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Nitrite as a nutrient for microfungi of the outer stem cortex of pine and spruce and its toxicity to *Fomes annosus*

Nitrit som näringsämne för lägre svampar i tallens och granens ytterbark och dess toxicitet mot Fomes annosus

by

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Introduction

A gateway to chemical control of *Fomes annosus* was opened when, in 1952, Rishbeth demonstrated that the invasion of conifer stands by this root-rot fungus could be prevented by the immediate treatment of freshly cut stumps with creosote. Now such stump treatment is general practice in Great Britain and Denmark, and is at the experimental stage in several other countries. The most popular chemical so far seems to be creosote, which contains phenol as its active constituent. However, since creosote has certain disadvantages, e.g. it is not selective, it leaves the stumps sterile for a long period and may eventually open the way for *Fomes* infection, other types of protectant have been tried, especially by British investigators. Thus, adequate protection has been obtained with preparations containing urea, disodium octaborate and ammonium sulphamate as their active constituents (RISHBETH, 1959 a). Infection by *Fomes annosus* has also been prevented by the artificial inoculation of stumps with oidia of *Peniophora gigantea*, whereby a biological antagonism type of protection is obtained (RISHBETH, 1963).

Recently, sodium nitrite under the trade name Fomesan, was introduced in Sweden as a chemical for stump treatment (GUNDERSEN, 1963). It has been shown to have a dual effect; first, it is highly toxic to *Fomes annosus* and may kill conidia at very low concentration, secondly, it may serve as a nitrogen nutrient for a variety of indigenous stump microfungi, of which several are antagonistic to *Fomes*. This secondary effect may possibly give permanent protection against the pathogen.

The effects of nitrite were further investigated in this laboratory, using stem disks of spruce and pine as stump models. The results of these experiments are described below.

Materials and Methods

The general procedure used for studying nitrite's effects was the soaking of thin stem disks of young pine and spruce trees in solutions of sodium nitrite, followed by an incubation period. Standard treatment time was 30 minutes; the incubation temperature was 23°C throughout.

Stem disks were also used as a medium for studying pure cultures of *Fomes annosus* (Fr.) Cke., and of microfungi isolated from nitrite-treated stem disks. For this purpose, freshly cut disks were placed in covered Petri dishes and exposed in closed jars of the vapour of propylene oxide, according to the method described by HANSEN & SNYDER (1947). Sterile stem disks were soaked in sterile solutions of nitrite or in sterile water (controls).

The stem disks were obtained from trees in the arboretum of Gothenburg Botanical Garden. Stem sections of pine (*Pinus silvestris* L.) and spruce (*Picea abies* (L.) Karst.) of 5—8 cm diameter were selected. When necessary, ca. 8 mm thick disks were cut with a band saw, and placed immediately in sterile Petri dishes. Precautions were taken to prevent the stems and disks from being contaminated, but perfect sterility was not attainable. Between the felling of the trees and the cutting of the disks, the stem sections were stored at 4°C in clean bags of heavy paper.

Because of the influence of pH on the degree of toxicity of the nitrite, it was always necessary to know the pH of the media, the solutions and the stem disk sap. The pH values of the phloem and bark (cortex) and of the xylem of selected trees used for the preparation of disks are given in Table 1.

In some experiments ordinary malt agar (pH 5.5) and Sabouraud agar (pH 6.0) were used. Stock cultures of *Fomes annosus* and the microfungi were also kept on these laboratory media.

The experiments with pH were made in a medium containing McIlvaine's citrate buffer as a base. The medium contained: $MgSO_4 \cdot 7\ H_2O$, 0.05 %; KCl, 0.05 %; $FeSO_4 \cdot 7\ H_2O$; sucrose 3 %; and dehydrated corn steep liquor, 0.25 %. The buffer was prepared from calculated proportions of the components: 0.1 molar citric acid, and 0.2 molar Na_2HPO_4. For a solid pH-medium, 2 % Ion-agar No. 2 (Oxoid) was added, but at pH 3.5 complete gelling did not take place.

Experiments made to determine nitrite-assimilation employed a medium similar to the pH-medium, but differing in that 0.1 % K_2HPO_4 was added, and in that the corn steep liquor content was reduced to 0.02 per cent.

Table 1. pH of bark and xylem of spruce and pine used for the preparation of stem disks.

Date of felling	Spruce			Pine		
	Age years	Bark pH	Xylem pH	Age years	Bark pH	Xylem pH
September 22.....	6	5.20	5.95	12	4.20	5.60
January 10.....	10	5.45	6.05	10	4.55	5.35
April 28.....	30	5.95	6.00	14	4.60	5.65

Instead of buffer, distilled water was used as a base. The pH of this medium was adjusted to 6.3. Solutions of NH_4NO_3 and $NaNO_2$ (adjusted to the same pH) were added after separate sterilization of the solutions and the basal medium.

Analytical grade chemicals, including $NaNO_2$, were used in all media.

Nitrite was determined colorimetrically in a Beckman Model C colorimeter, after colour development with sulphanilamide and 1-naphthylamine solutions.

Circular pieces (6 mm) of agar cultures of *Fomes annosus* and most of the microfungi, prepared as described by GUNDERSEN (1962), or spore suspensions in the case of the penicillia and *Aspergillus niger* served as inoculum. Suspensions of conidia of *Fomes annosus* were prepared by rinsing the surface of the welldeveloped growth of the fungus on a pine stem disk with a few ml of distilled water.

The area of the stem disks covered by mycelial growth was determined by tracing the disk outline and the visible outline of the growth on a tracing paper, cutting out the projections and weighing the pieces of paper on an analytical balance. From these weights the percentage cover of the growth could be calculated.

Other experimental details are described where appropriate in the experiments.

