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Effect of *Fomes annosus* on seedlings of *Picea abies* in the presence of *Boletus bovinus*

Fomes annosus' inverkan på groddplantor av Picea abies i närvaro av Boletus bovinus

by

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ABSTRACT

The mycorrhiza-forming fungus *Boletus bovinus* (L. ex Fr.) O. Kuntze was shown to protect seedlings of Norway spruce from the usually fatal attacks, which result from treatment with the root rot fungus *Fomes annosus* (Fr.) Cke. New techniques were developed for the culture and application of both species of the microorganisms. The investigation was performed under greenhouse conditions.

Introduction

Mycorrhiza-forming fungi isolated earlier from forest trees were found to inhibit the growth of *Fomes annosus* (Fr.) Cke when cross plated on malt extract agar or grown in nutrient solution (Hyppel 1968).

The results of these experiments suggested a possible protective effect for host plants of these fungi.

No report could be found in the literature concerning protective effect of mycorrhiza-forming fungi against *F. annosus*. There are however two recent publications dealing with such protective effect against other root pathogens. Marx (1967) found that *Phytophthora cinnamomi* Rands. failed to cause damping off in pine seedlings. This resistance occurred when an ectotrophic mycorrhiza was formed by a basidiomycete of the genus *Leucopaxillus* on the roots of the seedlings. Šašek (1967) reported that *Tricholoma saponaceum* (Fr.) Kumm. could protect Scots pine, *Pinus silvestris* L., seedlings from damping off *in vitro*, but no mention is made of mycorrhiza formation. Both of these investigations were performed with plants and fungi grown under sterile conditions. Finally Santoro and Casida (1962) hunting for new antibiotics, found that some species of *Boletus* and *Cenococcum graniforme* (Sow.) Ferd. and Winge produce bacteriostatic substances by cultivation in nutrient solution. These are without effect on yeast cells or fungi. They are however discussed as root protecting agents acting in the rhizosphere of forest tree seedlings.

The experiments described below were carried out in the greenhouse of the Department of Forest Botany at the Royal College of Forestry in Stockholm.

Material and Methods

A. General

The seed of Norway spruce, *Picea abies* KARST. used in this study was harvested from approved stand in the county of Västmanland, middle Sweden. The seed germination rate, determined in a Jacobsen apparatus, amounted to 82 %. All seeds were treated with a copper-base fungicide, 8-oxikinolate, to avoid damping off.

Shive's nutrient solution was applied once a week to the seedlings, which —in addition—were moistened with tap water as required.

The greenhouse microflora was tested by plating on malt agar and appeared to be dominated by a *Penicillium* sp.

The fungal cultures used in this study were both obtained from an area near Bogesund, northeast of Stockholm; the *Fomes annosus* strain, Sä 1, was isolated from a live Norway spruce and the mycorrhiza-forming fungus, *Boletus bovinus* (L. ex FR.) O. KUNTZE, was secured from a fruiting body growing there naturally.

Upon isolation, the fungi were cultivated in small flasks containing Norkrans' (1949) nutrient solution, and also glass beads to facilitate grinding of the mycelium. Incubation at 25° C was allowed for two weeks with *F. annosus* and one month with *B. bovinus* cultures. The mycelia were then ground and transferred to a barley medium, prepared by autoclaving 150 g of barley kernels with 200 ml distilled water. In addition a simpler method was used for *F. annosus* whereby conidia grown on malt extract agar plates were suspended in sterile distilled water. The suspension was added to the autoclaved barley medium. After three to eight weeks the kernels were penetrated completely by the hyphae. The kernels had become somewhat drier during the incubation and could be separated readily from one another. They were placed mostly in seed boxes and flasks as layers, one-kernel thick.

