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Biological control of *Fomes annosus* in Norway spruce (*Picea abies*) with immunizing commensals

Biologisk bekämpning av Fomes annosus i gran (Picea abies) med "immunzing commensals"

by

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ABSTRACT

All Norway spruces tested which survived prolonged exposure to *F. annosus* without damage were found to harbor microorganisms in their stem wood, while only 40 % of spruces tested at random yielded microorganisms. Several of the microbial isolates displayed antagonistic activities toward *F. annosus*, particularly *Trichoderma album* and *Coryne sarcoides*.

Artificial inoculations of immunizing commensals (IC) in fresh log sections of Norway spruce were successful only with *Trichoderma album*, *Coryne* sp. and bacteria. *Scytalidium* sp. FY strain could not be introduced successfully under those conditions. In only one instance did limited FY growth take place within the log, but it resulted in the formation of pathological zonation lines in the wood. Similar markings developed on the surface of dart-type inoculants, introduced in the live sapwood of Norway spruce.

The lethal action of *C. sarcoides* on *F. annosus* is due at least in part to the release of a water-soluble antibiotic by the former organism. No such substance appears to be released by *Trichoderma album*, although that latter fungus was able to immobilize or utilize in the malt broth one or more nutrients essential for the development of *F. annosus*. In addition, photomicrographs show that hyphae of *T. album* curl about the pathogen, while a lysis of the hyphal membrane in *F. annosus* and coagulation of its cytoplasm occur. *T. album* was effective against the pathogen at 18—22°C and at 5°C.

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1. Introduction

Biological control of forest tree pathogens has been studied for some years as a means of preventing or curing certain forest diseases. The results obtained so far have not been very encouraging, yet research efforts continue. The need is increasing for control measures which do not leave lingering chemical residues in the environment. New awareness has been shown of the hazards entailed by the indiscriminate use of chemical biocides such as chlorinated hydrocarbons, particularly DDT. Mercury salts have created serious waste disposal problems in Sweden, forcing the shut down of commercial fishing in certain coastal regions among other unfortunate repercussions.

With wood products biological control methods have been attempted also. Douglas-fir utility poles in service are infected at times with a wood destroyer *Poria carbonica* which was stopped with a wood commensal, *Scytalidium* sp. FY strain in Oregon, U.S.A. The role of FY in the ecosystem presented by the pole—or similar substrate—is defined as that of an *immunizing commensal* (Ricard & Bollen, 1968) abbreviated to IC in the following pages.

Misunderstandings have arisen in discussions about IC, particularly with respect to their identity and function. Some workers feel that IC and *Scytalidium* sp. are interchangeable terms while others see no difference between commensal and contaminant.

Whether or not a microorganism can be considered as an IC depends on (a) its effect on the substrate; and (b) the final use intended—by man—for that substrate. This latter consideration is represented by the symbol FUS in the abbreviated definition shown below. It depends further on the ability of the IC to prevent or stop the development of an harmful—in terms of FUS—organism in the substrate. This harmful organism, be it decay fungus or insect, is symbolized by HOS below while the letter ω symbolizes "*growth compatible* with" the symbol following immediately and "*incompatible* with" the lower symbol.

$$IC_d\ \omega\ \frac{FUS}{HOS}$$

IC_d represents a desirable IC, the growth of which is compatible with

FUS—the final use of the substrate as intended by man—and incompatible with HOS—an harmful organism, usually decay fungus. This summarized definition above is not intended in any way to pose as a strictly quantitized mathematical formula, but merely to function as an abbreviated definition for simplicity sake in subsequent discussion.

It is meant to illustrate that the very same strain of fungi may be an excellent IC for wood used in poles and yet act as a pathogen in the same wood—from the standpoint of anatomy—growing in a live tree intended for pulping. It should be stressed also that only microorganisms which *grow* in the substrate to be protected function as IC and not those which remain dormant or induce rejection reactions.

The original use of an IC_d, *Scytalidium* sp. FY strain was applied to the control of Douglas-fir pole decay in Oregon, U.S.A., caused by a basidiomycete, *Poria carbonica* (Ricard & Bollen, 1968; Ricard, Wilson & Bollen, 1969). Another potential use is reported here for the control of heartrot in the spruces growing in Sweden. This decay is caused by another basidiomycete, *Fomes annosus*. Its cost was estimated to 150 million Swedish crowns per year in 1955 (Rattsjö & Rennerfelt, 1955). There are, however, ways of limiting the loss by chemical pulping of damaged wood (Björkman, 1948; Björkman *et al.*, 1949; Björkman *et al.*, 1964).

2. Review of literature

Attempts at biological control for plant disease have centered largely on plant root environment, i.e. the soil. These attempts have been reviewed at length in a symposium on "Ecology of soil-borne plant pathogens" edited by K. F. Baker & W. C. Snyder (1965). With few exceptions, these attempts have not met with unqualified success. The many factors interacting in soil provide much buffering capacity and little lasting response to attempts at changing the predominant microbial populations.

Recently particular attention has been given to the manipulation of the mycorrhizal fungi. Certain mycorrhizae have been used to protect seedlings against various diseases in laboratory or greenhouse experiments (Hyppel, 1968; Marx, 1969).

Immunizing commensality attempts to establish protection within the substrate at hand rather than in its environment, distant or immediate. For wood, this concept rests simply on its following characteristics: (1) a culture medium unfavorable for many microorganisms because of its low nitrogen content; (2) a well protected substrate, enveloped in a contamination barrier, the bark; (3) a plentiful supply of energy in the form of several major carbon constituents, which are not all needed in the final uses of wood. Henningsson (1968) discusses the relative function of the various cell wall constituents with respect to microbial activities in some detail. It is also known from the elaborate studies of Professor W. Liese that certain fungi can penetrate the tracheid wall without significant damage to its structure (Liese & Schmid, 1964), while a recently developed technique for the measurement of wood deterioration by Sharp & Eggins (1968) list *Trichoderma viride* and *Gliocladium roseum* as fungi able to develop in wood without measurable effect on its mechanical properties. Etheridge (1957) reported no decay caused by *Coryne sarcoides* growing on wood blocks. Yet immunizing commensality has not received much attention as a concept in microbial ecology of living trees, perhaps because of the absence of microorganisms from certain tree species when sound. However various components of that concept have been studied separately at some length and can be reviewed accordingly.

2.1. Occurrence of microorganisms in stem wood of living trees

The early concept of sterility in sound wood has not always been borne out by recent studies. Basham (1966), Bier (1965), Bourchier (1961), Good (1962), Shigo (1965), Whittaker (1962) and others have reported the occurrence of microfungi and other organisms in wood of living trees. More recently bacteria have been found active in the lower pith column of Balsam fir by Etheridge & Morin (1967). Both sapwood and heartwood of certain sound living trees have been found to harbor microorganisms, though other species seem to remain sterile so long as the woody tissue is undamaged.

2.2. Inoculation of trees

Traditionally wood substrates are inoculated with a spore suspension or carrier supporting vegetative cells. The inoculants are often introduced in the opening left by the removal of a wood core. Details on various methods are given by Shigo (1958) and Roncadori (1962).

2.3. Interaction of fungi

This topic was reviewed recently by Baker (1968). Fungal antagonism is considered generally to occur as antibiosis, pre-empting or mycoparasitism.

Antibiosis from water-soluble metabolites is seen as inhibition zones which Hyppel studied extensively (1968) following the early work of Björkman (1949). Stillwell *et al.* (1969) characterized cryptosporiopsin a broad-spectrum antibiotic produced by a wood-inhabiting fungus, while Etheridge (1957) attributed the antagonism exhibited by *Coryne sarcoides* against wood-destroying fungi to an unidentified antibiotic substance. Mycoparasitism involves direct contact between hyphae of antagonistic fungi. Barnett (1964) reviewed this form of antagonism, citing *Gliocladium roseum* as a typical mycoparasite and pointing to details of its mode of action. Certain fungal species appear to attack basidiomycetes through both antibiosis and mycoparasitism. Weindling (1932) and subsequent workers reported the activities of *Trichoderma viride* as a mycoparasite, but also in certain strains, as the source of two antibiotics, gliotoxin and viridin. Not all strains of *T. viride* are equally active, some cultures did not release antibiotics. In addition low temperatures affect appreciably the physiological activities of certain active strains, Hyppel (1963) found that temperatures below 12°C affected adversely *T. viride,* while *Fomes annosus* would continue its development. Etheridge (1969) found also that low tem-

peratures favored another pathogen, *Stereum sanguinolentum* on fresh-wound surfaces over competing fungi such as *Peniophora cinerea, Alternaria tenuis* and *Ceratocystis piceae.*

Pre-empting by various fungi of essential nutrients can prevent development of decay organisms. Baechler (1956) reported on the effect of thiamin immobilization as a means to prevent decay in wood. In field studies, Cobb & Barber (1968) observed that "blue stain fungi occurred in many stumps and often appeared to limit colonization by *F. annosus*". Lai & Bruehl (1968) emphasize that relative sequence in colonization may be an important factor in determining the antagonistic effect of fungi: "the isolation of organisms isolated on the same stems and buried in soil for 5—8 months emphasized the importance of 'possession of substrate'. Each of the test fungi *(Trichoderma* sp. and *Cephalosporium gramineum)* that were placed first on straw exhibited some ability to dominate the substrate and to limit colonization of that substrate by challenging fungi." Hodges (1969) concluded from his extensive observations on southern yellow pines that, "All fungi which invade the root system ahead of *F. annosus* effectively block further extension of that fungus. This is irrespective of whether or not these fungi show antagonistic effects in pure culture against *F. annosus.*"

Yet Manka (1965) favors *Trichoderma album*—obtained from soil —as he indicates that from all *Trichoderma* sp., *T. album* showed the strongest antagonistic activity against *F. annosus.* This was observed when discs of healthy trunks of *Pinus silvestris* were inoculated artificially with mixtures of these fungi. The conclusion of this study was as follows: "a fully effective protection of discs of *P. silvestris* against infection by *F. annosus* at 23°C was obtained only when the discs were inoculated with spore suspensions of *T. album.* Other *Trichoderma* sp. were not so successful."

These reports contributed largely to the selection of *Trichoderma album* and *Coryne sarcoides* for more detailed study among the various fungi isolated from Norway spruces surviving prolonged exposure to *Fomes annosus.* Several other antagonistic microorganisms were isolated, but were not studied further as no information seemed available about them.

2.4. Field applications

Rishbeth (1957, 1959*a*, 1959*b*, 1959*c*, 1963, 1967) made extensive studies of the *F. annosus* problem in England. He demonstrated the effectiveness of a wood destroyer *Peniophora gigantea* to stop stump

infection during thinning in pine stands. The method has now been applied to several thousand acres of pine forest. Rishbeth made an invaluable contribution by obtaining successful results in the field for the control of *F. annosus* with a biological agent, avoiding the use of lingering poisonous substances such as boron and fluoride compounds.

3. Materials and methods

3.1. Microbial population in stem wood of Norway spruces

3.1.1. Isolation of microorganisms

The trees were sampled with an increment borer dipped in alcohol to prevent contamination. The wood cores were handled in the fashion described earlier by Ricard & Mothershead (1966) and plated in two Petri dishes containing respectively 2.5 % and 1.5 % malt extract agar.

