}$C]pyrazophos incubated for 48 h in medium. Figures in scans indicate radioactivity recovered in pyrazophos (PS), the phosphate analogue of pyrazophos (PO), 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine (PP), and non-identified metabolites present at the origin.

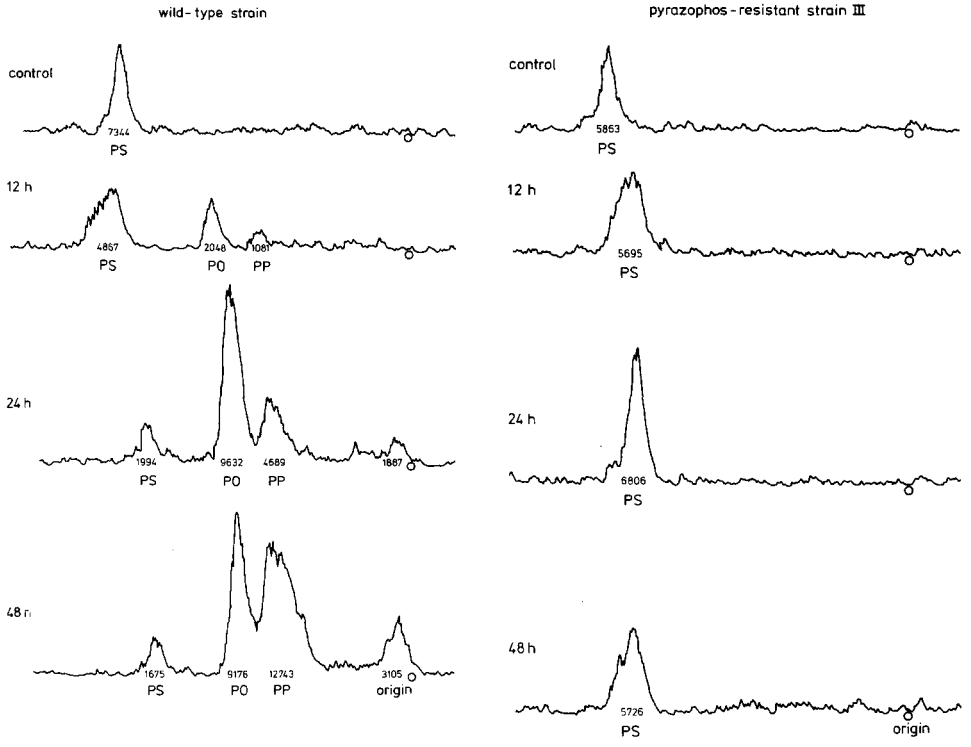


*Fig. 2. TLC-radioscans van cultuurmedia van het wild-type en de pyrazofos-resistante stam III van Pyricularia oryzae, geïncubeerd gedurende 12, 24 en 48 h met $10^{-3}$ M [$^{14}$C] pyrazofos in Gly medium. Controle: $10^{-3}$ M [$^{14}$C]pyrazofos gedurende 48 h in medium geïncubeerd. Getallen in de scans geven de radioactiviteit in dpm teruggevonden in pyrazofos (PS), de fosfaatanaloog van pyrazofos (PO), 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo (1,5-a)pyrimidine (PP) en in niet-geïndentificeerde metabolieten, aanwezig op de opbrengplaats.*

under UV-light and did not co-chromatograph with pyrazophos or anyone of its metabolites since no radioactivity in this spot could be detected. It might be due to picolinic acid or piricularin, toxins sometimes formed by *P. oryzae* (De Waard, 1974).

## Discussion

Resistance to pyrazophos in strains of *P. oryzae* isolated from sectors in colonies on pyrazophos-containing agar seems to have a genetic basis since it appeared to be stable for more than one year during culturing on fungicide-free medium. Resistance to pyrazophos has also been found in *Sphaerotheca fuliginea* (Dekker and Gielink, 1979)

Fig. 3. TLC-bioassays of culture media of the wild-type and pyrazophos-resistant strain III of *Pyricularia oryzae*, incubated for 12, 24, and 48 h with $10^{-3}$ M [$^{14}$C]pyrazophos. Solvent: benzene/acetone 3:1. Test fungus: *Cladosporium cucumerinum*. Abbrevations: as in Fig. 2.