Results

General effects of nitrite on the indigenous microflora of the bark of spruce and pine

A 30-minute soaking of stem disks of spruce and pine in solutions of sodium nitrite had a very striking effect on the microfungi which naturally inhabit the outer bark. After a few days in the incubator, it was apparent that most of the disk surface had been colonized by these fungi. Since this was not visible on pine disks soaked in water, and since only a few small mycelia appeared on spruce disks similarly treated, is was evident that the nitrite had stimulated the growth of the microfungi. Disks treated with 0.1 per cent sodium nitrite were dotted with a variety of fungal colonies, and treatment with one per cent nitrite resulted in most cases in the complete coating of the disk surface with heavy fungal growth (Plate 1). Predominant amongst the organisms were sporulating *Penicillium* and *Trichoderma* spp. of olive-green, grey-green and yellowish-green colour; also white, yellowish and dark-coloured colonies of other microfungi were mixed into the pattern.

Approximately one per cent sodium nitrite was found to be the optimal concentration for fungal development. At higher concentrations the toxicity of the nitrite became increasingly apparent, especially in spruce, where disks treated with two per cent nitrite became almost sterile. The microfungi of pine were less affected, and several colonies appeared after treatment with five to eight per cent nitrite. Ten per cent sodium nitrite caused complete inhibition, and the stem disks became brownish after the treatment, probably because of the nitration of the cellulose and other compounds of the xylem. In a few cases, a sterile mycelium began to grow from the cortex of these disks after some time and, in the absence of competition from other organisms, expanded rapidly over the surface (Plate 1, Fig. 6).

The percentage cover of spruce and pine stem disks by mycelium following treatment with different concentrations of sodium nitrite is shown in Figs. 1 and 2. As might be expected in experiments of this kind, there was always considerable variation in the degree of surface coverage, presumably attributable to differences in the distribution of fungal species and numbers in different disks. However, in several tests made at different times of the year, using disks from different trees, fungi always developed optimally at about one per cent sodium nitrite concentration, perhaps a little above this concentration in pine and a little below in spruce.

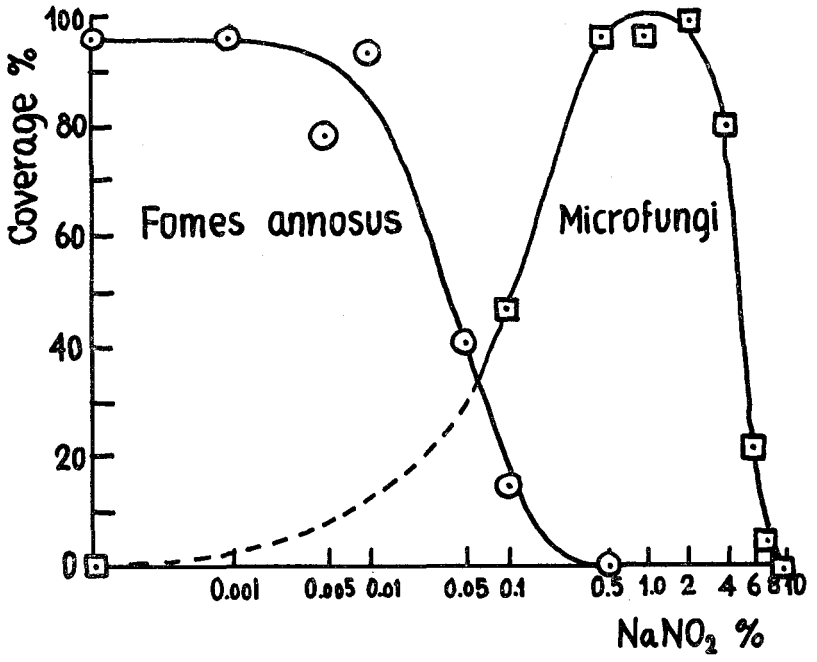

Fig. 1. Growth of *Fomes annosus* on sterilised, and microfungi on unsterilised stem disks of pine treated with solutions of increasing concentration of sodium nitrite. Incubation 10 days at 23° C.

The inhibition of *Fomes annosus* growth by nitrite on sterilized stem disks has been shown for comparison in Figs. 1 and 2. The toxicity of nitrite to *Fomes annosus* is further described in a later section.

The organisms of nitrite-treated pine and spruce disks

As mentioned previously, penicillia and *Trichoderma* spp. were the most common inhabitants of the nitrite-treated stem disks, although other microfungi were always present. But bacteria and yeasts were never seen, except for the occasional yeast-like *Candida* spp. No attempts were made to identify every organism present on the disks, but 16 different organisms were isolated in pure culture and identified, and their reactions to nitrite further investigated. The 16 microfungi are listed in Table 2. Three would sporulate neither on laboratory media nor on sterilised stem disks; these organisms are therefore referred to by their isolation numbers only.

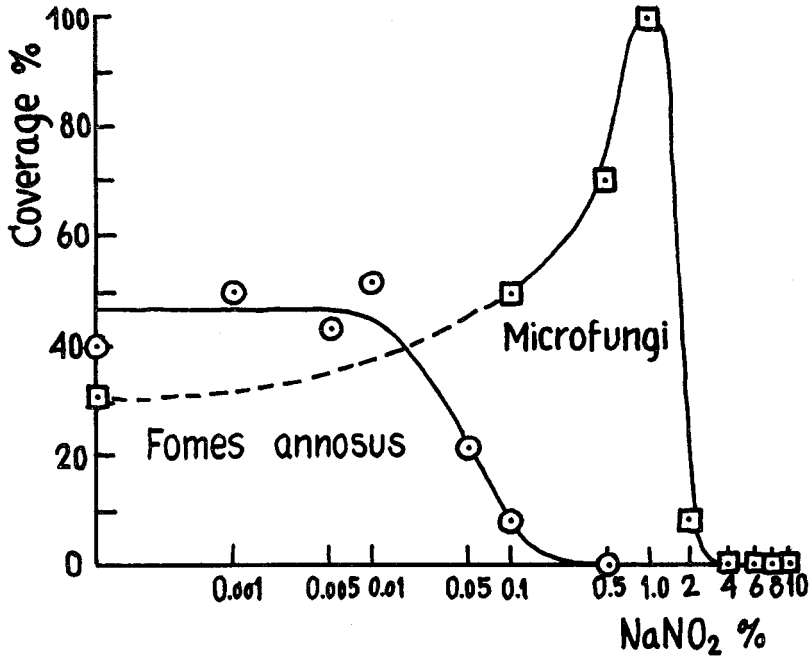

Fig. 2. Growth of *Fomes annosus* on sterilised, and microfungi on unsterilised stem disks of spruce treated with solutions of increasing concentration of sodium nitrite. Incubation 10 days at 23°.