3.1.2. Demonstration of antagonism

Fungi and actinomycetes yielded by the wood cores were transferred to the center of fresh 2.5 % malt extract plates and isolates of *F. annosus* from three different sources were placed near the edge of the medium, equidistant from one another (see Fig. 1). Bacterial isolates were streaked from the edge of the medium to the center along three lines in such a way as to mark off 3 wedge-shaped areas of similar size, as illustrated in Fig. 1.

3.1.3. Selection of strains

The *Scytalidium* sp. FY strain obtained from Oregon was used for the experiments described here. The *Trichoderma album* and *Coryne sarcoides* cultures were isolated from the Tönnersjöheden forest trees reported in Table III.

3.2. Introduction of antagonistic isolates in stem wood of Norway spruces

3.2.1. Inoculant preparation

One hundred-cm^3 Erlenmeyers received 25 cm^3 of (chain-)saw dust and 15 ml of liquid. Wood from three tree species were used: birch, Scots pine and Norway spruce. Water solution of malt extract, ammonium nitrate and ammonium tartrate were used separately for enrichment purposes. Two concentrations of malt extract were prepared: 0.25 % and 2.5 %; for ammonium tartrate 0.01 % and 0.1 %; with ammonium nitrate 0.1 %. Four replicates were prepared with each variable. Inoculations were made with the customary mycelium-covered agar blocks and incubation lasted 8 weeks at room temperature. Some of these cultures were used for inoculation of log sections by forcing wood particles covered with mycelium into an increment borer opening.

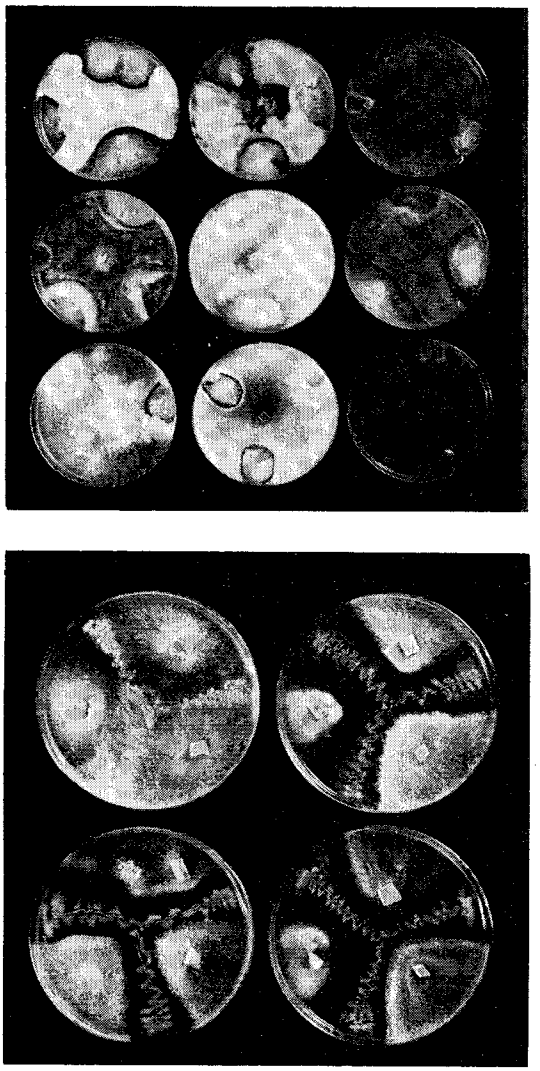

Fig. 1. Evaluation of antagonism between *Fomes annosus* and various microorganisms isolated from stem wood. Isolates from Tönnersjöheden and other forests were cross plated with three strains of *F. annosus* on malt extract agar and incubated at room temperature (18 to 22°C) for 30 days.
In the *upper* photograph, note the center plate where growth of *F. annosus* is unhampered, while the plates at both ends of the right hand row show almost complete inhibition of *F. annosus* by *T. album*.
The *lower* photograph illustrates screening of bacterial isolates.

With FY, other inoculants were used in addition to the sawdust: darts prepared with birch doweling and Douglas-fir veneer strips. The preparation of these inoculants was described earlier by Ricard, Wilson & Bollen (1969).

3.2.2. Log inoculation

Norway spruces sampled at Stensängen were cut in log sections approximately 35 cm long with a diameter ranging from 16 to 24 cm. As soon as cut the logs were placed temporarily in large plastic bags and taken to the greenhouse. All cut surfaces were then rubbed with ethanol and the logs were wrapped in tight-fitting plastic bags. The bags were then sealed with masking tape. For inoculation purposes and later to determine the extent of microbial development—if any—from the inoculant introduced, the surface of the plastic was rubbed with ethanol and the increment borer applied directly through the wrapping. Once the core was obtained, the opening was handled in the same fashion as usual though covered with masking tape as the last step.

Upon completion of the experiment, the log was split for visual observation of the inoculant behavior inside the log.

3.3. Interaction of certain IC's and *F. annosus*

3.3.1. Photomicrographs

For examination under the light and "Stereoscan" electron microscope, *F. annosus* and fungal antagonists were cross-plated on malt extract agar in the usual fashion. Although a glass coverslip was then placed aseptically between the two inoculants so that individual hyphae from known origin could be examined and photographed.

With the light microscope, the coverslip could be examined while in place by merely removing the cover of the Petri dish. For actual photography, the coverslip was removed from the agar and placed on a standard glass slide. A 10/0.22 objective was used with a standard Zeiss camera giving an approximate 600 × magnification in the prints.

With the "Stereoscan" electron microscope, the glass coverslip was first placed on the aluminium stub about 1 cm in diameter, provided for use in the unit and glued in place. The overhanging portion of the coverslip outside the metal stub was then broken off with tweezers. The specimen was then plated by gold vapors under vacuum and examined.

In contrast with the standard electron microscope, the "Stereoscan" does not allow differential penetration of the electrons, instead it "detects and displays information derived from the action of an ultra-fine electron probe scanning a specimen surface in a square raster. Processed information .... is imaged on crt screens scanned in synchronism with the electron probe..... Resolution depends upon the type of specimen and the operating mode and conditions, but .... it is always better than 300 Å and can be better than 150 Å. Depth of focus .... is always better than that of a light microscope for a similar magnification by a factor of at least 300. Magnification values .... are switchable from × 20 till × 100,000." Cambridge Instrument Company, Ltd., London SW 1, England.

A major difficulty experienced with the "Stereoscan" was in the focusing on particular hyphae spotted with the light microscope. After several unsuccessful attempts, a make shift technique proved effective. It consisted in building a ridge with household glue around the area of interest. Once thoroughly dried the glue could be gold plated as readily as fungal hyphae, though partial drying would result in undesirable bubbling action under vacuum.

Photographs were taken with a Land polaroid camera or on 24×36 mm film strips.

3.3.2. Cross plating and viability test

Interaction between *F. annosus* and various IC's was evaluated by cross plating on 2.5 % malt extract agar followed by transfer of the mixed mycelia from the two fungi tested to Kuhlman-Hendrix agar (1962). If *F. annosus* hyphae had been merely inhibited upon contact by antagonistic hyphae, then growth should have resumed on this medium favoring the pathogen. If *F. annosus* was killed upon contact with the IC, then no growth of the pathogen could take place even on the selective medium. Both *Trichoderma viride* and *Scytalidium* sp. FY strain can grow somewhat on the Kuhlman-Hendrix medium, but not as rapidly and profusely as *F. annosus*. Fig. 7 provided kindly by Mr. Delatour of the Centre National de Recherches Forestières in Nancy, France, illustrates growth of *F. annosus* on malt extract agar and the Kuhlman-Hendrix medium respectively.

3.3.3. Dilution experiments

Two and one half percent malt broth was inoculated with *Trichoderma album, Coryne sarcoides* and FY and incubated at room temper-

ature for 30 days on a mechanical shaker. It was then incubated, still, for another 60 days. The broth was then sterilized by filtration through a Millipore membrane with 0.22 micron openings. The relative fungal yield, dry weight, was 2.75 g per l for *T. album* and 1.17 g for *C. sarcoides*. The spent filtered broth was then distributed among a number of Erlenmeyers. Half of the flasks were diluted with sterile fresh malt broth while sterile distilled water was added to the flasks in the other half at the following concentrations: 2, 5, 10, 20, 30, 40, 50 and 100 % spent broth. The flasks were then inoculated with *F. annosus* and incubated for 7 weeks.

Upon completion of the experiment, the mycelia were collected, dried and weighed. The mass of the mycelia was then plotted against concentration of fresh broth or distilled water.

3.3.4. Low temperature interaction between F. annosus and T. album

Three sets of six flasks were used, one set was inoculated with *F. annosus* only, one set with *T. album* and the third set was inoculated with both fungi.

All eighteen 100 ml Erlenmeyer flasks containing 50 ml of 2.5 percent malt broth were inoculated and incubated for 5 days at room temperature. Then one half of the flasks was placed at 5°C while the other half remained at room temperature. The incubation continued for 3 weeks, the mycelium was then filtered off, dried and weighed.

3.3.5. Mass determination of the FY crystals

The crystals formed by FY were reported earlier as active against *Poria carbonica* (Ricard & Bollen, 1968). An attempt was made, through the cooperation of the Spectrometry Department of the Royal Caroline Medico-Chirurgical Institute under the guidance of Docent R. Ryhage to determine whether or not they represented a single chemical compound and, if pure, its mass.

Crystals were picked off the malt extract agar medium and placed directly in the syringe of the mass spectrometer. The graph obtained in the low resolution LKB unit indicated the purity of the compound present in the crystals and the high resolution Atlas MAT, model SMI, allowed computation of the exact mass.

3.3.6. Stump inoculation experiment

At the Rattsjöberg farm in Värmland, 21 Norway spruces about 18 to 25 cm in diameter at the stump were cut off and inoculated on 8 July, 1969.

The freshly exposed wood surface was immediately coated with a propagule suspension of *T. album* and *Coryne sarcoides,* single or mixed with *F. annosus* spores. The treatments were *T. album* alone, *F. annosus* alone, *F. annosus* and *T. album* mixed, *C. sarcoides* alone, *C. sarcoides* and *F. annosus* mixed. All treatments were made in triplicate, except the *T. album* treatment which was applied to six trees.

The IC propagule suspension was prepared as follows: One-month-old plates covered with spores were scrapped off and the fungal particles were collected in a sterile 0.2 % agar semi-gel. The mixture was ground aseptically in a Waring blendor for 45 seconds. The suspension was poured on top of the stump, then spread fairly evenly with a paint brush. No cover was put over the treated stumps as the weather was cool and moist.

The *F. annosus* propagules were suspended in water in a preparation supplied by Dr. Hyppel and applied with a plastic squeeze bottle.

4. Results

4.1. Microbial population in stem wood of Norway spruces

The culture on malt extract agar of wood cores from Norway spruces of various locations showed that the occurrence of microorganisms in the stem wood was a common event. Table 1 reports the number of trees sampled at various locations. A total of 546 trees were sampled one or more times and 274 of them showed microorganisms indicating an overall incidence of microorganisms in 50.4 % of the trees sampled. The incidence varied appreciably however, ranging from 100 % in the

Table 1. Occurrence of microorganisms in the stem wood of Norway spruce trees sampled at various locations in Sweden in late 1968 and early 1969.