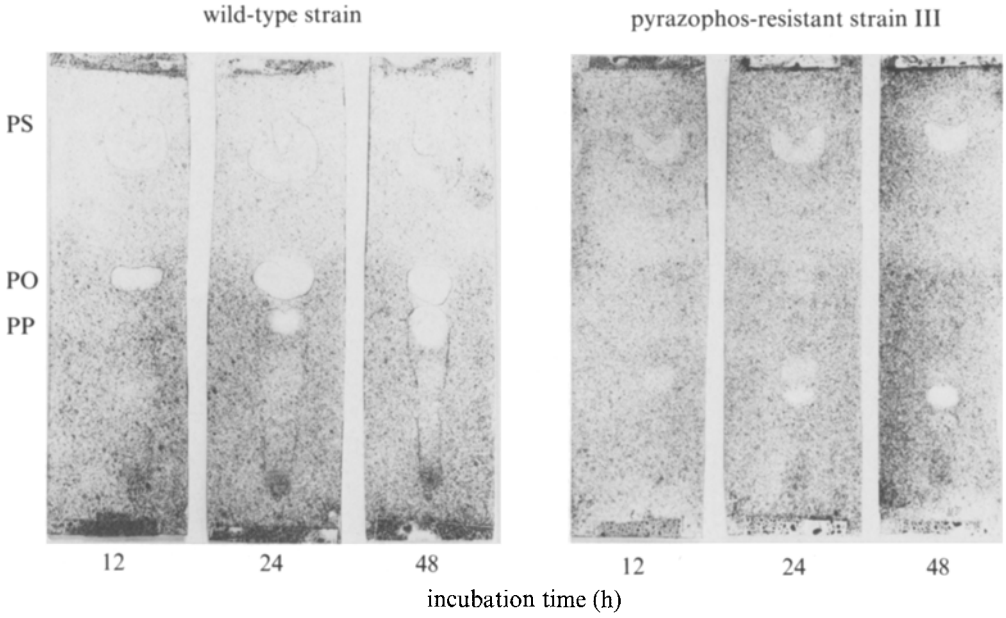


*Fig. 3. TLC-bioassays van cultuurmedia van het wild-type en de pyrazofos-resistente stam III van Pyricularia oryzae, geïncubeerd gedurende 12, 24 en 48 h met $10^{-3}$ M [$^{14}$C]pyrazofos. Loopvloeistof: benzeen/aceton 3:1. Testschimmel: Cladosporium cucumerinum. Afkortingen: als in Fig. 2.*

and to other organophosphorus fungicides such as IBP and edifenphos in *P. oryzae* (cf. Uesugi, 1978).

Pyrazophos-resistant strains of *P. oryzae* have a relatively low rate of in vitro growth and degree of sporulation indicating decreased fitness. Fitness and competitive ability of pyrazophos-resistant strains of *S. fuliginea* also appeared to be somewhat reduced (Dekker and Gielink, 1979). This might implicate that under field conditions emergence of resistance to pyrazophos only may occur at a high and continuous selection pressure of the fungicide. This is comparable with emergence of resistance to IBP and edifenphos in *P. oryzae*, which was only found after ten years of intensive use in rice fields (Uesugi, 1978).

