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Nitrite as a factor in the decline of Fusarium oxysporum f.sp. dianthi in soil supplemented with urea or ammonium chloride

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Abstract

Addition of 1 g urea or NH_4Cl per kg dry soil (0.1%) reduced the population of *Fusarium oxysporum* f. sp. *dianthi* in one of two soils tested. Ammonia does not seem to be the responsible factor since it accumulated similarly in both soils upon addition of NH_4Cl or urea. Addition of Nitrapyrine in combination with 0.1% urea or NH_4Cl increased ammonia concentrations in soil but decreased the population-declining effect. Addition of nitrate in amounts corresponding to those measured after decomposition of urea in soil had no effect on population development. Addition of nitrite in amounts corresponding to those measured during decomposition of urea in soil decreased the population of *F. oxysporum* f. sp. *dianthi*. In vitro, nitrite inhibited chlamydospore formation. Upon addition of 0.1% urea, nitrite accumulated 10 to 100 times more in the susceptible soil than in the not-susceptible soil. It is concluded that nitrite rather then ammonia is responsible for the decline effect of ammonia-generating compounds on populations of *F. oxysporum* f. sp. *dianthi* in soil.

Additional keywords: Ammonia, Nitrapyrine.

Introduction

A decline in populations of pathogenic soil fungi after the addition of organic materials to soil has been reported for various species (Tsao, 1979; Papavizas and Lumsden, 1980). This effect is frequently ascribed to liberation of ammonia during decomposition of the organic substances. Zakaria et al. (1980) demonstrated a correlation between ammonia amounts and a decline of *Fusarium oxysporum* and *Fusarium solani* in soil fortified with oilseed meals. According to these authors, alfalfa meal (1%, w/w) did not produce ammonia and its addition did not result in a population decline. Gilpatrick (1969), however, detected ammonia after addition of 5% alfalfa meal to soil. This coincided with a decrease in populations of *Phytophthora cinnamomi* on avocado roots. Chitin produced ammonia and decreased the *F. solani* population in soil (Schippers and De Weyer, 1972). Urea, which releases ammonia after microbial hydrolysis, suppressed populations of *F. oxysporum* f. sp. *cubense* (Sequeira, 1963), *Phytophthora cinnamomi* and *Ph. parasitica* (Tsao and Zentmyer, 1979), *Pythium ultimum, Thielaviopsis basicola* and *Macrophomina phaseolina* (Chun and Lockwood, 1985).

In soil, ammonia is oxidized to nitrite by bacteria of the genus *Nitrosomonas* and subsequently to nitrate by bacteria of the genus *Nitrobacter* (Fig. 1). It has been sug-

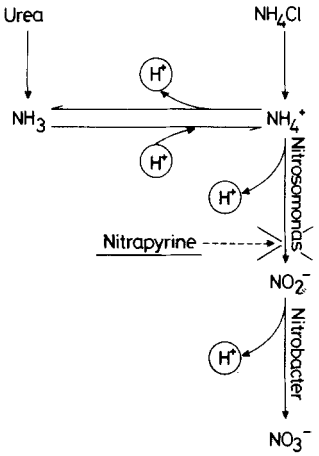

Fig. 1. Nitrification processes in soil upon addition of urea or NH_4Cl.

gested that nitrite, in addition to ammonia, is responsible for the population-declining effect of ammonia-generating coumpounds (Sequeira, 1963; Smiley et al., 1970; Tsao and Oster, 1981).

In previous work, decreased chlamydospore formation and enhanced lysis of chlamydospores of *F. oxysporum* in soil with ammonia-generating compounds was demonstrated (Löffler et al., 1986). However, since germination of macroconidia and germ tube growth of *F. solani* is stimulated by ammonia (Löffler and Schippers, 1984) and lysis of macroconidia of *F. solani* is delayed by ammonia (Schippers, 1972), chlamydospore population in soil enforced with ammonia-generating compounds does not necessarily reflect the total population of *Fusarium* in soil.