Location of trees					Age[a] in years	Characteristics of trees samples	No. trees sampled	No. trees yielding microorganisms
Community	County	Latitude[a]	Longitude[a]	Elevation in m[a]				
Simlångsdalen	Halland	56°43′	13°7′	190	66	surviving protracted exposure to *F. annosus*	10	10
Långmor	Gävleborg	61°58′	16°15′	300	40	random sampling	58	18
					60	mature healthy trees	7	7
					30	high nitrogen fertilization	33	15
					96	old healthy trees	18	18
North Dellen	”	61°55′	16°40′	42	60	very high water table	12	11
Skuleberget	Västernorrland	63°7′	18°25′	150	40	random	59	45
					40	alkaline soil streak pH 7.5—8.0	19	16
Garpenberg	Kopparberg	60°18′	16°10′	230	40	(pole)	(1)	(0)
					40	random sampling	4	4
Stensängen	Stockholm	59°25′	18°13′	30	60	random sampling	325	130
Total							545	274

[a] Approximately or average

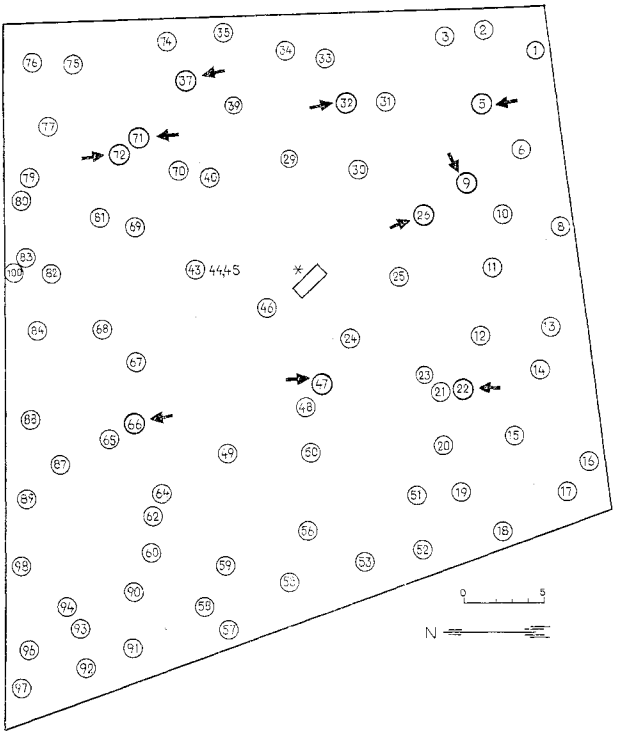

Fig. 2. Location of 66-year-old trees surviving exposure to *F. annosus* for more than 14 years in the Tönnersjöheden forest near Simlångsdalen in the Halland province. The plot held 100 trees originally. The trees unaffected by *F. annosus* are shown by arrows. The numbers missing represent fallen trees.
Diagram prepared by Mr. Sture Johansson, Simlångsdalen 1968 from experimental plot No. 14. Stora Skärsjön (Rennerfelt, 1958). See Table 3 for details on microorganisms isolated.

"surviving" trees of Simlångsdalen to a low of 40 % in the 325 trees sampled at random in the Stensängen area.

This finding (Comparable results have been reported in northeastern France by Delatour, 1970) was somewhat unexpected and verification of the detection technique took place on two different occasions: once by the sampling of a pole in Garpenberg (which yielded no microorganism) and later by a return trip to Simlångsdalen. This second trip was intended mainly to ascertain the origin of the fungi obtained earlier in terms of heartwood or sapwood. Several fungi were found to occur in the heartwood (see Table 3). In one tree, *Coryne sarcoides* was found only in the heartwood, at the second sampling.

Table 2. Frequency distribution of common microorganisms isolated from stem wood of Norway spruces at various locations in Sweden during late 1968 and early 1969.

Microorganisms	No. of trees	Notes
Bacteria	110	
Coryne sp.	40	Fungi listed as *Coryne* sp. showed hyphal glomerules and other morphological characteristics of the genus but no purple coloration. *C. sarcoides* displayed the typical pigment.
Coryne sarcoides	31	Six of these or about 18 % of the total found were isolated from trees fertilized with nitrogen in the Långmor area at the rate of 200 kg per ha.
Cephalosporium sp.	28	
Fomes annosus	28	
Ceratocystis piceae	3	
Trichoderma album	1	*T. album* was probably recovered from several other trees, but it could not be identified positively for lack of spores.

The situation in Simlångsdalen is particularly interesting since the trees sampled there are known clearly to have been exposed extensively to *F. annosus* for at least 14 years. As shown in Fig. 2, the trees, in a certain experimental plot there, have been sampled every 4 years to determine the location of *F. annosus* in the stems. The position of the "advancing front" of the pathogen is marked on the outside of the stem by a colored band.

One hundred trees were present in the experimental plot originally, 13 have disappeared already. In most stems each quadriennial check reveals further advance of the pathogen. However in several trees, the situation is different; either the pathogen has stopped as in tree No. 5 or it never started as in tree No. 71. All these unusual trees were sampled, all of them yielded fungi antagonistic to *F. annosus*.

Two of the isolates from Simlångsdalen were identified as *Trichoderma album* by Miss L. Beyer, Royal College of Forestry, Stockholm and Dr. C. S. Hodges, U.S. Forest Service Research Laboratories, Research Triangle Park, North Carolina, U.S.A. and *Coryne sarcoides* by Dr. J. A. von Arx, Centraalbureau voor Schimmelcultures, Baarn, Netherlands. Both of these fungi or related species have been studied by other research workers and reported as harmless to the wood (Sharp & Eggins, 1968) though antagonistic against various wood destroying fungi (Etheridge, 1957; Manka, 1965).

Coryne sarcoides was isolated from spruces at other locations as well and was particularly common in the "high nitrogen" trees of the Långmor area. The frequency of occurrence for the more common microorganisms is listed in Table 2.

In addition to published reports, the merits of various isolates were evaluated by cross plating with three different strains of *F. annosus* (see Fig. 1). In some instances the pathogen would completely overwhelm the isolate as seen in the center plate, but in other cultures *F. annosus* could barely begin to develop from the inoculant before it was stopped by the isolate. With bacteria, inhibition zone was the more common event if antagonism was to develop as shown in the lower photograph of Fig. 1. Another difference between fungal and bacterial isolates was that fungi seem to react equally to the various strains of *F. annosus,* while bacteria would be more active against certain strains than others.

Another frequent observation was that fungi freshly isolated from a tree would effectively hold back *F. annosus,* but that very same culture after a few transfers on malt extract agar would often become defenseless against the pathogen. This was noticed repeatedly with *Ceratocystis piceae* and *Cephalosporium* sp. These observations were consistent with Hodges' comments and Cobb's findings to the effect that antagonism in plates is not a necessary prerequisite for preventive action in the field, particularly in the case of *Ceratocystis* (see the section on review of literature for further details). Variation in performance can also result from prolonged exposure to laboratory culture conditions.

Such variation was experienced earlier with *Scytalidium* sp. FY strain and reported accordingly (Ricard, Wilson & Bollen, 1969). It is expressed both physiologically (pigment release) and morphologically (spore occurrence and mycelium texture).

As a result of the findings shown in Table 3, the following organisms were selected for further investigation: *Trichoderma album, Coryne* sp., *Ceratocystis piceae, Cephalosporium* sp. and bacteria. *Scytalidium* sp. FY strain was used also on the basis of its performance in Oregon, U.S.A. No *Scytalidium* isolate was obtained from the 546 trees sampled which resembled FY in terms of pigment release or antagonistic properties.

It should be emphasized that many other antagonistic microorganisms were isolated from spruce wood, but were discarded because no information was available about them. Sometimes not even a mere description. This happened with several fungi isolated from the Skule-

Table 3. Microbial population present in stems of healthy Norway spruces of the Tönnersjöheden forest surviving 14-year exposure to *F. annosus* propagules; samples obtained from experimental plot No. 14. Stora Skärsjön in the autumn of 1968.

Tree No. painted on trunk	Condition of the *F. annosus* infection	Microbial population			
		Location[a]		Occurrence of *F. annosus*[b]	Predominant type[c]
		Sapwood	Heartwood		
32	Stopped 12 years ago	+	++	+	*Coryne sarcoides*
71	never started	+	+	—	*Ceratocystis piceae*
47	V-shaped trunk; one branch infected, the other is intact	+	?	+	*Cephalosporium* sp.
66	never started	+	+	—	*Cephalosporium* sp.
72	stopped 12 years ago	+	+	+	probably *Trichoderma album*
9	never started	root samples		—	*Geotrichum* sp.
26	never started	root samples			bacteria
22	V-shaped trunk, infection stopped 2.5 m above ground in one branch, 0.3 m in the other	+	+	—	*Coryne* sp.
5	stopped 12 years ago about 2 m above the ground	+	+	+	*Trichoderma album*

a + = present; ++ = present in abundance; — = absent.
b Below the line painted on the stem on the basis of earlier sampling.
c Several microbial species were obtained occasionally from the same core as shown in Fig. 2. These different organisms would grow from different portions of the wood core, never as mixed cultures. All the organisms shown here were obtained above the line painted on the stems.

berget area, particularly with fungi releasing a distinctive green water-soluble pigment in the malt extract agar.

4.2. Introduction of antagonistic isolates in stem wood of Norway spruces

The various enrichments tried affected the development of nearly all the isolates used as reported in Table 4. Only *Ceratocystis piceae*—of the Simlångsdalen isolates—was able to grow on all substrates.

The sawdust cultures showing the more profuse microbial develop-

Table 4. Preparation of inoculant with potential IC for introduction in green spruce log section. The inoculation material was incubated at room temperature (18—22°C) for 60 days.

Culture material	Microorganisms used				
	C. piceae	*Cephalo-sporium*	FY	*C. sar-coides*	*Bacteria*[b]
Spruce[a] and water	1[c]	0	1	0	0
,, ,, malt extract:					
0.25 %	3	1	2	2	0
2.5 %	4	4	4	4	0
,, ,, NH_4 tartrate:					
0.01 %	2	1	3	2	0
0.1 %	2	1	3	2	0
,, ,, NH_4NO_3					
0.1 %	1	1	2	1	0
Pine[a] and water	2	1	1	1	0
,, ,, malt extract:					
0.25 %	2	2	2	1	0
2.5 %	4	4	4	4	0
,, ,, NH_4 tartrate:					
0.01 %	2	2	2	1	0
0.1 %	2	0	3	1	0
,, ,, NH_4NO_3					
0.1 %	1	1	3	0	0
Birch[a] and water	1	1	2	0	2
,, ,, malt extract:					
0.25 %	1	0	2	2	3
2.5 %	3	2	3	4	3
,, ,, NH_4 tartrate:					
0.01 %	2	0	4	1	3
0.1 %	2	0	3	1	3
,, ,, NH_4NO_3					
0.1 %	1	0	3	1	3

a As (chain-)sawdust.

b The lack of growth of bacteria on spruce and pine may have been caused by oxygen tension rather than chemical composition of the substrate. The birch was divided in smaller particles than the other wood substrates and allowed less air to circulate between the particles.

c At conclusion of experiment, each flask was rated as follows: 0, no growth, based on absence of mycelial and spore development for fungi or cells as seen under the microscope for bacteria; 1, scant growth; 2, light growth; 3, moderate growth; 4, profuse growth.