Nitrite as a nutrient

It was tempting to draw the conclusion that nitrite was utilized as a nitrogen source by the fungi which spontaneously colonized the nitrite-treated stem disks. The addition of any nitrogen compound to the carbon-rich, nitrogen-poor xylem of the conifers increases its value as a substrate for cellulose-decomposing micro-organisms, provided that the nitrogen compound is neither toxic nor enzymatically unassailable. Thus the addition of a utilisable nitrogen source may result in increased protein synthesis, followed by accelerated growth and increased cellulolytic activity.

Although nitrite is basically a toxic compound, it is not equally toxic to all organisms, so these considerations could not immediately be invalidated. A series of experiments was therefore carried out to discover whether sodium nitrite was utilizable as a nitrogen source by the 16 microfungi. The organisms were grown in pure culture on sterilised stem disks treated with different concentrations of sodium nitrite, and in the liquid nitrite medium described under the heading Methods. Growth with equimolar amounts (in

Table 2. Microfungi isolated from nitrite-treated stem disks of spruce and pine.

Isolation number	Species	Isolated from Disk—$NaNO_2$
G B2-2	*Mucor hiemalis* Wehmer	Spruce 0.1 %
G B2-3	*Candida sp.*	
T B2-1	*Cladosporium herbarum* Link ex Fr.	Pine 0.1 %
T B2-21	*Penicillium chermesinum* Biorge, var. 1	
T B2-22	*Penicillium chermesinum* Biorge, var. 2	
T B2-41	*Penicillium frequentans*-series	
T B2-42	*Penicillium implicatum*-series	
G B3-2	*Botrytis cinerea* Pers. ex Fr.	Spruce 1 %
G B3-3	*Sordaria fimicola* (Rob.) Ces. & De Not	
G B3-4	*Trichoderma lignorum* (Tode) Harz	
T B3-1	*Penicillium raistrickii*-series	Pine 1 %
T B3-3	*Aspergillus niger*-series	
T B3-41	Sterile mycelium	
T B3-42	*Trichoderma koningii* Oudemans	
G B4-1	Sterile mycelium	Spruce 10 %
T B4-1	Sterile mycelium	Pine 10 %

terms of N) of ammonium nitrate was determined for comparison, as was also growth in the absence of an inorganic nitrogen source. A small amount of organic nitrogen (less than 0.002 per cent), mainly in the form of amino acids and vitamins from the added corn steep solids, was available in the liquid medium. The results of these experiments are shown in Tables 3 and 4.

Growth was generally increased when nitrite was added as a nitrogen source. On the stem disks treated with nitrite, 20 mg % N was equivalent to an equal amount of ammonium nitrate for most of the fungi. 200 mg % nitrite-N was more or less growth inhibitory. However, a few of the organisms, including *Botrytis cinerea* and the two sterile mycelia G B4-1 and T B4-1, grew luxuriantly at the higher concentration and only sparsely in the absence of a nitrogen source.

In the liquid medium, nitrite was assimilated by ten of the microfungi in amounts varying from 6 to 100 per cent of the nitrogen available. A toxic effect of the nitrite was observed only in two of the fungi, viz. *Penicillium implicatum* and *Aspergillus niger*. This was unexpected, since both organisms grew well with nitrite on stem disks, and *A. niger* has previously been found to assimilate nitrite (KOSTYTSCHEW & TSWETKOWA, 1920). However, since considerable amounts of acid were produced by both fungi in the liquid medium, the toxicity of nitrite may have increased rapidly (cf. Fig. 3 and the section on the effect of pH on nitrite-toxicity) and finally reached a level at which it was no longer tolerable to the organisms.

Table 3. Growth of microfungi on sterilized stem disks treated with water solutions of ammonium nitrate or sodium nitrite before inoculation. Incubation 11—15 days at 23° C.

Organism	Disk	Water	NH_4NO_3 N = mg%		$NaNO_2$ N = mg%	
			20	200	20	200
Mucor hiemalis	Spruce	+ ±	+ + ±	± + +	+ +	±
Candida sp.	»	∓	∓	—	± +	—
Botrytis cinerea	»	±	—	+ ±	—	+ + +
Sordaria fimicola	»	+	+	∓	+ +	0
Trichoderma lignorum	»	± +	± +	+ +	+ +	+
G B4-1 sterile mycelium	»	∓	—	+ + +	—	+ + +
Cladosporium herbarum	Pine	+ +	∓ +	+ + +	± +	0
Penicillium chermesinum 1.	»	+ +	+ ∓	+ + +	+ +	+
P. chermesinum 2	»	− +	+ +	+ + +	+ ∓	∓ +
P. frequentans	»	+ ±	+ +	± +	+ +	∓
P. implicatum	»	± +	+ ±	± +	± +	0
P. raistrickii	»	+	—	+ ±	—	± +
Aspergillus niger	»	+ +	+ + +	+ +	+ + +	0
T B3-41 sterile mycelium	»	+ ±	+ ±	+	+ + ∓	0
Trichoderma koningii	»	+	—	+ ±	—	+ +
T B4-1 sterile mycelium	»	±	—	∓	—	+ +

— means: not tested

The fact that some of the fungi, e.g. *Sordaria fimicola*, *Trichoderma lignorum*, *Aspergillus niger* and the sterile mycelium T B3-41, failed to grow, or grew less well in pure culture on stem disks treated with one per cent sodium nitrite (= 200 mg % N) than on the one per cent sodium nitrite-treated disks from which they were isolated, suggests that metabiotic effects prevail in the mixed populations. The transformation products of nitrite in the mixed disk cultures were not investigated, but is seems very probable that the nitrite is rapidly transformed to non-toxic products by some of the organisms, thereby becoming assimilable by those of the organisms which cannot utilize nitrite directly.