The purpose of this work was to examine the effect of ammonia and its nitrification products on the population of *F. oxysporum* in two different soils. Concentrations of ammonia, nitrite and nitrate and soil pH were followed for 6 weeks. Whether ammonia or nitrification products of ammonia were responsible for the observed effects was examined by selective inhibition of *Nitrosomonas* spp. by addition of Nitrapyrine, nitrate or nitrite to soil (Fig. 1). Unless otherwise specified, the term 'ammonia' is used for the mixture of NH_3 and NH_4^+ throughout this paper.

Materials and methods

Test fungus. *Fusarium oxysporum* f. sp. *dianthi* (Prill. & Delacr.) Snyder & Hansen (isolate A78403) was used in this study. Macroconidia were produced as described previously (Löffler and Schippers, 1985).

Soils. Characteristics of the Baarn soil and the 's-Gravenzande soil were described earlier (Löffler et al., 1986). The original pH of the Baarn soil (5.7) was raised with 3.75 g $CaCO_3$ per kg soil to 7.0 to approximate the pH of the 's-Gravenzande soil (7.3). Soils were stored outside and sieved with a 4-mm sieve prior to use. In nitrification experiments, the American soils Boyer, Brookston and Capac were also used. Characteristics of these soils are described by Zakaria and Lockwood (1980).

Determination of the population of F. oxysporum f. sp. dianthi in soil. Soil samples (5 g) were shaken in 40 ml of sterile water for 10 minutes with a Griffin flask shaker (maximum speed) at 4 °C. After dilution, 100 µl of the soil suspensions were evenly spread over selective agar plates. For this purpose, the *F. oxysporum*-selective Komada medium (Komada, 1975) was slightly modified. Instead of 0.05% oxgall, the better soluble and equally effective Solacol (0.2%) was added before sterilization (Gams and Van Laar, 1982). Each soil suspension was tested in two dilutions on three agar plates. Colonies were counted after 4 and 10 days of incubation at 20 °C.

Determination of ammonia, nitrate and nitrite in soil. Soil samples (5 g) were shaken in 40 ml of 2.0 M KCl for 1 h with a Griffin flask shaker (maximum speed) at 4 °C. Soil suspensions were filtered through filter paper (Schleicher & Schüll no 595) and frozen at −20 °C for further use. To determine nitrite concentrations, 4.5 ml of filtered soil suspensions were mixed with 100 µl 0.025 M Na_2EDTA, 250 µl 6 mM Sulfanilamide and 10 µl 6 M HCl. After 3 minutes, 50 µl 4 mM N-(1-Naphtyl)-ethylen-diaminedichloride (Merck) was added. After at least 15 minutes, the absorption was measured at 542 nm with a Coleman spectrophotometer. Nitrite concentrations were calculated using calibration solutions containing 0.01 to 1 µg NO_2^- ml^{-1}. Concentration of ammonia and nitrate in soil and soil-pH were determined as described previously (Löffler et al., 1986).

Effects of urea, NH_4Cl, $NaNO_2$ and $NaNO_3$ on the population of F. oxysporum f. sp. dianthi in soil. Macroconidial suspensions of *F. oxysporum* f. sp. *dianthi* were dispersed over air-dried soils to give 10^5 macroconidia g^{-1} soil. Powdered urea, NH_4Cl, $NaNO_2$ or $NaNO_3$ was mixed thoroughly through the soil in proper concentrations. In one experiment, 0.042 ml of a 1 M Nitrapyrine solution (2-chloro-6-trichloromethylpyridine, Dow Chemical) kg^{-1} soil was added (10 mg kg^{-1}). Soilwater potential was adjusted to −0.12 bar with distilled water as measured with a Nieuwkoop tensiometer. Glass petri dishes (13 cm) were filled with 80 g of soil, sealed with parafilm and incubated at 23 °C for up to 6 weeks. At intervals, the number of propagules was determined for each condition in four separate dishes. Ammonia, nitrite and nitrate concentrations and soil pH were determined in two of the four dishes.