B. Application of barley kernel inoculants

The arrangement used in the seed boxes is diagrammed in figure 1. The boxes were made of foamed plastic measuring $250 \times 130 \times 120$ mm. The bottom was covered with a layer of heat sterilized perlite 10 mm thick. The perlite is a granulated, porous and chemically inert rock undamaged by heat sterilization. The sterile perlite was then saturated with Shive's nutrient solution and a layer of fungus permeated barley kernels was applied, then covered with another layer of perlite 50 mm thick. A second layer of fungal inoculant was then placed in the box and covered with another layer of perlite 30 mm thick. The surface of the perlite was leveled off and 100 seeds of

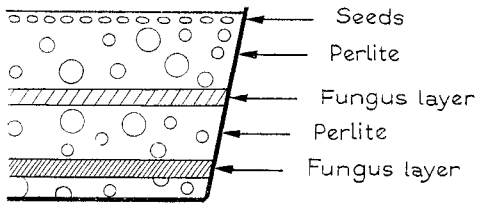

Fig. 1. The principles of applying seeds and fungi in boxes for protection tests.

Norway spruce were placed in five evenly spaced rows. The seeds were then covered with additional perlite and placed under glass sheets in the greenhouse. Germination occurred after 6 to 10 days, the glass sheets were then removed and the boxes exposed to photoperiods 16 hours long at 2000 lux at plant level. These periods were spaced with dark intervals of 8 hours each and provided with the temperature conditions shown in figure 2. The influence of outdoor temperatures prevalent in the spring and fall was avoided by performing the experiment during the winter.

The purpose of the interspersed layers in the seed boxes was to bring the roots of the seedlings in contact with the layer of mycorrhiza fungi inoculants before exposure to the pathogens. True mycorrhizal symbiosis with *B. bovinus* was not recognized during the experiment time. Besides, the fungus was isolated from a pine stand.

C. Exposure to variables in rapid succession

In order to evaluate the relative effects of *F. annosus*, *B. bovinus* and autoclaved barley, singly and in various combinations, on spruce seedlings, sixteen different experimental designs were considered and eleven were selected for actual use. These designs are diagrammed in figure 3. Related combinations are listed under the same letter and numbered at random.

Group A was intended to evaluate the effect of autoclaved barley kernels in comparison with *F. annosus* and *B. bovinus* permeated kernels respectively. All three "A" combinations had a bottom layer of *F. annosus* permeated kernels, only the top layer varied in composition: A-1 had *F. annosus* permeated kernels, while A-2 had *B. bovinus* there and in A-3 only plain autoclaved barley was present in that layer.

In the B group, only one layer of barley kernels was used, near the bottom of the seed box. B-1, B-2 and B-3 contained *F. annosus*, *B. bovinus* and plain autoclaved barley respectively. This group included the same variables as the preceding one, but exposure of the roots took place after they reached a later stage of development.