The effect of pH on the toxicity of nitrite to Fomes annosus

Nitrite may be fairly well tolerated by many organisms, both higher plants and micro-organisms, when it is present in a neutral or alkaline solution (Mevius & Dikussar, 1930; Cochrane & Conn, 1950; Sakaguchi & Wang, 1934). However, toxicity increases rapidly with increasing hydrogen-ion concentration of the nitrite medium (Mevius, 1928). The toxicity of nitrite has been ascribed to the undissociated nitrous acid molecule, HNO_2, which is formed in acid solution, and not to the nitrite ion, NO_2^-, which is present in neutral-alkaline solution:

Table 4. Growth (mg dry weight) and nitrite assimilation by microfungi in liquid medium in the absence of inorganic nitrogen, or with ammonium nitrate (N = 30 mg%) or sodium nitrite (N = 30 mg%) added. Incubation 4 weeks at 23° C.

Organism	No inorg. N		NH_4NO_3		$NaNO_2$		NO_2-N ass. mg%
	mycelium mg	final pH	mycelium mg	final pH	mycelium mg	final pH	
Mucor hiemalis	18	6.50	17	6.35	14	6.50	0
Candida sp	19	6.35	21	6.10	21	6.35	1.8
Botrytis cinerea	377	3.20	780	2.45	563	4.40	7.2*
Sordaria fimicola	16	6.55	17	6.45	13	6.60	0
Trichoderma lignorum	48	5.45	52	5.75	43	5.80	3.2
G B4-1 sterile mycelium	32	6.25	37	6.05	35	6.80	0
Cladosporium herbarum	196	6.15	99	5.55	167	6.40	2.6
Penicillium chermesinum 1	61	5.55	507	4.60	251	6.00	19.0
P. chermesinum 2	30	3.30	322	2.80	65	4.75	2.1*
P. frequentans	48	5.75	507	4.45	194	6.15	15.0
P. implicatum	45	2.60	531	2.50	6	4.15	0
P. raistrickii	50	3.80	897	4.30	874	5.05	30.0*
Aspergillus niger	22	2.25	393	1.90	5	3.80	0
T B3-41 sterile mycelium	42	5.60	34	5.65	71	6.70	10.4
Trichoderma koningii	27	4.85	42	3.45	64	5.55	5.6
T B4-1 sterile mycelium	37	6.35	35	6.10	40	6.95	0

* corrected for non-biological decomposition of the nitrite

$$HNO_2 \overset{H^+}{\rightleftharpoons} NO_2^- \overset{OH^-}{\rightleftharpoons} NaNO_2$$

At 25°C the dissociation constant of nitrous acid is 4.5×10^{-4}, and the corresponding pK of the acid is 3.35. According to SIMON & BEEVERS (1952), changes of pH will not influence the toxicity of weak acids, e.g. nitrous acid, at pH-levels below the pK of the acid, but at higher pH-levels the concentration required for inhibition increases rapidly, causing a decrease in toxicity.

The toxicity of nitrite to *Fomes annosus* seems to follow this rule as far as pH-values between 4 and 6 are concerned (Fig. 3). In both liquid medium and on agar medium containing a fixed concentration of sodium nitrite, viz. 0.005 per cent, maximum toxicity was obtained close to pH 4, and decreased rapidly towards pH 6.

Contrary to what was expected, *Fomes annosus* was little affected by the nitrite at pH-values below 4. However, this was shown to be due to non-biological decomposition of the nitrite, mainly to nitrous oxide (N_2O_3), at the lower pH-values and not to a decrease in the toxicity *per se*. The chemical decomposition of nitrite at four different pH-values is shown in the

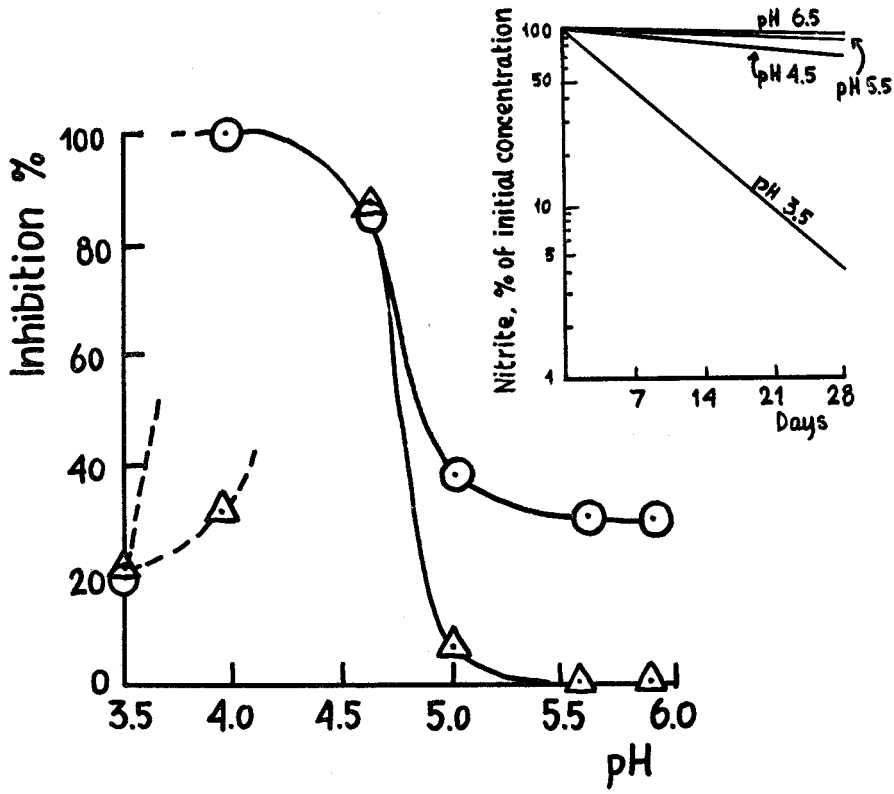

Fig. 3. Effect of pH on the toxicity of sodium nitrite to *Fomes annosus*. Legend: ○, linear inhibition on agar medium (7 days, 23°); △, inhibition calculated from dry weight of mycelium produced in liquid medium (4 weeks, 23°).
Inserted: Non-biological decomposition of nitrite in buffer solutions of different pH, temp. 23° C.

inserted graph in Fig. 3. At pH 3.5 less than five per cent of the nitrite initially present was left in a buffer solution kept at 23°C for four weeks, whereas more than 70 per cent remained in a solution of pH 4.5.