Determination of nitrification in soil. Nitrification was compared in five different soils. Some experiments were performed in Michigan (USA) with three American soils, some in the Netherlands with two Dutch soils. Air-dried soils were mixed with 0.1% urea and wetted with distilled water to give a soilwater potential of −0.12 bar. Glass petri dishes (13 cm) were filled with 80 g of soil, sealed with parafilm and incubated at 23 °C. Nitrite concentrations were determined at intervals.

Effect of nitrite on germination, viability, chlamydospore formation and mycelium growth. Macroconidia of *F. oxysporum* f. sp. *dianthi* were incubated in 10 ml of a 50 mM TRIS-HCl medium with 0.02 mM $MgCl_2$ (pH 7.5) and 0, 1, 5, 10 or 20 mM $NaNO_2$ in 25 ml erlenmeyer flasks at a concentration of 10^5 ml^{-1} at 23 °C. After 24 h, drops of the suspensions were fixed on glass slides and percentages of germination were determined microscopically.

Viability was determined by transferring a drop of the suspension to a 13-mm Sartorius membranefilter (pores 0.2 µm) on top of a filter paper. The incubation-medium

was thus absorbed by filterpaper while conidia remained on the membranefilter. Conidia were washed with a drop of water. After incubating the filters for 16 h on Czapek Dox medium (pH 3.5), germination percentages were determined microscopically.