Table 5. Results of IC inoculation in Norway spruce logs, obtained from Stensängen forests. The trees used were either infected naturally with *F. annosus* or free from any microorganism as determined by the sampling reported in Table 1.

Microorganism inoculated	No. of logs infected with *Fomes annosus*		No. of logs free from fungi	
	Inoculated	Successful implants	Inoculated	Successful implants
Scytalidium sp. FY strain	7	0	2	0
Coryne sarcoides	6	0	3	0
Coryne sp.	—	—	2	1
Cephalosporium sp.	4	0	3	0
Trichoderma album	1	0	2	2
Ceratocystis piceae	2	0	—	—
Bacteria	2	1	—	—
Total	22	1	12	3

ment were then introduced in the log sections. These cultures were consistently those enriched with malt extract broth at 2.5 % concentration. The spruce wood was preferred since the logs to be inoculated were of the same wood.

The result of the inoculations are listed in Table 5. By and large these results were not encouraging, though somewhat expected from previous experience of other workers and the writer in that area. *Stereum sanguinolentum* and other fungi developed abundantly on the cut ends of the logs, but the internal portion of the logs remained remarkably free from contaminants as demonstrated by the many cores obtained after incubation of the logs which yielded no microbial growth upon plating on malt extract agar.

Not one successful implant was obtained from inoculation with fungi in infected logs, though bacteria became established in one of the two logs in which they were introduced. In the logs free from infection, inoculations were somewhat more effective, particularly when *Trichoderma album* was used.

It was particularly interesting to find that fungus well established, since it had been included in that experiment somewhat as an afterthought because of the delays experienced in its identification (Fresh isolates of *T. album* did not sporulate readily making their identification difficult for a number of weeks). In order to include *T. album* in the log experiment, inoculations were made simply with strips of malt extract agar overgrown with the mycelium. Theoretically, these inoculants were far less effective than the sawdust or the doweling sections where the mass of propagule introduced is substantially

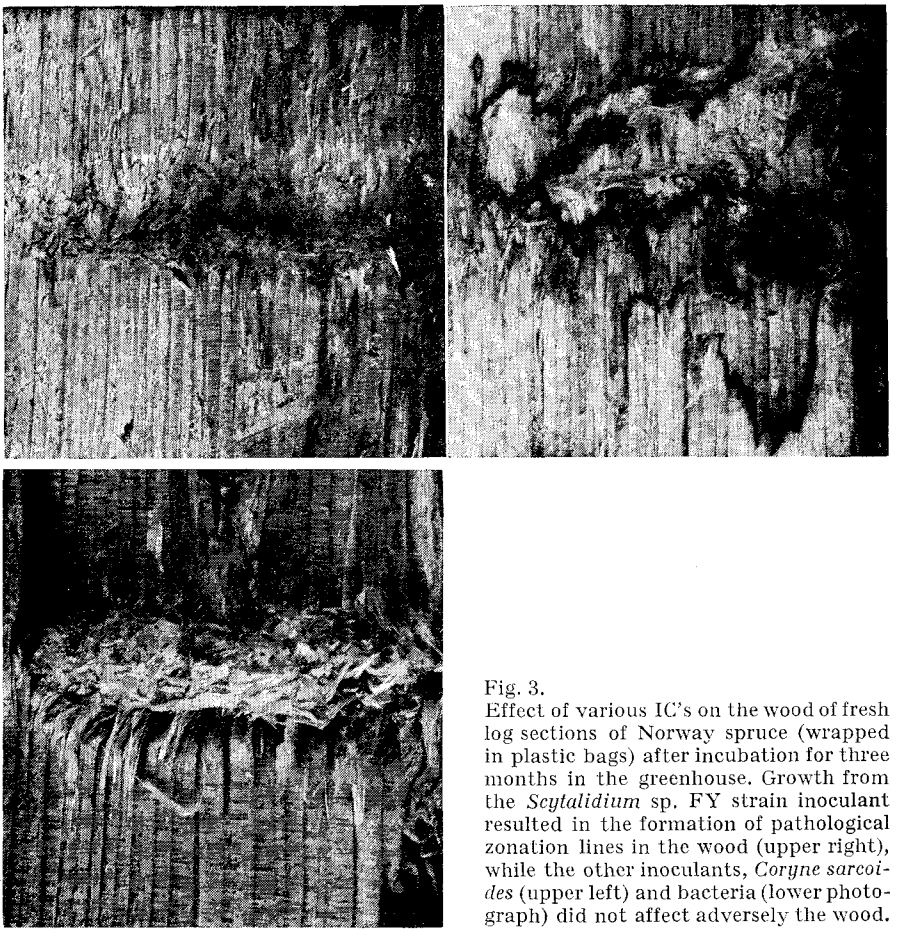

Fig. 3.
Effect of various IC's on the wood of fresh log sections of Norway spruce (wrapped in plastic bags) after incubation for three months in the greenhouse. Growth from the *Scytalidium* sp. FY strain inoculant resulted in the formation of pathological zonation lines in the wood (upper right), while the other inoculants, *Coryne sarcoides* (upper left) and bacteria (lower photograph) did not affect adversely the wood.

greater. Furthermore the substrate used for culture of the inoculant is much more similar to that found in the log for sawdust or other wood type inoculants. Outside of the *T. album,* only *Coryne* sp. could be recovered in the log sections.

FY was conspicuous by its failure to become established readily in the wood in spite of its inoculation in a total of 9 logs.

This failure was not due to a lack of viable propagules in the inoculants as even after incubation in the logs, the FY inoculants would yield the desired fungus when plated on agar medium. An explanation was obtained when the logs were split and pathological zonation lines were found within the wood (Fig. 3).

No pathological zonation lines were found with any of the other microorganisms introduced. Since *Coryne sarcoides* implant was not

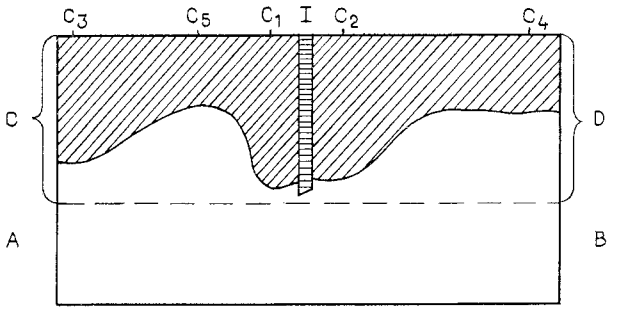

Fig. 4. Cross section of a Norway spruce log inoculated with *Trichoderma album*. The shaded area represents the portion of log where *T. album* became established. A and B represent the cut-off ends of the log. The log portion between C and D was tested through cores taken at various points, shown as C_1 to C_5. The original boring was used for introduction of inoculant (I).

available, a naturally inoculated tree was examined. Stem wood surrounding the sampling area of a wood core extensively permeated with *C. sarcoides* showed no pathological zonation lines.

For FY the results of the log inoculation experiment were consistent with the findings of the stem wood sampling. That organism does not seem to prosper in live Norway spruce, where it should be considered as a pathogen since (1) it induces the formation of pathological zonation lines; (2) related species cause blue stain (Klingström & Beyer, 1965); (3) it uses xylan, which is an important component of pulp (Ricard & Bollen, 1968); (4) it causes significant mass loss (Klingström & Beyer, 1965).

The occurrence of bacteria in the log sections was quite interesting but was not studied further in this investigation.

The qualitative recovery of *T. album* from logs led to further sampling for an assessment of its distribution within the log. The results of the additional samplings are presented in the diagram of Fig. 4. The mycelium of *T. album* was found to grow both in heartwood and sapwood. Its distribution may have been influenced by oxygen tension though not very strongly. Its is obvious however that development of *T. album* mycelium in the wood does not proceed in as uniform fashion as it does on malt extract agar. This also was somewhat expected from the earlier observations made in Oregon on the development pattern of FY in Douglas-fir poles.

4.3. Interaction of certain IC's and *F. annosus*

4.3.1. Hyphal morphology

The photomicrographs of Fig. 5 show clearly that side by side occurrence of *Trichoderma album* or *Scytalidium* sp. FY strain and *F. annosus* seem to have little detrimental effect on the first two fungi. On the other hand, *F. annosus* hyphae are obviously in poor condition. In the upper photomicrograph several holes in the hyphal membrane *F. annosus* can be seen. In addition the contents of the punctured hyphae have no longer the homogeneous appearance of healthy cytoplasm, but instead have taken the appearance of coagulated proteins.

In the lower photograph, individual gaps in the hyphal membrane cannot be seen so readily, but the contents of the hypha appear to be in an advanced stage of decomposition.

In contrast with the condition of the pathogen, the hyphae of *T. album* and FY both appear to be quite healthy judging from the continuity of the hyphal membrane and the uniformity of the contents of the hyphae.

The identity of the respective hyphae can be assessed readily from the occurrence of conidia on the FY and *F. annosus* hyphae, besides the relative diameter of the hyphae involved. No *F. annosus* conidia are seen in the photomicrographs presented in Fig. 5, but they were found frequently in the older portions of the mycelium. FY spores are obvious in the lower photomicrograph. In Fig. 6 condition of a *F. annosus* hypha lying side by side with an FY hypha is shown as seen with the "Stereoscan" electron microscope.

4.3.2. Viability

The cross plating experiments followed up by plating on Kuhlman-Hendrix medium provided perhaps the most conclusive single bit of evidence to the effect that FY and *T. album* do kill *F. annosus* and not merely inhibit it.

The Kuhlman-Hendrix medium is a selective culture material intended to encourage specifically the development of *F. annosus* while it is much less favorable to most other fungi, particulary the *Trichoderma* sp. The upper photograph of Fig. 7 shows typical growth of *F. annosus* on Kuhlman-Hendrix medium in comparison with its development on the malt extract agar.