Effect on conidia of Fomes annosus

The inhibiting effect of nitrite on the germination of conidia of *Fomes annosus* was studied both in a suspension of conidia and on sterilised stem disks pre-treated with solutions of sodium nitrite.

In the first experiment, 1.8 ml of phosphate buffer (pH 5.5), containing approximately 10^3 conidia/ml, was pipetted into sterile tubes, and 0.2 ml of sterile sodium nitrite solutions (in the same buffer) was added to make a

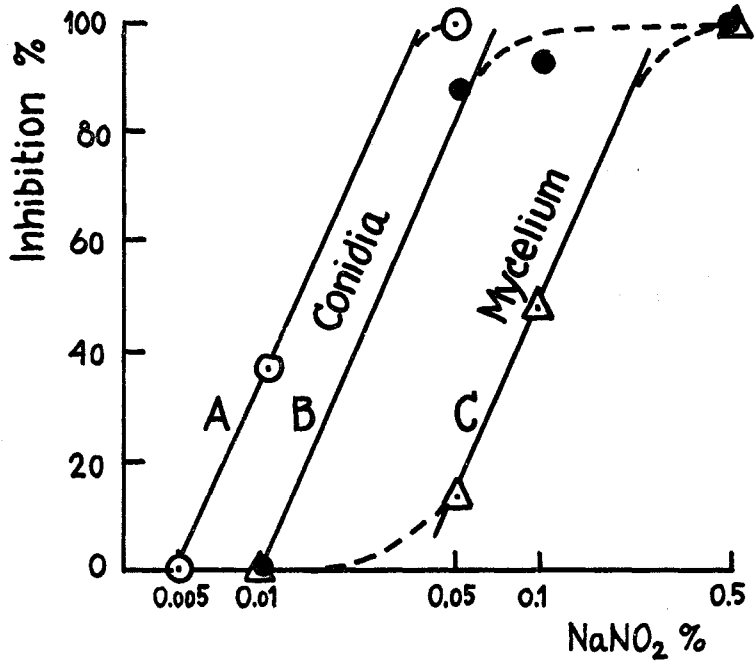

Fig. 4. Inhibition by sodium nitrite of germination of conidia and of areal growth of mycelium of *Fomes annosus*. Legend: Curve A, conidia in phosphate buffer pH 5.5; curve B, conidia on pine stem disks pH 5.4; and curve C, mycelium on spruce stem disks pH 6.0.

concentration series containing from 0.0005 to 0.1 per cent sodium nitrite. The tubes were incubated at 23 °C, and after three days the percentage of conidia which had grown was determined microscopically. The results are shown in Fig. 4, curve A. When exposed to 0.01 per cent nitrite, about 50 per cent of the viable conidia failed to grow and, at a concentration of 0.05 per cent sodium nitrite, inhibition was complete.

Essentially the same results were obtained on pine stem disks which had first been soaked in nitrite solutions and then sprayed evenly with a suspension containing less than 10^3 conidia/ml. The number of micro-colonies in ten fields was counted stereomicroscopically after five days' incubation at 23 °C, and the percentage inhibition calculated from the control disks (Fig. 4, curve B). The slightly lesser degree of inhibition of conidial germination on stem disks than in suspensions may possibly be ascribed to the dilution effect of the stem disk volume.

Table 5. Effect of sodium nitrite on the growth of some common stump fungi on sterilized pine stem disks. Incubation 7—10 days at 23°.

Organism	Growth inhibition, % of control $NaNO_2$		
	0.01 %	0.1 %	1 %
Fomes annosus	0	85	100
F. pinicola	0*	47	100
Trechispora Brinkmanni	0*	60	100
Peniophora gigantea	0	25	100
Coniophora puteana	0	0	100
Stereum sanguinolentum	0	0	100
Polyporus abietinus	0	42	100
P. borealis	19	44	100
P. circinatus	17	88	100
Lenzites sepiaria	0	0**	100

* growth stimulation
** somewhat reduced aerial mycelium

Growth inhibition of Fomes annosus mycelium

Because of the suitability of stem disks, especially of pine, as a substrate for *Fomes annosus*, and presumably because of the rapid diffusion of the nitrite-ion in the wood sap, the growth response of mycelium to different concentrations of nitrite could be measured with considerable accuracy. Areal growth curves of *Fomes annosus* are shown in Figs. 1 and 2. Between approximately 0.005 and 0.5 per cent sodium nitrite, inhibition was always fairly linear. Another nitrite response curve, drawn from 17 day-old cultures on spruce stem disks, is shown in Fig. 4, C.

As the inhibition curves show, nitrite effects the growth rate of *Fomes annosus*, but the mechanism by which the nitrite interferes is not known. Under certain circumstances nitrite may act as a mutagen (COCHRANE, 1958), but there is no evidence that mutations have occurred in *Fomes annosus* after contact with nitrite, neither has increased resistance to nitrite been observed.

The toxicity of nitrite to some common stump fungi

As none of the fungi isolated from the nitrite-treated stem disks was listed among the stump fungi investigated by KÄÄRIK & RENNERFELT (1957), it was of interest to determine to what degree nitrite would interfere with the growth of some of the common Swedish stump colonizers. The usual sterilized, nitrite-treated stem disks were used, and the degree of inhibition was calculated from the growth areas of the fungi on untreated disks, and

on disks soaked in solutions of 0.01, 0.1 and 1 per cent sodium nitrite. Nine common stump fungi, taken from the Department's pure culture collection, were tested. The results are shown in Table 5.