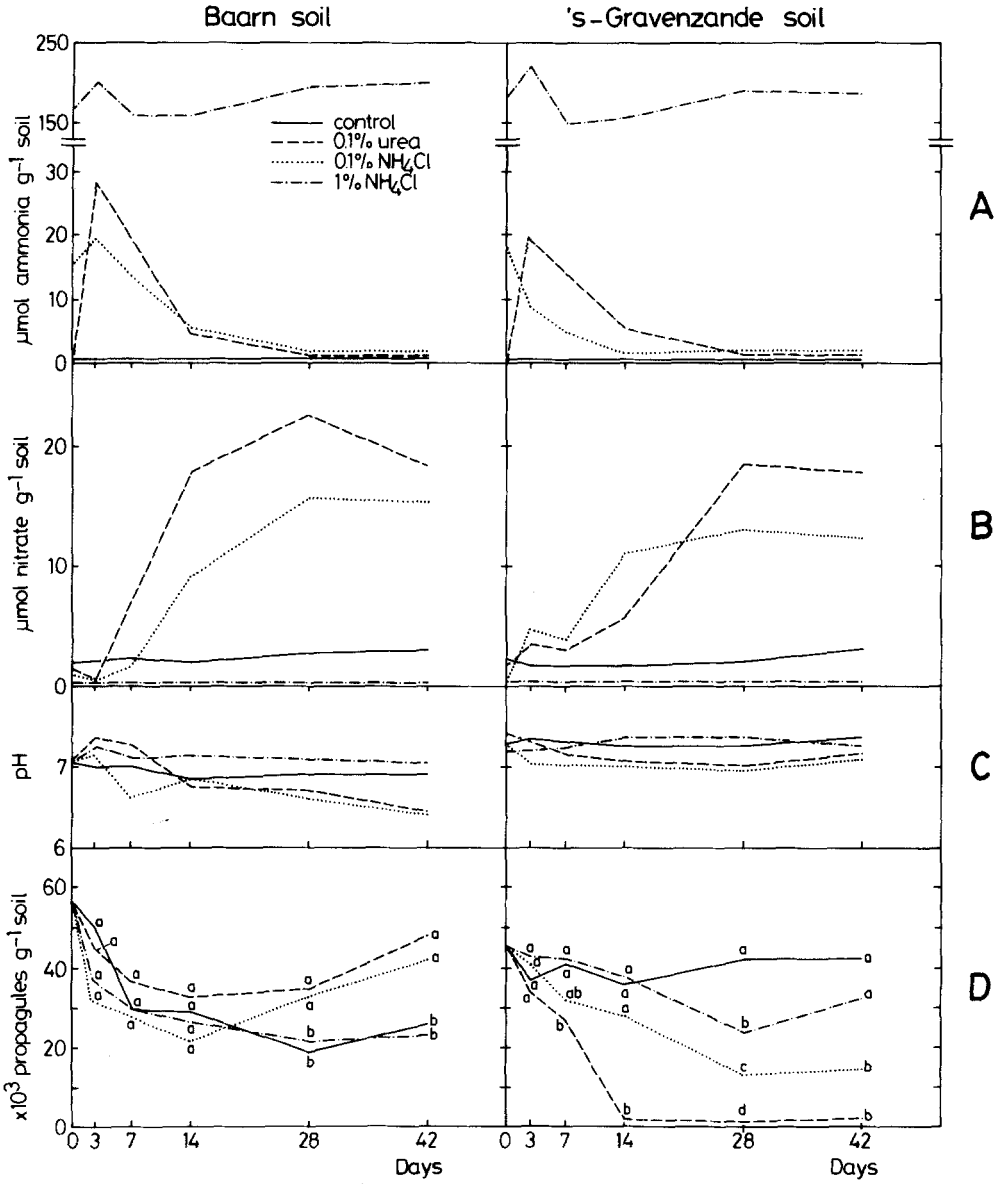

Fig. 2. Development of the population of *F. oxysporum* f. sp. *dianthi* in Baarn and 's-Gravenzande soil after addition of 0.1% or 1% NH_4Cl or 0.1% urea (D). At each sampling date, concentrations of ammonia (A) and nitrate (B) and the soil pH (C) were measured. Different letters at one sampling date indicate a significant difference (n = 4, p = 0.05) between the treatments according to the T-method.

The effect of nitrite on chlamydospore formation was tested with pregerminated macroconidia incubated in TRIS-HCl buffer containing 0.02 mM $MgCl_2$ (pH 7.5) and 0, 1, 5, 10 or 20 mM $NaNO_2$. After 10 days, the number of chlamydospores was determined according to Löffler et al. (1985).