The lower photograph on the right of that same Fig. 7 indicates the relative growth of *T. album* and *F. annosus* on Kuhlman-Hendrix medium when inoculated simultaneously. These two fungi were inocu-

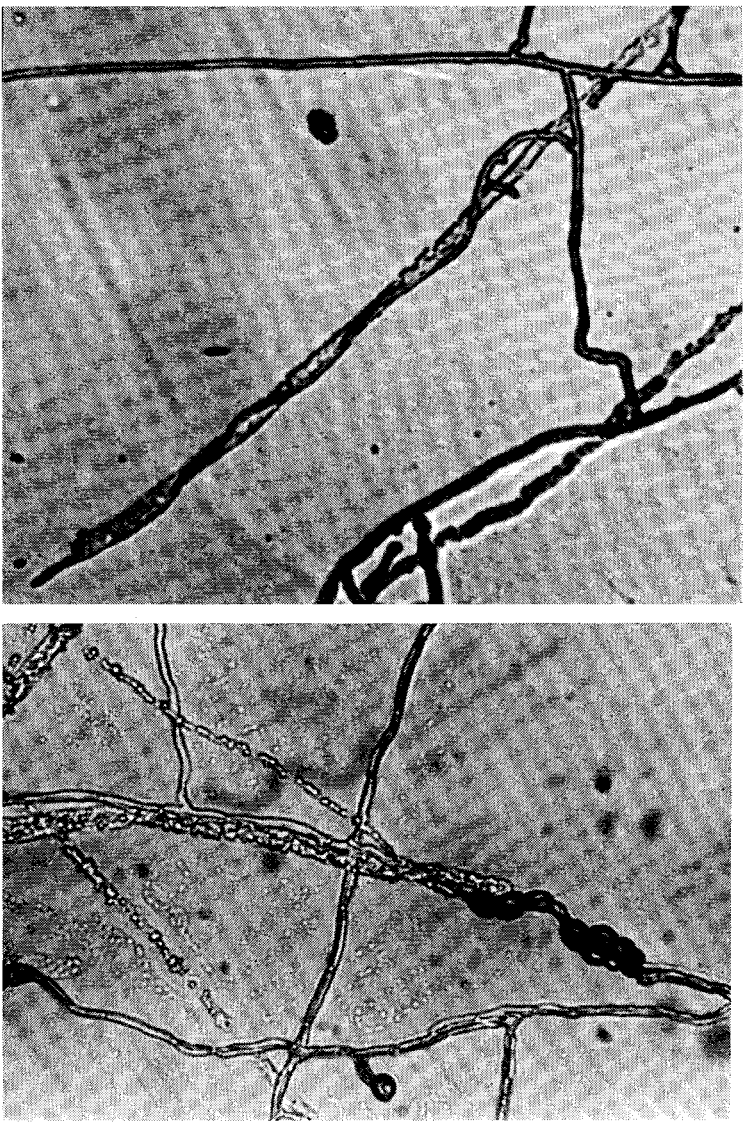

Fig. 5. Interaction between hyphae of two IC and *Fomes annosus*. In the *upper* photograph, *Trichoderma album* is seen curling about *F. annosus*. Holes appear in the membrane of the pathogen, as expected of polyene type toxins (Kinsky, 1967) the cytoplasm of *F. annosus* appears to be denatured.
In the *lower* photograph, FY shows the typical spores of the *Scytalidium* sp. and developed side by side with an hypha of *F. annosus* in an advanced stage of decomposition. Both FY and *F. annosus* were inoculated in the plate at the same time.
Magnification 528 ×.

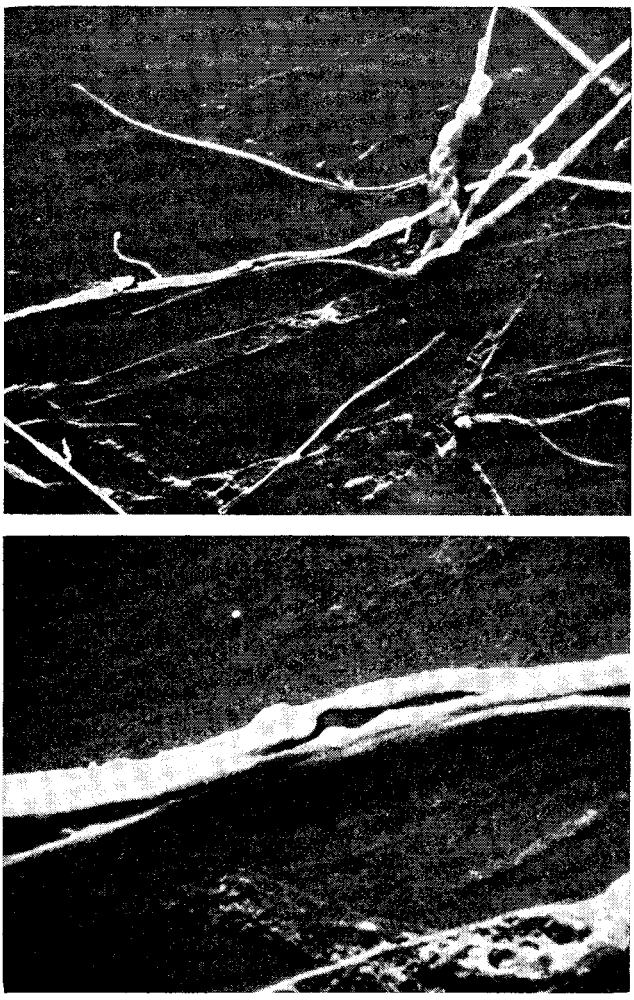

Fig. 6. "Stereoscan" electron microscope view of mixed hyphae of *Scytalidium* sp. FY strain (note the typical spores shown in the upper photograph 570 ×) and *F. annosus*. The lower photograph (2300 ×) may be composed of a partially lyzed hypha of *F. annosus* alongside a normal hypha of FY. This interpretation would be consistent with the findings shown in Fig. 5. The Geological Survey Institute of Sweden provided the "Stereoscan" microscope, related facilities and technical assistance.

lated in the »*F. annosus*» and »IC» segments of the plate. While the *F. annosus* inoculant yielded abundant growth, the *T. album* barely developed. Similarly in the unmarked segments of the plate on the left-hand side, relative growth of FY and *F. annosus* can be seen. Although

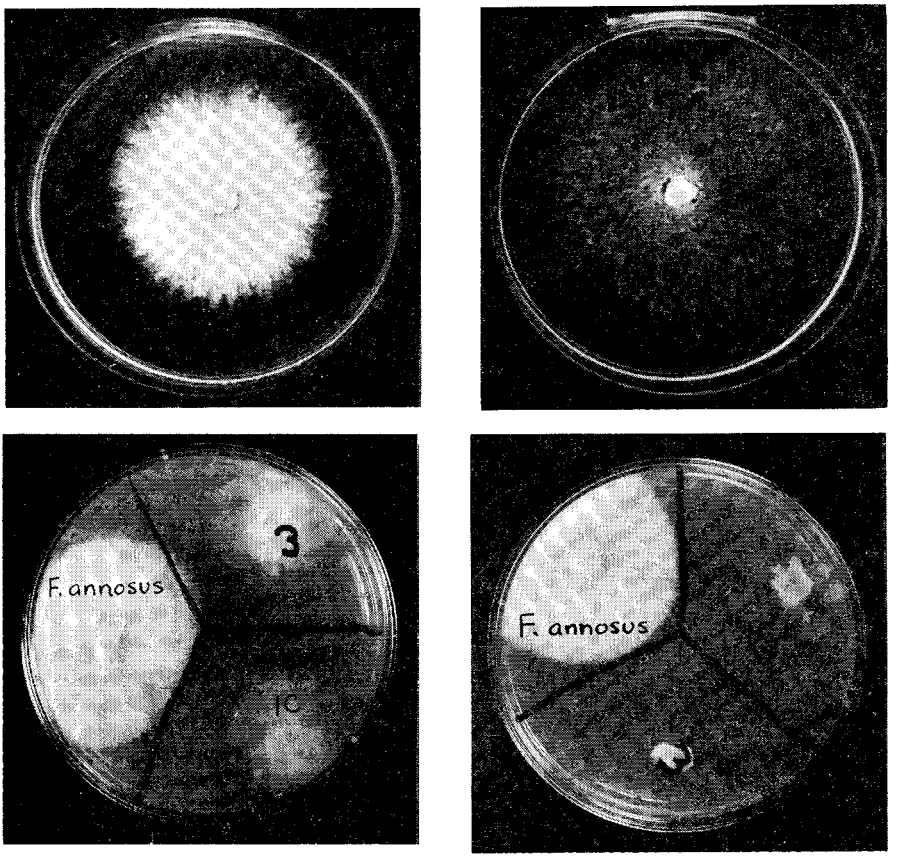

Fig. 7. *Upper* photographs: *Fomes annosus* colonies on Kuhlman-Hendrix selective medium (left) and malt extract agar (right). These photographs were received from Mr. Delatour of the Centre National de Recherches Forestières, Nancy, France.
Lower photographs: Viability test for *F. annosus* exposed to *Trichoderma album* (left) and *Scytalidium* sp. FY strain (right). Kuhlman-Hendrix selective medium was used in the plates. The inoculants, identified with an ω mark, were obtained from mixed colonies of IC and *F. annosus*. The other two colonies shown are controls.

FY fares better on Kuhlman-Hendrix medium than *T. album*, its growth is distinctly poorer than that of *F. annosus*.

In the third segment of the two plates shown in the lower photographs of Fig. 7, in the part marked with an ω, mixed inoculant was placed. This inoculant was obtained from the area of a malt extract agar plate where mycelia from two different colonies had met and intermingled: *F. annosus* and *T. album* in one instance; *F. annosus* and FY in the other. In both instances, the mixed mycelium inoculant

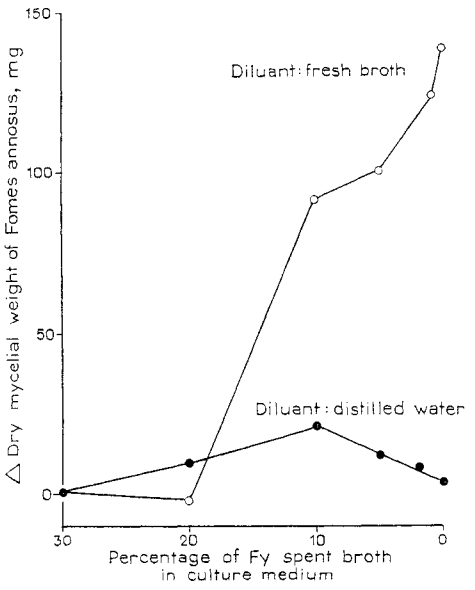

Fig. 8. Effect of FY metabolic activities in malt extract broth on subsequent growth of *F. annosus*. The broth was first inoculated with FY and incubated at 18—22°C for 90 days. The broth was then sterilized by filtration, diluted with distilled water or fresh broth and inoculated with the pathogen. The mycelial weight was determined after incubation for 50 days. Difference in mycelial weight is shown in the graph so as to make it possible to use the same scale with both diluants. Absolute values are shown in Table 6.

failed to yield any *F. annosus* even though it was placed on a medium favoring strongly the pathogen.

In both plates on the lower photographs of Fig. 7, all three inoculants were placed in the plates at the same time.

4.3.3. Metabolism

Dilution experiments were performed with distilled water and fresh malt extract broth on FY, *T. album* and *C. sarcoides* spent liquid media separately to study the mode of interaction between these fungi and *F. annosus*.

Whenever a microorganism (A) affects unfavorably the development of a second organism (B), the cause may be intoxication or starvation.

A standard experiment (Marr, 1956) to answer that question is to dilute the broth used for the culture of organism A with distilled water

Table 6. Effect of FY metabolic activities in malt extract broth on subsequent growth of *Fomes annosus*, expressed in milligrams of dry mycelial weight. These figures show the absolute values used in relative terms for the ordinate of Fig. 8.