None of the stump fungi would grow on wood treated with one per cent nitrite, and only *Coniophora puteana*, *Stereum sanguinolentum* and *Lenzites sepiaria* grew unaffected at 0.1 per cent nitrite concentration. The difference between the degrees of inhibition of *Fomes annosus* and the remaining six stump fungi was small and, at least in the case of *Trechispora brinkmanni* and *Polyporus circinatus*, not significant. Of special interest was the reaction of *Peniophora gigantea*, the important competitor and antagonist of *Fomes annosus* in the colonisation of fresh stumps under natural conditions (RISHBETH, 1959 b). This organism has previously been found to be somewhat less sensitive to nitrite than *Fomes annosus* (GUNDERSEN, 1963) but, considering nitrite as a fungicide selective to *Fomes annosus*, the difference in sensitivity is probably not sufficiently large to favour *Peniophora gigantea* under natural conditions.

Antagonistic properties of the nitrite-assimilating microfungi

A large number of the micro-organisms common in forest soils has been found to be antagonistic to *Fomes annosus* in tests on laboratory media (BJÖRKMAN, 1949; RENNERFELT, 1949; NISSEN, 1956; KLINGSTRÖM & BEYER, 1965). However, very little is known about the nature of these antagonisms, and only in a few cases have true antibiotics been shown to be involved (RENNERFELT, 1949; ENEBO, 1949; GUNDERSEN, 1961). It is also not known whether the antagonisms are operative in the soil under natural conditions, although the failure of several workers to obtain growth of *Fomes annosus* in unsterilised forest soil may support this supposition.

As has been shown above, *Fomes annosus* was completely inhibited by 0.5 per cent sodium nitrite in stem disks, but the same concentration induced a heavy growth of microfungi, of which several were shown to be capable of assimilating nitrite. As a result of the action of these organisms on the nitrite, and because of the dilution of the nitrite by the wood sap, chemical destruction, adsorption, or other factors, the nitrite concentration will rapidly decrease, and will eventually reach a level at which *Fomes annosus* is no longer inhibited. Thus, the nitrite-treated stem disk may finally again become suitable as a substrate for *Fomes annosus*. However, assuming that one or more of the microfungi already established on the disk were antagonistic to *Fomes annosus*, a secondary barrier against the invasion of *Fomes annosus* would operate at the time when the nitrite was no longer an obstacle to its growth.

Table 6. Antagonism of microfungi against Fomes annosus. Legend: 0, antagonism not demonstrated; (+), growth of antagonist and F. annosus meeting without inhibition zone; +, slight inhibition of F. annosus; ++, moderate inhibition; and +++, strong inhibition of F. annosus.

Organism (antagonist)	Inhibition of *F. annosus*		
	stem disk	agar medium	other effects observed on agar
Mucor hiemalis	++	+÷	
Candida sp.	0	+	
Botrytis cinerea	+++	0	
Sordaria fimicola	++	+	
Trichoderma lignorum	+++	++	
G B4-1 sterile mycelium	÷++	++	slight necrosis of *F. annosus*
Cladosporium herbarum	0	(+)	
Penicillium chermesinum 1	+	+	
P. chermesinum 2	0	+	slight mutual inhibition
P. frequentans	0	−+	mutual inhibition
P. implicatum	0	++	
P. raistrickii	÷	+÷	
Aspergillus niger	++÷	++	
T B3-41 sterile mycelium	0	+÷	mutual inhibition
Trichoderma koningii	+++	++	
T B4-1 sterile mycelium	+++	++	necrosis of *F. annosus*

The following experiment was performed to investigate this possibility: Non-sterilised stem disks of pine were treated with a low concentration of sodium nitrite, viz. 0.05 per cent. After seven days' incubation, scattered colonies of microfungi had developed on the disks. At this point, pieces of agar-inoculum of *Fomes annosus* were placed on spots where the disk surfaces were apparently devoid of microfungal mycelium. Similar inocula were placed on sterilized pine disks, both on those which had been treated with 0.05 per cent nitrite, and on untreated disks. To prevent drying, and to dilute the remaining nitrite, 5 ml of sterile water was added to all disks. The incubation was then continued, and after ten days the following observation could be made:

On both kinds of control disks, *Fomes annosus* had formed well-developed colonies which were rapidly expanding from the agar inocula, but on the disks colonized by microfungi, *Fones annosus* had been completely suppressed. The sparse tufts of mycelium which could be seen on a few of the agar blocks disappeared during continued incubation, and it was obvious that one or more of the microfungi was very strongly antagonistic to *Fomes annosus*. Disks from this experiment are shown in Plate 2, Fig. 1.

The next series of experiments investigated separately the antagonistic properties of the 16 previously isolated microfungi. This was done on steri-

lized stem disks on which *Fomes annosus* and one of the suspected antagonists were inoculated opposite each other, approximately 5 cm apart.

In this test, six of the microfungi failed to show any antagonistic properties, but of the remaining ten, some were powerful antagonists capable of suppressing the growth of *Fomes annosus* almost completely. Amongst the strongest were the two species of *Trichoderma*, and *Botrytis cinerea* (Plate 2, Figs. 2, 3 and 4, and Table 6).

This experiment was repeated with agar media (Sabouraud and malt agar) which, however, unveiled a somewhat different picture of the antagonisms (Table 6). For example, the penicillia, which in the disk test had shown hardly any antagonism, were strong antagonists on agar. The same was the case with the sterile mycelium T B3-41 and *Candida* sp. The opposite was found with *Botrytis cinerea*, which did not suppress *Fomes annosus* on agar but did so well on the stem disk.

Discussion

Nitrite is a compound of considerable interest and importance in biology. It is formed by the oxidation of ammonia by some of the nitrifying bacteria, and utilized as an energy source by other nitrifiers. Furthermore, nitrite is formed as an intermediate in the biological reduction of nitrate by a diversity of micro-organisms, higher plants and animals, and may be utilized as the only source of nitrogen, for instance by several fungi (WANG, 1936; SAKAGUCHI & WANG, 1936; TALLEY & BLANK, 1942; MORTON & MACMILLAN, 1954). In spite of its central position in nitrogen metabolism, nitrite is a compound of great potential toxicity. It may react spontaneously with many organic compounds whose chemical properties are thereby drastically changed. For instance, nitrite may cause deamination of the purines and pyrimidines in both DNA and RNA, and bring about serious disturbances in the functions of the genetic apparatus (CLOWES, 1963).