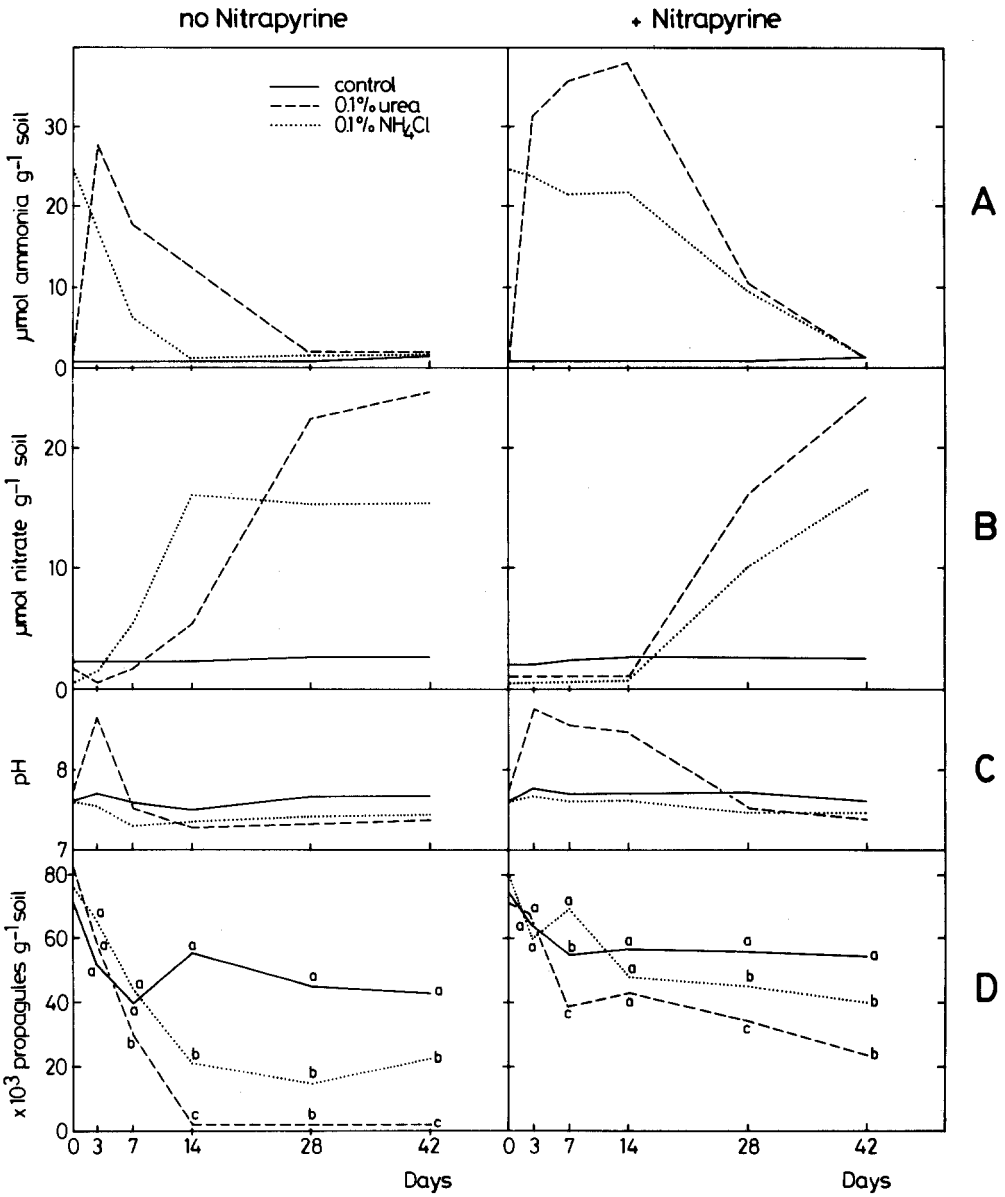

Fig. 3. Development of the population of *F. oxysporum* f. sp. *dianthi* in 's-Gravenzande soil after addition of 0.1% NH_4Cl or 0.1% urea with or without 10 mg Nitrapyrine kg^{-1} (D). At each sampling date, concentrations of ammonia (A) and nitrate (B) and the soil-pH (C) were measured. Different letters at one sampling date indicate a significant difference (n = 4, p = 0.05) between the treatments according to the T-method.

The effect of nitrite on mycelial growth was determined with actively growing mycelium on a 5 mm agar disk placed in the centre of Tchernoff agar plates (Tchernoff, 1965) supplemented with 0, 1, 5, 10 or 20 mM $NaNO_2$. After 5 days colony diameters were measured.

All experiments were run in triplicate.

Statistics. Results were analyzed by Analysis of Variance. The T-method was used to calculate the Minimum Significant Difference (MSD) according to Sokal and Rohlf (1981).

Results

Upon addition of 0.1% urea or NH_4Cl, the population of *F. oxysporum* f. sp. *dianthi* declined in 's-Gravenzande soil, but not in Baarn soil (Fig. 2D). In both soils, decomposition of ammonia (Fig. 2A), accumulation of nitrate (Fig. 2B) and changes in soil pH (Fig. 2C) were comparable. In 's-Gravenzande soil, the effect of 1% NH_4Cl on population decline was less pronounced than that of 0.1% NH_4Cl (Fig. 2D).

Addition of 10 mg Nitrapyrine kg^{-1} to 's-Gravenzande soil inhibited the nitrification as is demonstrated by delayed ammonia decomposition (Fig. 3A), delayed nitrate accumulation (Fig. 3B) and higher soil pH (Fig. 3C). The population-declining effect of urea and NH_4Cl was less in soil with Nitrapyrine than in soil without Nitrapyrine (Fig. 3D). In vitro, 50 mg Nitrapyrine kg^{-1} had no effect on germination of macro-