	Percentage of FY spent broth present						
Diluant used	40	30	20	10	5	2	0
Distilled HOH	6.1	7.8	17.1	28.9	20.0	16.0	11.4
Fresh malt extract broth	—	210.8	209.1	302.6	331.3	335.1	350.1

in one set, and in another set with fresh culture medium; sterilize by filtration and inoculate with organism B.

If starvation is the main cause of the detrimental effect observed, namely the depletion of some essential nutrient, such as thiamine for example (Henningsson, 1968), then obviously the addition of distilled water to the spent broth is not going to stimulate the growth of organism B. However the addition of fresh culture medium will bring about an increasingly positive response until such concentration level that the depleted nutrient is no longer limiting factor to the growth of the second organism.

On the other hand, if a water-soluble, toxic metabolite has been released by the first microorganism (FY, *T. album* or *C. sarcoides*), which affects adversely the growth of the second organism *(F. annosus)*, then the addition of distilled water to the spent medium will have some helpful effect on the growth of the *F. annosus*. This effect will last until such dilution level that the limiting factor to the growth of *F. annosus* is no longer the presence of toxic metabolite, but the lack of certain nutrient. Conversely, the lower levels of fresh medium additions to the spent broth will not be particularly helpful, though the higher dilution levels will bring a response from the second organism, *F. annosus* in this instance.

This is precisely what happened when the spent broth of *Scytalidium* sp. FY strain was diluted with distilled water or fresh broth. The results are presented in the graph of Fig. 8 and Table 6. The first 3 dilutions of the spent broth with *distilled water* at 70, 80, and 90 % distilled water corresponding to 30, 20 and 10 % FY spent broth respectively resulted in a directly proportional increase in the mycelial weight of *F. annosus*. Beyond the 90 % dilution however, the response of the pathogen became negative, as could be expected from the excessive dilution of essential nutrients in the culture medium approaching

the composition of plain distilled water. Dilutions with fresh broth had the expected opposite effect: at the lower dilutions the concentration of FY toxic metabolite was still significant and almost no difference in mycelial weight was obtained between the 30 and 20 % FY spent broth concentrations (corresponding to 70 and 80 % fresh malt extract broth additions respectively). Beyond that dilution however rise in mycelial yield occurred in direct proportion of the amount of fresh broth introduced in the FY spent medium, showing consistently through the 10, 5, 2 and 0 % concentrations.

In this instance also, FY demonstrated its ability to provide an excellent textbook illustration of established principles of microbial physiology. Earlier, its release of antibiotic substance was found to be typical of inducible enzyme catalyzed reactions and the occurrence of the antibiotic is conveniently "marked" by the release of an unmistakable water-soluble yellow pigment. Pigmentless colonies are also active against certain wood-decay fungi but such colonies are generally less antagonistic than the pigmented colonies. Ideally in the dilution experiment just discussed several additional dilutions should have been used, especially in the 80—90 % dilution range (i.e. 20—10 % FY broth concentration). The implication of this experiment however is consistent with the observations with the light microscope, the selective Kuhlman-Hendrix medium and more precisely with the mass spectrometry data reported later in the report.

The values obtained with *C. sarcoides* spent broth are graphed in Fig. 9. There again the occurrence of a toxic metabolite in the spent medium is suggested strongly. A straight line-of-best-fit is obtained when plotting the dry mycelium weight values on a *logarithmic scale* against the percentage of *C. sarcoides* spent medium present in the substrate. This is typical of lethal agent action.

With the *T. album* spent broth the pattern is appreciably different (Fig. 10). Addition of distilled water results consistently in a smaller amount of mycelium. This suggests that the concentration of water-soluble toxic metabolite in *T. album* spent broth is not significant, but rather that nutrient shortage is the limiting factor to *F. annosus* growth. This consideration is reinforced by the effect of the fresh broth additions. Every level of addition results in a further increase of the yield of *F. annosus* mycelium, up to the 90 % addition level (i.e. 10 % *T. album* spent broth concentration) when the yield of mycelium levels off for all practical significance. Again additional dilution in the 0 to 50 % range would have been helpful to make a firmer conclusion possible, it seems however that with *T. album* no large amount of

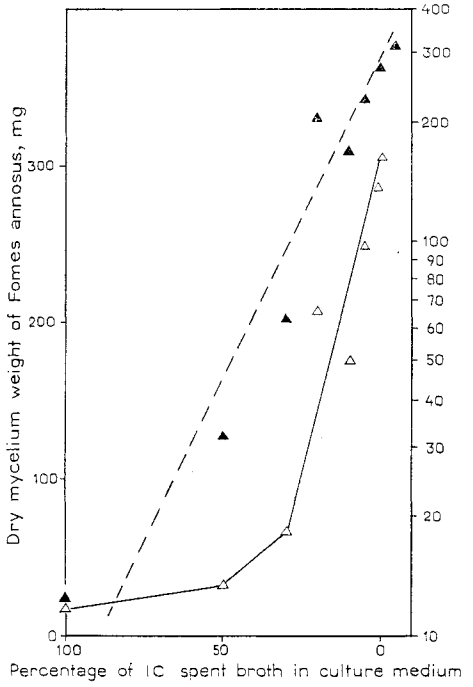

Fig. 9.
Effect of metabolic activities of *Coryne sarcoides* in malt extract broth on subsequent growth of *F. annosus*. (Diluant: fresh malt broth).

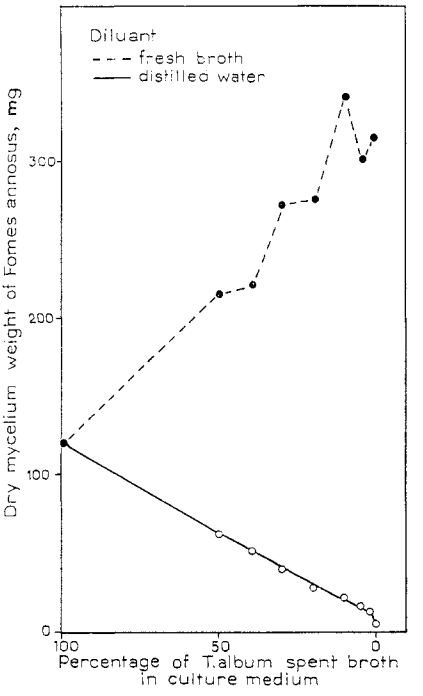

Fig. 10.
Effect of metabolic activities of *Trichoderma album* in malt extract broth on subsequent growth of *F. annosus*.

water-soluble toxic metabolite is released although a significant depletion of essential nutrient may well occur in the spent *T. album* malt extract broth.

The suggestions of the *F. annosus* yield curves on the various concentrations of culture media first used to grow these various IC's are consistent with the absolute values obtained in the 100 % spent broth cultures. These values are represented by a bar graph in Fig. 11. *F. annosus* yields on 100 % fresh malt extract broth and 100 % distilled water are shown as positive and negative controls respectively. The values obtained between the two controls though are more nearly similar to the distilled water yield. This is particularly true for the *C. sarcoides* spent broth which produced 13.7 mg of *F. annosus* mycelium, while distilled water yielded 11.1 mg. The FY spent broth resulted in a value of 28.4 mg but *T. album* spent broth allowed a growth of 106.8 mg of *F. annosus* dry mycelial weight.

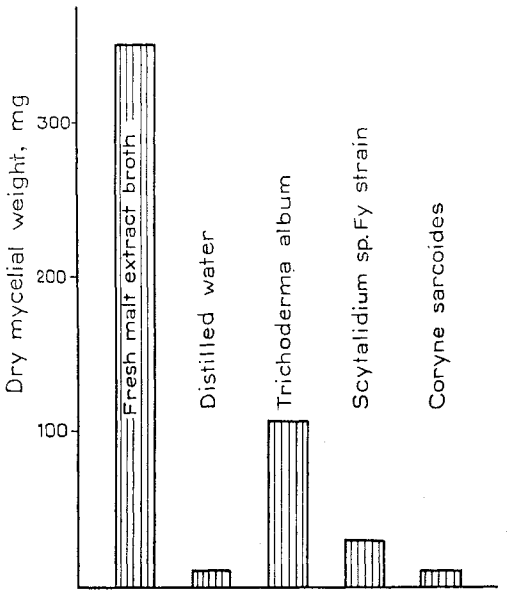

Fig. 11. Yield of *Fomes annosus* on sterile filtrates from IC cultures, incubated for 30 days at 18—22°C. The IC used are shown above the corresponding bars; distilled water and malt extract broth were used as negative and positive controls respectively.

This finding about *T. album* is not inconsistent with the other observations made about the fungus in this study. Its action on *F. annosus* appears to be lethal according to the photomicrographs and the cross-plating experiments, but it appears also to require close contact between the hyphae of *T. album* and the pathogen. It would be quite likely for a true mycoparasite to exert its antagonistic activities by non water-soluble exoenzymes just as obligate phytoparasites, which show very localized enzyme action in most instances. There are exceptions also, *Stereum purpureum* for example and its water-soluble metabolites translocated to the "silver" leaves, typical of the disease (Deverall, 1969).

Interaction of *T. album* and *F. annosus* was studied also at + 5°C since Hyppel (1963) observed that a related fungus *T. viride* lost its antagonistic activities toward the pathogen at low temperatures. The results obtained are shown in Table 7. Hyppel's gravimetric experiment was duplicated. The relationship in the results are expressed in a

Table 7. Effect of temperature on antagonism of *T. album* against *F. annosus*, as determined by incubation in triplicates at 5°C and 18—22°C of pure and mixed cultures inoculated in malt extract broth.

Inoculant	Dry mycelium weight in mg		
	5°C	18—22°C	% ΔM_t [a]
F. annosus	124.2	283.1	56.2
T. album	66.7	419.4	84.9
Mixed mycelia	82.3	427.3	80.6

[a] $\% \Delta M_t = \frac{(M_{RT} - M_5)}{M_{RT}} \times 100$

where M_{RT} = mycelial mass at 18—22°C
M_5 = " " at 5°C

somewhat different fashion stressing the differential levels between the values obtained. The weight difference between the monofungal cultures of *T. album* at room temperature and at + 5°C correlates closely with that of the mixed mycelia: 84.9 % vs. 80.6 %, while the comparable difference for *F. annosus* is only 56.2. These values suggest strongly that the limiting factor for growth in the mixed mycelia culture was *T. album* and not *F. annosus*.

In a comparison between the various IC's studied, *T. album* appears to have intrinsic merits as well. Its relative rate of growth is substantially greater than that of both FY and *C. sarcoides* as illustrated by Fig. 12. In addition it displayed a stability in its antagonistic activities quite superior to that of *Scytalidium* sp. FY strain. There are, however, variations in the appearance of *T. album* colonies, though less obvious than in the case of FY.

These variations involve pigmentation and the formation of crystal-like substances in the substrate. These features cannot help but bring to mind the well known controversies of the antibiotic-producing strains of *T. viride* vs. the non antibiotic-producing cultures. It is perhaps an asset for *T. album* to be able to perform as a true mycoparasite against *F. annosus* regardless of action from a water-soluble toxic metabolite.