The toxicity of nitrite is determined by at least two factors: its concentration, and the hydrogen-ion concentration of the solution in which it is present. As was said above, the hydrogen-ion concentration influences the degree of dissociation of the nitrite. The negatively charged nitrite-ion, formed at neutral and alkaline reactions, does not readily pass through the cell membrane, whereas the uncharged nitrous acid molecule does (MEVIUS, 1928). It is not known whether nitrite has any harmful effects on the cell membrane itself, but if it is allowed to pass into the interior of the cell, as it does in acid solutions, it may well be able to interfere adversely with metabolic processes.

The purpose of the present investigation was to look further into the action of nitrite on a mixed population of micro-organisms. In the first place, nitrite was found to depress, or even completely inhibit the growth of the parasitic basidiomycete *Fomes annosus*. In the second place, nitrite was found to serve as a nitrogen source for several indigenous saprophytic microfungi present in a substrate of low nitrogen content. The discovery that several of the microfungi were strong antagonists of *Fomes annosus* was an unexpected result. On the treated stem disks, *Fomes annosus* was thus twice prevented from establishing itself, initially by the toxic action of the nitrite, later by the antagonistic action of microfungi. In considering nitrite as a fungicide, it is a question whether this secondary effect may not be the more important, since from the moment the antagonistic fungi begin to grow on the wood, nature itself takes over the control measures.

It might be objected that the stem disk method applied in this investigation is not fully representative of events which take place under field conditions. However, when used on fresh stumps, nitrite appears to have very much the same effects as on stem disks: the germination of *Fomes annosus* conidia is inhibited, and colonization by a variety of microfungi is highly stimulated by nitrite (GUNDERSEN, 1963). The species composition of the microflora is adventitious; it probably depends entirely on the kind and number of spores and mycelia present in and on the tree and stump bark at the moment of felling, and on the kind of micro-organisms which may enter the fresh stump from the air before and after the nitrite treatment. The composition of the microflora may differ considerably even on disks cut from the same section of a stem. This may be made clear by a comparison of the disks shown in Figs. 2 and 3 of Plate 1. The two disks were both cut from the same stem and were treated simultaneously with the same concentration of nitrite. Under field conditions, the strength of the nitrite solution used, the pH of the stump tissues, and even climatic conditions may be factors which determine the species composition of the stump microflora. Since several of the more common stump fungi were shown to be as sensitive to nitrite as *Fomes annosus*, a stump microflora different from the usual might be expected on nitrite-treated stumps.

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Summary

Solutions of sodium nitrite were found to promote the growth of microfungi present as spores or mycelia on stem disks of spruce and pine. The soaking of disks in one per cent sodium nitrite solution resulted in the almost complete overgrowth of both pine and spruce disks by sporulating *Trichoderma spp*, *Penicillium spp.*, *Botrytis cinerea*, *Sordaria fimicola* and other fungi. Sixteen different species of microfungi were isolated from nitrite-treated disks. Amongst these, ten were shown to assimilate the nitrite. Several of the microfungi were strong antagonists of *Fomes annosus* in stem disk tests.

Fomes annosus conidia and mycelium were very sensitive to nitrite, germination and growth being completely inhibited by 0.5 per cent sodium nitrite at pH 5.5—6.0. Of nine common Swedish stump fungi, only *Coniophora puteana* and *Stereum sanguinolentum* were considerably less sensitive to nitrite than *Fomes annosus*.

The pH of the medium was shown to influence the toxicity of nitrite to *Fomes annosus*, maximum toxicity being obtained near pH 4.

The efficacy of nitrite as a preventive fungicide against *Fomes annosus* infection of fresh stumps is discussed.

Fig. 1

Fig. 2

Fig. 3

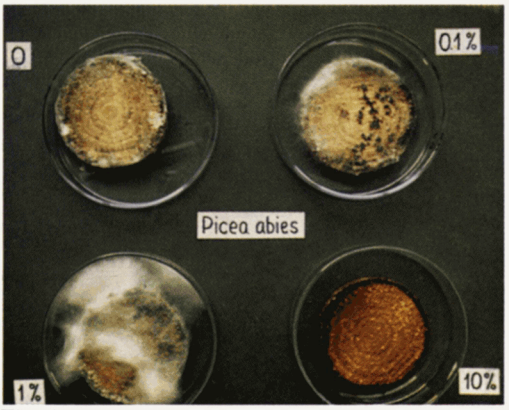

Fig. 4

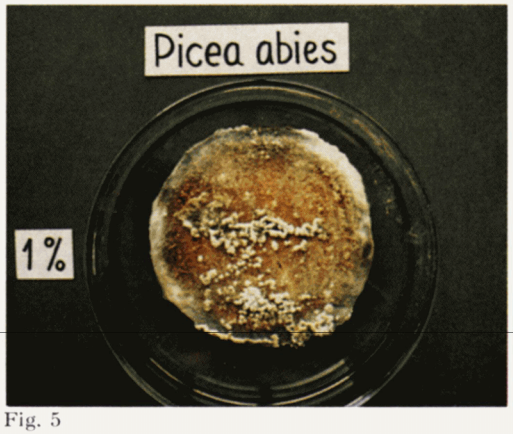

Fig. 5

Fig. 6

Fig. 1. Stem disks of pine treated with solutions of different concentration of sodium nitrite.

Fig. 2 and 3. Two different stem disks of pine almost completely covered by colonies of microfungi as a result of treatment with 1 % sodium nitrite.

Fig. 4. Stem disks of spruce treated with solutions of different concentration of sodium nitrite.

Fig. 5. Spruce stem disk after treatment with 1 % sodium nitrite.

Fig. 6. A sterile mycelium expanding on spruce stem disk treated with 10 % sodium nitrite. Note the brownish discoloration of the xylem.

All cultures were incubated for 8 days at 23° C.