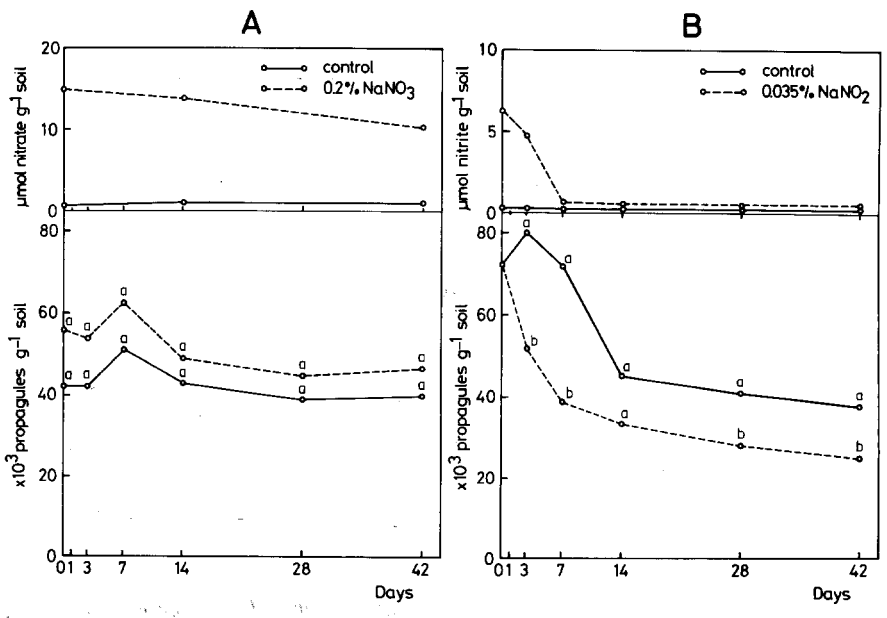

Fig. 4. Development of the population of *F. oxysporum* f. sp. *dianthi* and the nitrate and nitrite concentrations in 's-Gravenzande soil with 0.2% $NaNO_3$ (A) or 0.035% $NaNO_2$ (B), respectively. Different letters at one sampling date indicate a significant difference (n = 4, p = 0.05) between the treatments according to the T-method.

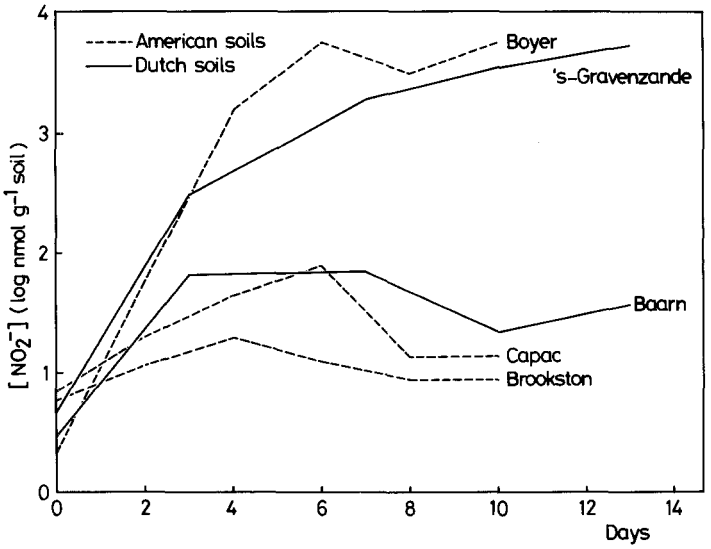

Fig. 5. Nitrite accumulation upon addition of 0.1% urea in 5 different soils.

conidia and colony growth of *F. oxysporum*. In soil, 10 mg Nitrapyrine kg^{-1} had no effect on the population of *F. oxysporum* f. sp. *dianthi* (Fig. 3D, controls).

Addition of 2 g $NaNO_3$ kg^{-1} to 's-Gravenzande soil had no effect on the population development of *F. oxysporum* f. sp. *dianthi* (Fig. 4A). Most of the nitrate was still present after 6 weeks of incubation. Addition of 0.35 g $NaNO_2$ kg^{-1} to 's-Gravenzande soil, however, decreased the population of *F. oxysporum* f. sp. *dianthi* significantly (Fig. 4B). After 7 days of incubation, nitrite was no longer detectable.