In connection with these antibiotic substances, Fig. 14 presents a typical FY plate yielding crystals of substance active against *F. annosus* (Ricard & Laird, 1968) and *Poria carbonica* (Ricard & Bollen, 1968). Fig. 13 shows the appearance of one of these crystals magnified about 50 ×. The mass of such crystals was calculated at 348.0 at the Mass

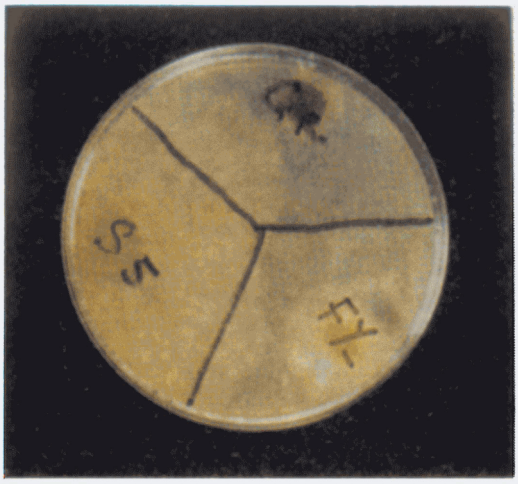

Fig. 12. Relative speed of growth of various IC. *Trichoderma album* (S 5) occupies a third of the plate, *Coryne sarcoides* (GR) has barely started to develop, *Scytalidium* sp. FY strain is intermediate in development between the other two colonies. All three inoculants were introduced in the plate at the same time. The first two isolates were obtained from Norway spruce stems in the Tönnersjöheden forest. FY was obtained in Oregon, U.S.A.

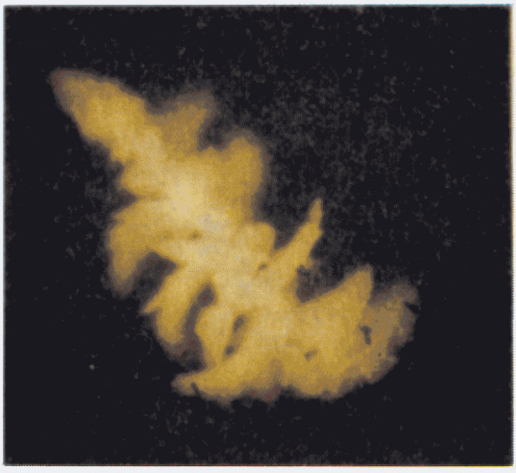

Fig. 13. FY crystals as seen under the stereoscope. Similar crystals were used for mass spectrometer analyses and also found to stop growth of *Poria carbonica* (Ricard & Bollen, 1968). Photograph was taken by J. L. Overholser at Oregon State University, Corvallis, Oregon, U.S.A.

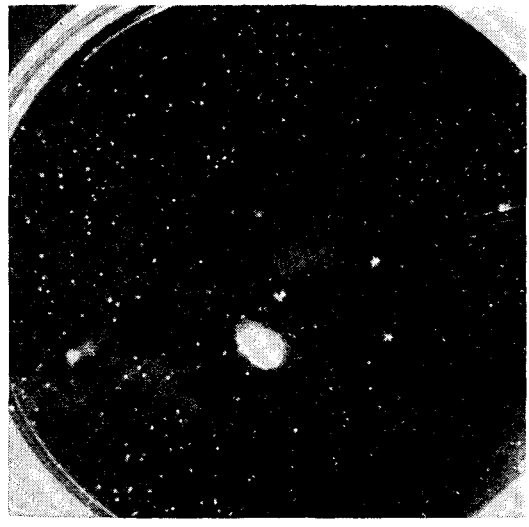

Fig. 14. FY crystals as seen in a Petri dish, after prolonged culture on malt extract agar.

Spectrometry Dept. of the Royal Caroline Medico-Chirurgical Institute and later at the Nobel Institute for Analytic Chemistry. The exact mass was established at 348.42809 at the former Institute. These findings are consistent with the observations made by Schroeder (1969) on the same substance.

5. Discussion

An important finding in this study was the occurrence of live microorganisms in the stem wood of healthy Norway spruces. This suggests that IC may be as significant for the control of *F. annosus* in stems or roots of Norway spruces as it is in Douglas-fir poles. However the more suitable IC species are likely to be different in spruce and involve *Trichoderma album* and *Coryne sarcoides* rather than *Scytalidium* sp. Definite conclusion, however, will have to wait for results from field trials and demonstration of the effectiveness of the IC considered in terms of Koch's postulates, just as it was made in more than 50 Douglas-fir poles in service in Oregon.

The field trials now under way are concerned primarily with the selection of species to be used for application to live forest trees, but the usefulness of IC is suggested strongly by the occurrence of a variety of such organisms in healthy survivors of prolonged exposure to *F. annosus.*

IC can be expected to find applications in all tissues where microbial commensals grow. This is implied in the expression mentioned in the introduction:

$$\text{IC } \omega \frac{\text{FUS}}{\text{HOS}}$$

The main practical question concerns the microbial strain (IC_d) to be used in a given situation. In both problems studied so far, the first term defined was FUS (i.e. poles in Oregon and wood chips in Sweden); the second term HOS was *Poria carbonica* abroad and *Fomes annosus* here.

Scytalidium sp. FY strain was found to be a suitable IC_d in Oregon, and current field trials are assessing the potential of other IC_d for the Swedish problem. Should the presently tested organisms fail, there are quite a number of other IC_d candidates available.

The IC concept has not met with universal acceptance let alone use and a number of conflicting viewpoints have been voiced. Some of the more frequently heard arguments are reviewed here under the heading of a few key words followed by amplification into a typical comment about IC:

1. Universality of *Scytalidium* sp. usefulness: "*Scytalidium* sp. are useful in poles, trees and chips for preventive and curative applications".

This situation would be very desirable for simplicity sake in the treatment of various wood substrates. Unfortunately it has been demonstrated in field tests as yet for only one application, poles (Ricard, Wilson & Bollen, 1969), while in others the information available suggest quite the contrary (Klingström & Beyer, 1965).

These authors reported that *Scytalidium album* causes a weight loss of 1 % in the dry weight of Norway spruce wood blocks incubated 45 days at 25°C. Comparing this figure with those cited in the recent review of Assarsson (1969) on wood chip storage, places *S. album* among the wood chip decay fungi. This conclusion is supported further by the findings of Ricard & Bollen (1968) that *Scytalidium* sp. utilizes xylan, an important component of wood pulp. In terms of the FUS factor in $IC_d \omega \frac{FUS}{HOS}$, *Scytalidium* sp. seems rather indesirable in wood chips or live trees grown as a source of such chips.

On the other hand xylan and the other compounds included in the hemicelluloses are not appreciably significant in the mechanical properties of wood, so that *Scytalidium* sp. can be used effectively in poles.

2. Antagonism and release of water-soluble antibiotics are synonyms:

"only those microorganisms which produce an inhibition zone when plated on agar plates are antagonistic."

For many years antibiosis has been measured in terms of inhibition zone width. It is convenient and very effective for many purposes. However as a measure of antagonism it is sometimes misleading as it does not measure such well known antagonistic phenomena as mycoparasitism or starvation, not to mention the occurrence of water-*insoluble* antibiotics. Standard methods for measuring antagonism among microorganisms have been described by Johnson *et al.* (1959).

As early as 1944 Waksman discovered "chaetomin", a water-*insoluble* antibiotic formed by a genus known to include several wood inhabiting species. Barnett has studied mycoparasitism with various coworkers for a number of years and reviewed the subject in 1963. The importance of nutrient shortage in the determination of predominant microbial species during decomposition of plant material is well-known (Garrett, 1951), particularly with respect to the relative ability of the microorganisms involved to utilize simple or complex carbon compounds.

The effectiveness of nutrient shortage in regulating fungal growth is so obvious that it has been used as means to control the development of wood-destroying fungi (Baechler, 1956). Although thiamin shortage can be brought about by chemical means, it can also result from the

preventive growth of an IC as a pre-empter. This ability of IC to function preventively as well as curatively—by introduction in a substrate at the incipient decay stage (Ricard, Wilson & Bollen, 1969)—suggests a need for further characterization of IC.

Preventive IC (=IC_p) are rather numerous (Hodges, 1968) while curative IC (=IC_c) appear to be much scarcer. In contrast with the IC_p, IC_c must be relatively insensitive to metabolites released by the competing wood-destroying fungus. IC_c such as *Scytalidium* sp. FY strain are not affected adversely by certain competing fungi, but to the contrary, competition is required for their effective biosynthesis of antagonistic substances. The typical yellow water-soluble pigment released by FY occurs only when it is—or has recently been—exposed to certain competing microorganisms. But neither pigment nor antibiotic substances are released (Ricard & Laird, 1968) in full, when FY is grown as a monofungal culture. Antibiotic production of FY can be assessed readily as it crystallizes (Fig. 14) in relatively pure and active form (Ricard *et al.*, 1969; Ricard & Bollen, 1968).

The use of IC_p can be symbolized as follows:

$$IC_p + S \rightarrow \boxed{IC_p \cdot S}$$

$$\boxed{IC_p \cdot S} + HOS \rightarrow \boxed{IC_p \cdot S} + HOS \downarrow$$

where the terms used earlier—in the introduction—have the same meaning as before while S is substrate and $\boxed{IC_p \cdot S}$ is the stable complex formed by commensal and substrate without damage to the final use of the substrate. The arrow pointing downward after HOS, represents elimination from the system.

In the curative use of IC, the sequence can be represented as follows:

$$HOS + S \rightarrow (HOS \cdot S)$$

$$(HOS \cdot S) + IC_c \rightarrow \boxed{IC_c \cdot S} + HOS$$

where (HOS · S) is the temporary complex formed by the HOS and S during the incipient decay stage. This complex is temporary since in time it leads to the destruction of the substrate utilized and the formation of HOS propagules.

3. Preventive use of FY:

"*Scytalidium* sp. FY strain cannot be used for preventive purposes since its antagonistic properties require the presence of competing microorganisms."

It has been observed that various sub-strains of FY vary in their antagonistic properties. The degeneration of FY cultures can be ob-

served readily under laboratory conditions as the loss of antagonistic ability and yellow pigment release are usually simultaneous. Regeneration of both functions can also be observed readily by cross plating of yellow pigment free cultures with various wood-destroying fungi. Not all species are equally useful for that purpose. The following gradient in effectiveness for FY regeneration was observed:

Poria carbonica > *Fomes annosus* > *Poria vaporaria*

with induction of pigment release by *P. carbonica* before the competing colonies meet; several days after the colonies meet with *F. annosus;* and not at all with *P. vaporaria.* However the FY pigment release is maintained in presence of the latter organism if it were active at the time the competing colonies meet, but no FY regeneration takes place under those conditions.

There are however several simple ways to maintain the release of FY active metabolites such as simultaneous inoculation in wood with another IC, *Trichoderma album* for example.

4. Wood preservation requires chemicals:

"creosote, pentachlorophenol, arsenic and other inorganic salts can be used, but not biosynthetic substances."