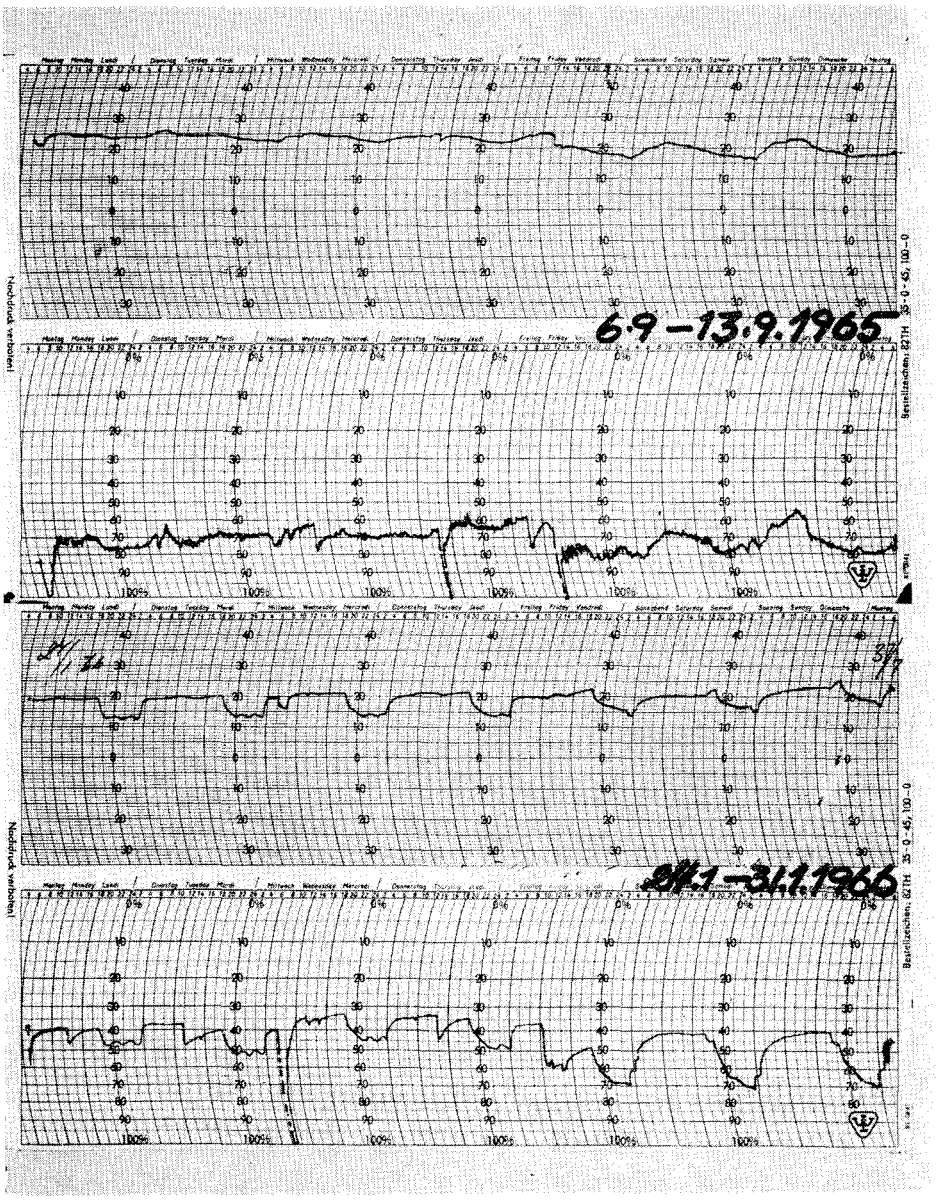

Fig. 2. Thermohygrograms from the greenhouse at two different seasons.

The group C included only two combinations C-2 and C-3. In C-2 both layers of barley kernels were permeated with *B. bovinus*, and in C-3 only plain autoclaved barley was present in both layers. These arrangements were

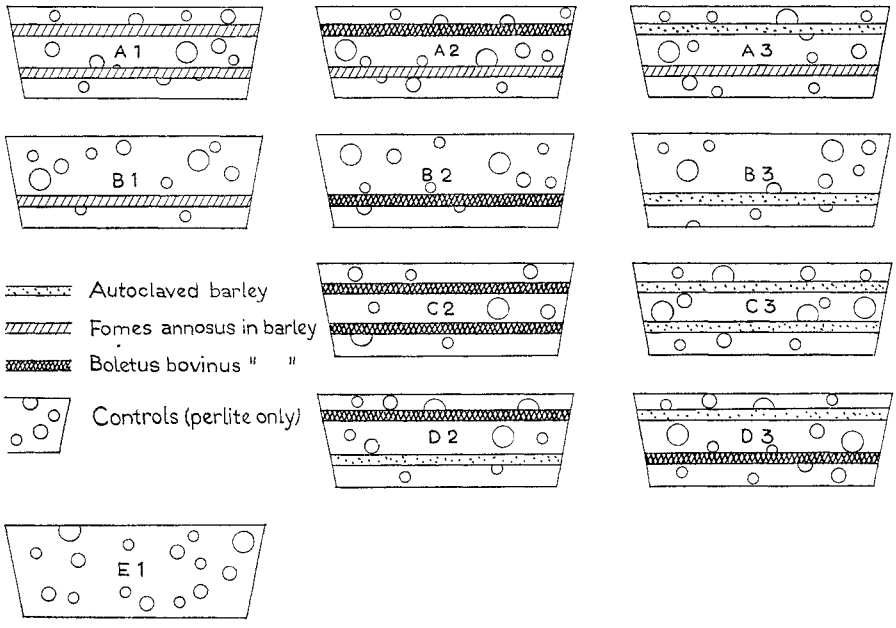

Fig. 3. The combinations used with *Boletus bovinus* and *Fomes annosus* grown on barley kernels, and the controls.

comparable with A-2 and A-3 in that the layers present were identical in composition, but inverse in their relative position.