Plate 2

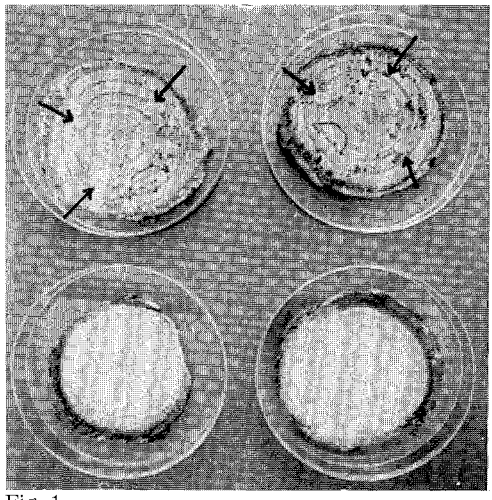

Fig. 1

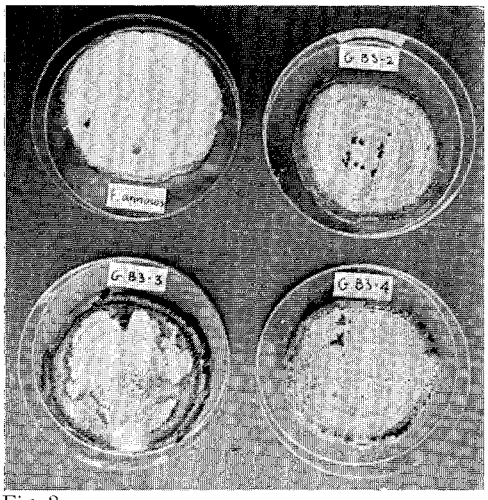

Fig. 2

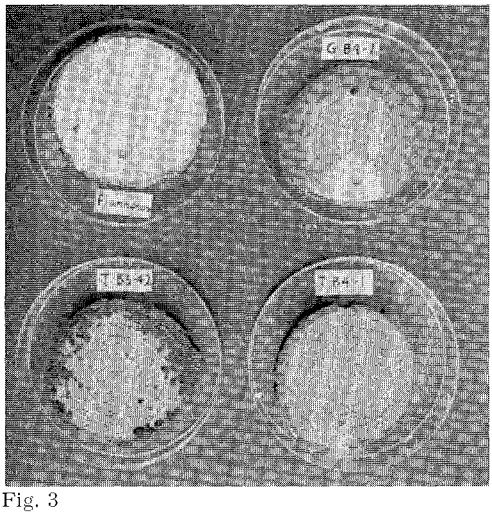

Fig. 3

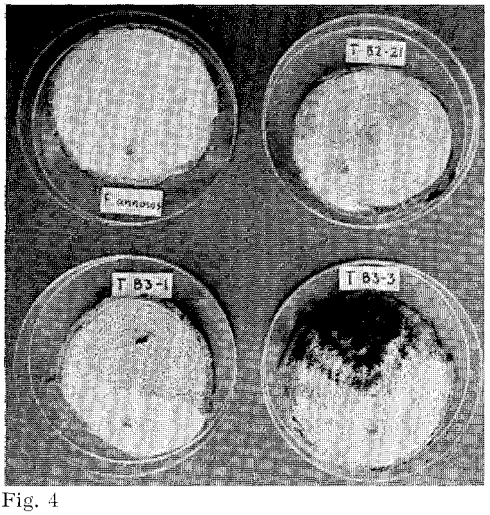

Fig. 4

Fig. 1. Antagonistic inhibition of *Fomes annosus* growth by microfungi which have colonised spontaneously pine stem disks treated with 0.05 % sodium nitrite (*two upper disks*). The arrows point to 6 mm agar pieces constituting the *F. annosus* inoculum. *Lower left: F. annosus* on sterilised stem disk treated with 0.05 % sodium nitrite; *lower right: F. annosus* on sterilised, untreated stem disk. The disks were inoculated with *F. annosus* one week after the nitrite treatment, the photographs were taken three weeks later.

Figs. 2, 3 and 4. Antagonism against *F. annosus* exerted by pure cultures of stem fungi on sterilised pine stem disks (antagonist at the upper part, *F. annosus* at the lower part of the disks). Consult Table 2 for identification of the microfungi. Incubation 2 weeks at 23° C.

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Sammanfattning

Nitrit som näringsämne för lägre svampar i tallens och granens ytterbark och dess toxicitet mot Fomes annosus

Vattenlösningar av natriumnitrit har visat sig stimulera tillväxten hos lägre svampar som förekommer på stamtrissor från gran och tall. Trissor av de nämnda trädslagen som doppats i en-procentig natriumnitritlösning invaderades nära nog fullständigt av sporbildande svampar som *Trichoderma*- och *Penicillium*-arter, *Botrytis cinerea*, *Sordaria fimicola* och andra. Sexton arter av lägre svampar kunde isoleras från de trissor som behandlats med nitrit. Tio av dessa visades kunna assimilera nitrit. Åtskilliga av svamparna hade en starkt antagonistisk effekt på *Fomes annosus* i denna typ av test.

Rotrötesvampens, *Fomes annosus*, konidier och mycel visade stor känslighet mot nitrit. Groning och tillväxt hämmades fullständigt vid en nitritkoncentration av 0,5 % vid pH 5,5—6,0. Av nio undersökta, på stubbar allmänt förekommande svampar, kan endast *Coniophora puteana* och *Stereum sanguinolentum* betecknas som påtagligt mindre känsliga mot nitrit än *Fomes annosus*. Den större tolerans mot nitrit som den konkurrerande *Peniophora gigantea* uppvisar i jämförelse med *Fomes annosus* är sannolikt alltför subtil för att praktiskt kunna utnyttjas eftersom man besprutar stubbskären med upp till 10 %-iga lösningar av natriumnitrit.

Lösningens pH-värde visades ha inverkan på nitritets giftighet (toxicitet); maximum uppnåddes vid ett pH-värde nära 4.

Användbarheten av nitrit mot *Fomes annosus* i förebyggande syfte diskuteras.

Electronic version

Edited by J.G.K.Flower-Ellis