During decomposition of urea, 10 to 100 times more nitrite accumulated in 's-Gravenzande and Boyer soil than in Baarn, Brookston and Capac soil (Fig. 5).

In in vitro experiments, nitrite concentrations as low as 1 mM stimulated the germination of macroconidia after 6 and 24 h. No inhibition was found with concentrations up to 20 mM. Viability and colony growth were not affected by nitrite at the tested concentrations. Chlamydospore formation, however, was greatly reduced by nitrite. The reduction increased with increasing concentrations of nitrite (Table 1).

Table 1. Effect of nitrite on germination and survival of macroconidia, on mycelial growth and on formation of chlamydospores in pregerminated conidia. In brackets: incubation periods.

$NaNO_2$ mM	% Germination[1] (24 h)	% Viability[1] (24 h)	Colony diam.[2] in mm (5 dg)	Chlam./100 con.[1] (10 dg)
0	12	100	58	505
1	85	100	61	285
5	89	100	64	81
10	86	100	64	38
20	82	100	59	19

[1] After incubation in 50 mM TRIS-HCl buffer solution (pH 7.5).
[2] After incubation on Tchernoff agar.

Discussion

The declining effect of urea and NH_4Cl on populations of *F. oxysporum* in soil does not seem to be induced by ammonia. During decomposition of 0.1% urea and NH_4Cl, ammonia concentrations developped equally in Baarn- and 's-Gravenzande soil. Since the pH of both soils was comparable, the NH_3 concentrations were similar in both soils. In 's-Gravenzande soil, contrary to Baarn soil, the *F. oxysporum* f. sp. *dianthi* population declined rapidly upon addition of 0.1% NH_4Cl or urea. Moreover, in 's-Gravenzande soil, 1% NH_4Cl liberated much more ammonia than 0.1% NH_4Cl, whereas 0.1% NH_4Cl seemed to be more effective (Fig. 2A, D). Apparently, ammonia concentrations in soil obtained with 1% NH_4Cl inhibited nitrifying bacteria and thus oxidation of ammonia to nitrate (Fig. 2B). This suggests that a nitrification product of ammonia (nitrite or nitrate) rather than ammonia was responsible for the population decline. The effect of 10 mg Nitrapyrine kg^{-1} to soil with 0.1% urea sustains this supposition: Ammonia concentrations increased in the presence of Nitrapyrine, but this increase was not reflected in an enhanced decline of the population of *F. oxysporum* f. sp. *dianthi*. During nitrification, first nitrite and thereafter nitrate is formed. Addition of nitrate in amounts corresponding to those measured after total decomposition of 0.1% urea (i.e. 0.2% $NaNO_2$) to 's-Gravenzande soil did not affect the population of *F. oxysporum* f. sp. *dianthi* (Fig. 4). Moreover, nitrate concentrations in Baarn and 's-Gravenzande soil developped similarly after addition of 0.1% urea or 0.1% NH_4Cl (Fig. 2B), whereas populations of *F. oxysporum* f. sp. *dianthi* did not. Addition of nitrite in amounts corresponding to those measured during decomposition of 0.1% urea (i.e. 0.035% $NaNO_2$) decreased the population of *F. oxysporum* (fig. 4B). In this experiment, the population decline in the control is stronger than in most other experiments. We ascribe this to the presence of about 50% microconidia in the inoculum, whereas the inocula used in the other experiments contained mainly macroconidia. Survival of single-celled microconidia can be expected to be less than that of 4 to 5-celled macroconidia. Within 7 days of incubation, all nitrite had disappeared, apparently because of oxidation of nitrite by *Nitrobacter* spp. The population decline was less drastic than in soil supplemented with 0.1% urea, probably since in soil with urea, in addition to oxidation of nitrite, formation of nitrite by oxidation of ammonia occurs. Thus in soil suplemented with 0.1% urea, nitrite was present over a longer period than in soil supplemented with 0.035% $NaNO_2$. In separate experiments, much more nitrite accumulated during decomposition of 0.1% urea in 's-Gravenzande soil than in Baarn soil (Fig. 5). Thus nitrite accumulation is correlated with the population declining effect of urea and NH_4Cl in those soils. The American soils used in this study are known to support the declining effect of ammonia-generating oilseed meals on populations of *F. oxysporum* to different degrees. Population reductions are less in Capac soil than in Boyer soil and absent in Brookston soil (Zakaria and Lockwood, 1980). Upon addition of 0.1% urea, nitrite accumulation was highest in Boyer soil and lowest in Brookston soil (Fig. 5). Hence, in the American soils the population declining effect of ammonia-generating compounds was also correlated with nitrite accumulation. Zakaria and Lockwood (1980) demonstrated an inverse relation between responsiveness and organic matter contents in soils. This is also found in the Baarn and 's-Gravenzande soil (Löffler et al., 1986). The relation between nitrite accumulation and organic matter content in soil is not clear.