The effect of barley on *B. bovinus* was tested with the D group, as this enrichment material could be expected to stimulate the natural microflora present in the greenhouse. Such stimulation could then result in secondary effects on *B. bovinus*.

Controls were provided by the E-1 group where seedlings developed in moist perlite.

Each combination was prepared in quadruplicates with 100 seeds in each replicate. The results were expressed as percentage of surviving seedlings.

D. Delayed exposure to *F. annosus*

This study was performed by culturing the seedlings in boxes inoculated with *B. bovinus* only, using the barley kernel method. Seven weeks later, *F. annosus* was introduced in the rhizosphere of the seedlings by forcing a few kernels of barley permeated with the pathogen down from the surface of the seed box. This practise eventually caused a less uniform inoculation than the continuous layer technique, but it avoided disturbance of the roots, besides

providing for control over the time interval between contact with *B. bovinus* and exposure to *F. annosus*.

Six seed boxes were used, two with each of three combinations: B-2, with one *B. bovinus* layer at the bottom; B-3 with plain autoclaved barley at the bottom; and controls with perlite only.

E. Mode of preventive action by *B. bovinus*

For this investigation, the Norway spruce seedlings were grown under the same conditions as in the barley kernel method described above with one half of the seed boxes containing a single bottom layer of *F. annosus* permeated barley kernels and the other half containing perlite only.

For irrigation, a water extract of *B. bovinus* was applied to one half of the boxes in each combination, while tap water was used with the other boxes. The extract was obtained by addition of 500 ml distilled water to a flask containing a two-month old culture of *B. bovinus* prepared according to the method described above. Twenty-four hours later, the liquid phase was poured off and sterilized by filtration. The sterile filtrate was applied in aliquots of 50 ml per box twice a week. Shive's nutrient solution was applied as needed. The experiment lasted 4 months.

Results

Among the seedlings exposed to the variables used in rapid succession, A-2 was observed with particular attention since it provided for preliminary contact with *B. bovinus* followed shortly by exposure to *F. annosus*. A protective effect was apparent for 4 weeks (fig. 4). It was no longer noticeable after about 8 weeks, when more than 90 % of the seedlings were dead. In A-3 where the top layer contained autoclaved barley kernels only with *F. annosus* in the bottom layer, the lethal effect developed still more quickly. In that instance also, more than 90 % of the seedlings were dead by the end of the seventh week.

When both layers of barley kernels contained *F. annosus* as in A-1, only 10 % of the seeds were able to show some development. After just 5 weeks, all seedlings were dead.

In the controls (E-1), 83 % of the seeds had germinated after 3 weeks and produced viable seedlings. A slight increase took place in the following weeks with a final count at about 85 %.

The other combinations resulted in varying effects on the seedlings. The combinations with two layers of plain autoclaved barley as well as those with