Resistance to pyrazophos in *P. oryzae* is probably due to lack of conversion of the fungicide into PP, which is regarded as the fungitoxic principle of pyrazophos (De Waard, 1974). Even after 48 h of incubation no detectable amounts of PO-pyrazophos and PP were found in the culture medium. Pyrazophos-resistant strains also displayed a slightly increased sensitivity to PP. These data indicate that resistance is not related to the mechanism of action of the toxic principle PP, but rather with the formation of the lethal breakdown product. Apparently, development of resistance by prevention of the lethal synthesis of PP is more feasible than development of resistance by a change of the target site(s) of PP. This is in agreement with the multisite inhibitory effects of PP (De Waard, 1974) and the failure to induce resistance to PP in *Ustilago maydis* (Geor-

gopoulos et al., 1975). The acquired mechanism of resistance in the pyrazophos-resistant *P. oryzae* strains is identical with the natural mechanism of resistance in pyrazophos-insensitive fungi such as *P. debaryanum* and *S. cerevisiae* (De Waard, 1974). It is also comparable with the mechanism of resistance to 6-azauracil in strains of *C. cucumerinum* which do not convert this chemical into 6-azauridine-5′-phosphate; this conversion-product is regarded as the intrinsic fungitoxicant (Dekker, 1967).

The cross-resistance found between pyrazophos and EBP or edifenphos is hard to explain, since no attempts were undertaken to study the metabolism of the latter two fungicides. Inability to break down EBP or edifenphos would enhance their fungitoxicity because metabolites of these fungicides are not fungitoxic (Uesugi and Tomizawa, 1971; Tomizawa and Uesugi, 1972). This does not hold since no negative cross-resistance with pyrazophos was found. It might be that in resistant strains the enzyme involved in conversion of pyrazophos into its phosphate analogue mutates in such a way that at the same time affinity for pyrazophos decreases and for EBP or edifenphos increases. A low degree of resistance to EBP and edifenphos has indeed been ascribed to an increased conversion rate of the fungicides (cf. Uesugi, 1978). Strains of *P. oryzae* with a high degree of resistance to IBP metabolize the fungicide at a normal rate (Tomizawa and Uesugi, 1972). In that case the tentative explanation for resistance to IBP, coupled with collateral sensitivity to BPA as given by Uesugi and Sisler (1978) might apply. According to this explanation in IBP-resistant strains an enzyme critical for fungal growth and responsible for breakdown of BPA may change in such a way that substrate affinity for this chemical decreases (resulting in collateral sensitivity), while affinity to inhibitors of the enzyme such as IBP also decreases. Similarly, decreased substrate affinity of such an enzyme for pyrazophos (resulting in decreased sensitivity) may be coupled with decreased affinity to inhibitors of the enzyme such as EBP or edifenphos. Another explanation might be that a phosphate group is required for transport of the fungicides. Resistant strains might lack such a transport system through which pyrazophos does not reach the enzymes which metabolize it and EBP or edifenphos do not penetrate to the target site. Further investigations are necessary to prove whether this explanation really applies or whether other mechanisms of cross-resistance are involved.

**Acknowledgments**

The authors thank Dr J. Dekker, Dr A. Fuchs and Dr H. D. Sisler for a critical reading of the manuscript.

**Samenvatting**

*Resistentie-mechanisme tegen pyrazofos in Pyricularia oryzae*

Pyrazofos-resistente stammen van *Pyricularia oryzae* werden geïsoleerd van sectoren in kolonies op een pyrazofos-houdend medium. In tegenstelling tot de wild-stam zetten deze resistente stammen pyrazofos (Afugan, Curamil) niet om in zijn fosfaat-analoog en 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine (PP), dat als het fungitoxische principe van pyrazofos wordt beschouwd. De gevoeligheid van de resistente stammen voor PP was iets hoger dan die van de wild-stam. Deze gegevens wijzen

er op dat het resistantie-mechanisme geen verband houdt met een verandering van de aangrijpingsplaatsen van PP, maar met het onvermogen om pyrazofos in PP om te zetten. De pyrazofos-resistente stammen vertoonden kruisresistentie met andere organische fosforfungiciden als EBP (Kitazin) en edifenfos (Hinosan). Een verklaring voor deze kruisresistentie kan nog niet gegeven worden.

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