In vitro, the effect of nitrite on survival was tested in concentrations corresponding with those in soil. Maximum nitrite concentrations in 's-Gravenzande soil with 0.1% urea approximated 5000 nMol g^{-1} soil. Since soil moisture was about 25% (−0.12 bar), the nitrite concentration in soil water was 20 mM. It must be considered, though, that all chemical determinations reflect mean values in soil and not necessarily the situation in microsites. Concentrations of nitrite may be higher in situ. Moreover, since part of the soil water will be bound, the nitrite concentration in the free water may be higher. Nitrite decreased chlamydospore formation in vitro; germination, viability and radial growth, however, were not affected or even stimulated (Table 1). Nitrite has also been suggested to enhance lysis of chlamydospores in soil (Löffler et al., 1986). Because in soil, *F. oxysporum* survives mainly as chlamydospores, nitrite seems to be an important factor counteracting survival of this fungus. In conclusion, addition of NH_4Cl or urea to one of two soils tested, decreased the population of *F. oxysporum* f. sp. *dianthi*. This reduction was due to a decreased chlamydospore formation and an enhanced lysis of chlamydospores by nitrite rather than by ammonia.

Acknowledgements

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Samenvatting

Nitriet als factor in de afname van de populatie van Fusarium oxysporum f.sp. dianthi in grond waaraan ureum of ammoniumchloride is toegevoegd

Toevoeging van 0.1% ureum of 0.1% NH_4Cl verminderde de populatie van *F. oxysporum* f. sp. *dianthi* in een van de twee getoetste gronden. Ammoniak lijkt niet verantwoordelijk voor deze afname, aangezien ammoniak in beide gronden gelijkelijk ontwikkelde na toevoeging van ureum of NH_4Cl. Toevoeging van Nitrapyrine tesamen met ureum of NH_4Cl aan de 'actieve' grond verhoogde de concentratie ammoniak in de grond, maar verlaagde het remmend effect. Toevoeging van nitraat in hoeveelheden die overeenkomen met die welke gemeten worden na volledige omzetting van ureum had geen effect op de populatie van *F. oxysporum* f. sp. *dianthi*. Toevoeging van nitriet in hoeveelheden die overeenkomen met die welke gemeten worden na volledige afbraak van ureum verminderde de populatie wel. In vitro remde nitriet de chlamydosporevorming van *F. oxysporum* f. sp. *dianthi*. Toediening van 0.1% ureum aan de grond gaf een 10 tot 100 maal hogere nitrietaccumulatie in de 'actieve' grond dan in de 'niet-actieve' grond. Daarom wordt de conclusie getrokken, dat nitriet veeleer dan ammoniak verantwoordelijk is voor de vermindering van de populatie van *F. oxysporum* f. sp. *dianthi* in grond waaraan ammoniak-genererende verbindingen zijn toegevoegd.

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