It has been said that one of the prime dividend of contemporary biology is to have demonstrated the ubiquity of fundamental mechanisms in countless different forms of life, for example glycolysis for the release of energy and DNA for transmission of the genetic code. However, when the control of wood-destroying fungi is discussed, some wood technologists insist on ignoring the experience developed in disciplines traditionally oriented toward microorganisms rather than toward wood. Microbiology has abandoned the use of mercury and arsenic salts for most applications in human pathogens. Antibiotics—the "miracle drugs"—were found to be far superior in terms of disease control and ease of application. Still the difference of substrates involved creates a barrier on the presumed response of microorganisms. The provincialism reflected by these views is particularly unexpected in the light of criteria already accepted for the demonstration of cause to effect relationships in various substrates.

Koch's postulates were formulated originally to determine the cause of various human diseases. Yet as microorganisms were studied in other substrates, the application of the postulates expanded, virtually unchanged until viruses became known. In spite of the complications raised by these non-saprophytes, Koch's postulates have remained widely accepted.

Particularly now that pollution of the environment is recognized

as a significant problem it should be possible for antibiotics and other biosynthetic compounds to be considered for substitution of arsenic and other man-made salts forming harmful residues.

5. Incipient decay stage damage:

"when a fungus is detected in wood its mechanical properties are damaged so badly that it must be discarded."

This viewpoint has been heard from an engineer with administrative responsibilities. Simple solutions have always their attractiveness. In this instance however they are not supported by numerous studies made on the effect of a variety of wood inhabiting fungi. Some of these studies date as far back as 1942 (Chidester).

Current findings on the ubiquity of fungi in the stem wood of healthy forest trees cast further doubt on the validity of that opinion.

6. Single vs. multiple decay agent theories:

"decay in wood is induced not by just one fungal species, but by the successive activities of a variety of microorganisms resulting in ecological conditions similar to those prevailing in soil."

This could be a major problem since biological control agents in soil have not been particularly successful, as mentioned in the literature review.

Shigo (1969) has confirmed his earlier findings that decay in the hardwood species of the Northeastern section of the United States involve synergic activities of several microbial species somewhat like the process converting plant material into humus. It is increasingly obvious however that microbial populations vary considerably from one species of trees to the next, for example in Sweden the occurrence of fungi in the stem of healthy Norway spruce appears to be considerably higher than in Scots pine or birch (Ricard, 1970).

Sequence of the wood-decay process can be observed readily, though interpretation of findings may present difficulties. Microscopic examination of Douglas-fir tracheids exposed to *Poria carbonica* (Ricard, 1966) suggest that more than just one microbial attack is required to break down the tracheid wall. Quite often the bore holes made by the fungal hyphae are empty while on occasions the hyphae present seem partially autolyzed. Yet at other times normal hyphae can be seen within the bore holes. Obviously additional microbial activity is required in order to disintegrate a tracheid wall showing only 4 or 5 emply holes. One alternative could be that microorganisms of different species would then become involved in the next phase of the breakdown. Liese (1969) has studied this possibility and concluded that in softwoods different phases of disintegration sometimes

involved only several successive attacks, spaced by appreciable time intervals, of hyphae belonging to the same fungal species. This explanation is consistent with the cyclic physiological characteristics of the perennial *Polyporaceae* in particular, and their ability to resume growth after a dormant period.

Both in Douglas-fir poles and Norway spruce trees the bulk of the wood-disintegration process seems to involve only one organism, *Poria carbonica* and *Fomes annosus* respectively. After visible evidence of decay has developed, a variety of fungi imperfecti and bacteria are found commonly to be active (Ricard & Mothershead, 1966).

So long as only one fungus is involved in the more critical phases of the decay process, it should be possible to avoid the undesirable fungal activities with just one IC. It is still standard practise in wood preservation to rely on only one active compound to prevent decay (as for example in the Cellon process) in spite of the experience with resistant mutants in related fields such as insect control (DDT-resistant houseflies, cyanide-resistant aphids, etc...).

However IC do not have to be used singly. Multiple inoculations of such different species as *Scytalidium* sp. FY strain, *Trichoderma album* and *Coryne sarcoides* offer several advantages in wood used for construction purposes, for example, as suggested by the results described above:

(a). these various organisms can influence decay fungi through different mechanisms. In order for a wood destroyer to become resistant to the various IC mentioned it would have to develop simultaneous mutations for nutritional deficiencies (induced by *T. album*), membrane lysis (induced by *Scytalidium* sp. FY strain) and water-soluble toxic metabolites (released by *C. sarcoides)*. The probability of such mutant ever developing is rather low.

(b). these various IC have the ability to induce antagonistic activities in one another thereby providing for continuous biosynthesis of toxic substances.

(c). the growth rate of these IC vary considerably so that they do not maintain their peak metabolic activities during the same period in a given proportion of the substrate, providing partial protection quickly and more thorough treatment later on.

(d). Sapwood and heartwood support growth of these IC to varying extent. *Coryne sarcoides* develops more extensively in the heartwood of spruce while *Trichoderma album* favors the sapwood. As a result combined inoculation of the two IC is needed for maximum permeation of both wood types.

7. Application of IC to forests:

"inoculation of forest trees is impractical considering the number of individual trees in forests and the time required for inoculation of each tree."

This consideration is quite realistic for trees already placed in their final location, but it does not apply equally well to seeds, seedlings or nursery stock. The inoculation of forest trees at these various stages is a relatively simple matter. It is known from such reports as Tveit & Wood (1955) and Ricard (1967) that IC such as *Chaetomium* and *Scytalidium* sp. do not affect adversely the development of oat and Douglas-fir seedlings respectively though they would not necessarily be the best IC for these respective purposes.

Since most reforestation work uses seeds or seedlings obtained under controlled conditions, the inoculation of such reforestation stock with IC would not be impractical, if it can be demonstrated that IC introduced at that stage develop within the wood as it is formed by the tree.

Regardless of their ultimate significance, these various objections have had a stimulating effect on the evaluation of IC for practical purposes. This is possible only when such objections are raised openly and discussed freely.

Summary

A survey was made of the microbial population occurring naturally in 546 spruces located in various Swedish forests: Tönnersjöheden, Skuleberget, Långmor, Stensängen, N. Dellen. This survey was made on the assumption that immunizing commensals (IC) could be equally significant in the control of *Fomes annosus* in live Norway spruces as for the control of *Poria carbonica* in Douglas-fir poles in service.

Fifty percent of the trees sampled showed microorganisms ranging from 40 percent in trees sampled at random in Stensängen to 100 percent in healthy trees surviving prolonged exposure to *Fomes annosus* in Tönnersjöheden forest. The microorganisms isolated included fungi, bacteria and actinomycetes. Some of the fungi obtained had been reported already for their antagonistic activities, particularly *Coryne sarcoides* and *Trichoderma album*. Less actively antagonistic fungi such as *Ceratocystis piceae* and *Cephalosporium* sp. were obtained repeatedly. Bacteria were by far the most common type of microorganisms obtained, with occurrence in 20 percent of the trees. Several of the bacterial isolates showed typical water-soluble antibiotic activity against *Fomes annosus.*

The artificial inoculation of fungi in logs presented the same difficulties as were experienced earlier with pole sections. Only *Trichoderma album* and *Coryne* sp. were introduced successfully in logs, besides bacteria. *Scytalidium* sp. FY strain failed to become established readily in the wood and induced the formation of pathological zonation lines. This latter feature is consistent with the previously reported ability of *Scytalidium* sp. to utilize xylan and to induce significant weight losses in wood blocks. Interaction of certain IC's and *Fomes annosus* was observed in detail: (1) under the light microscope; (2) after cross plating on malt extract agar followed by transfer to selective Kuhlman-Hendrix medium, and (3) after culture of *F. annosus* on various dilutions of spent broth inoculated previously with IC. *Scytalidium* sp. FY strain and *Coryne sarcoides* appear to release a water-soluble metabolite toxic for *Fomes annosus. Trichoderma album* has a lethal effect on *Fomes annosus* at room temperature and at 5°C. This action is not due apparently to a water-soluble substance, suggesting that *T. album* functions rather as a mycoparasite.

Immunizing commensality appears to be a significant factor in the survival of healthy Norway spruces exposed to *Fomes annosus* and potential IC were isolated. Confirmation of the effectiveness of the IC isolated is waiting for results of field trials now under way, in keeping with the requirements set by Koch's postulates.

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Sammanfattning

Biologisk bekämpning av *Fomes annosus* i gran *(Picea abies)* med "immunizing commensals"

En undersökning har utförts av den mikrobiella artsammansättningen i 546 granar från skilda delar av Sverige.

Undersökningen gjordes med arbetshypotesen att »immunizing commensals» (IC) skulle kunna vara lika betydelsefulla för att undertrycka *Fomes annosus* hos gran som för att bekämpa *Poria carbonica* hos stolpvirke av douglasgran.

Som helhet visade sig 50 % av de insamlade proven hysa mikroorganismer av något slag; varierande från 40 % av slumpvis utvalda granar i Stensängen till 100 % räknat på friska träd inom starkt rotröteangripna granbestånd i Tönnersjöheden.

De isolerade mikroorganismerna omfattade både svampar, bakterier och aktinomyceter. Några av de isolerade svamparna har tidigare omtalats för sin antagonistiska aktivitet, så i synnerhet *Coryne sarcoides* och *Trichoderma album*. Mindre utpräglat antagonistiska svampar såsom *Ceratocystis piceae* och *Cephalosporium sp.* erhölls upprepade gånger under isoleringsarbetet. Bakterier var också i hög grad vanliga med en förekomst av 20 % i de undersökta träden.

Många av bakterieisolaten hade även en karakteristisk, vattenlöslig antibiotisk effekt gentemot *Fomes annosus*.

Den experimentella inokuleringen av svamp i stammar visade sig vara lika svår som vid tidigare försök med stamsektioner av douglasgran. Förutom bakterier kunde endast *Trichoderma album* och *Coryne sarcoides* med framgång fås att etablera sig. *Scytalidium* sp., stam FY, kunde icke omedelbart fås att växa i ved utan i stället utbildades patologiska zoneringar. Denna senare egenskap är helt i överensstämmelse med den tidigare rapporterade förmågan hos *Scytalidium* sp. att utnyttja xylan och att orsaka icke obetydliga viktsförluster i träklossar.

Antagonismen mellan vissa IC och *Fomes annosus* studerades också i detalj genom: (1) undersökningar i ljusmikroskop (2) samympning på maltextraktagar och efterföljande överföring till selektivt Kuhlman-Hendrix-medium, samt (3) kulturförsök med *Fomes annosus* i medium innehållande varierande koncentrationer av substrat som tidigare inokulerats med IC.

Scytalidium sp., stam FY, och *Coryne sarcoides* synes frigöra en vattenlöslig metabolit med toxisk effekt på *Fomes annosus*.

Trichoderma album har letal inverkan på *Fomes annosus* vid rumstemperatur och vid +5°C. Denna påverkan är synbarligen ej orsakad av någon vattenlöslig substans, utan beror måhända på att *Trichoderma album* verkar som mykoparasit.

»Immunizing commensality» synes vara en faktor av betydelse för fortlevnaden av friska granar i rotröteinfekterade bestånd, och potentiella IC har också isolerats.

En bestämning av den naturliga aggressiviteten hos de isolerade IC, i enlighet med de krav som Koch's postulat kräver, kan förväntas efter utvärdering av resultaten från pågående fältförsök.