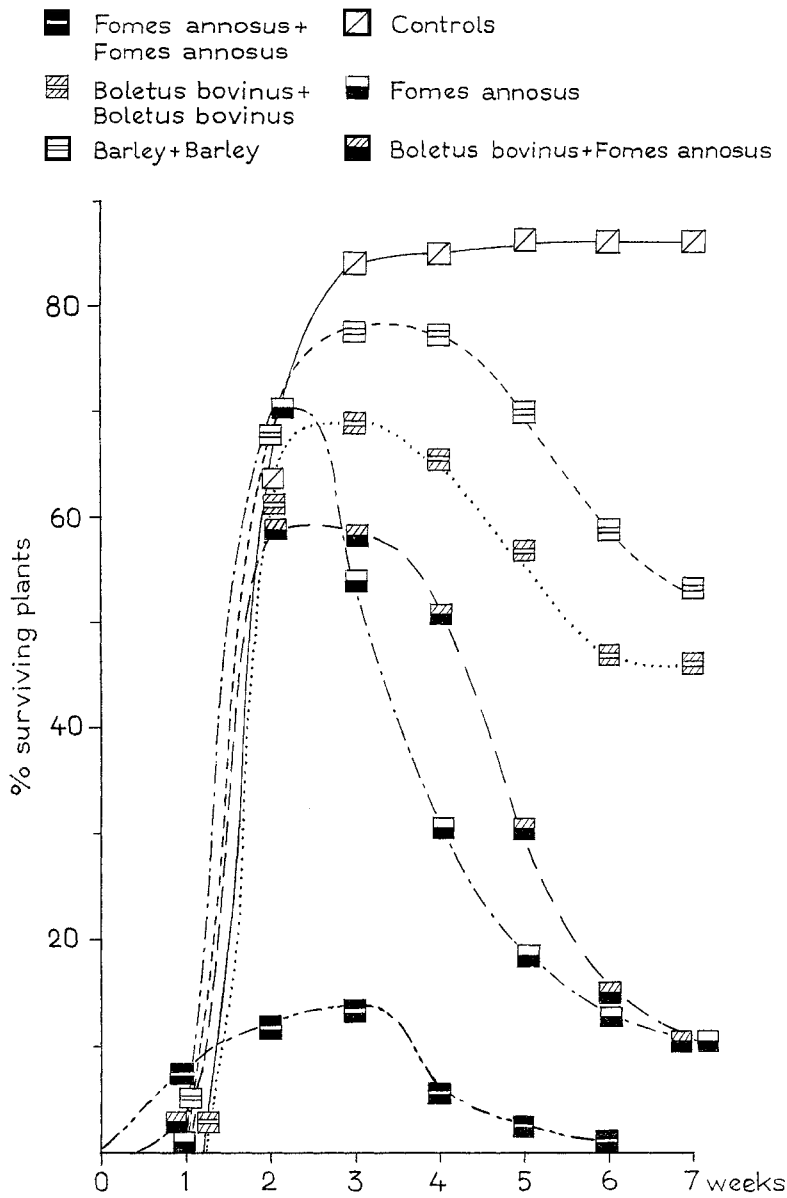

Fig. 4. Survival of spruce seedlings with different combinations of *Boletus bovinus*, *Fomes annosus* and autoclaved barley kernels. The symbols represent layers in the experiment boxes.

two layers of *B. bovinus* had a detrimental effect on the viability of the plants with only 50 % of germination and seedling development. This effect was more obvious yet when a layer of barley was combined with a layer of *B.*

bovinus. In that instance only 20 % survival could be observed in the seedlings. When *B. bovinus* was present in the top layer and plain barley was in the bottom layer, the damage could be expected to occur on the young roots lacking protective cork tissue. On the other hand, when the barley was placed on top and *B. bovinus* at the bottom of the seed box, a protective effect could have occurred from the natural microflora present in the greenhouse.

The greatest variation between replicates took place where only a single layer of barley or fungus penetrated barley was present. When *F. annosus* was placed at the bottom, the lethal effect was less pronounced than in the boxes where two layers of *F. annosus* were present. However as time went on, the survival rate eventually reached zero.

One layer of plain barley at the bottom caused a noticeable decrease in viability with only 50 % survival in the seedlings. It seems that a contamination factor may have occurred also in the D 3 combination mentioned above.

A favorable effect on the seedlings was observed when one layer of *B. bovinus* was present at the bottom of the boxes. The survival rate was equal or greater than in the controls; furthermore the seedlings appeared to be taller and greener in this group.

In conclusion, these experiments indicated no protective effect from *B. bovinus* on the Norway spruce seedlings, when the mycorrhiza fungus was placed as a top layer and *F. annosus* at the bottom of the seed box (fig. 5).

Fig. 5. *Fomes annosus* (one layer in the bottom) | *Fomes annosus* (bottom layer) and *Boletus bovinus* (top layer) | Controls (no fungi, no barley)
Five weeks spruce seedlings in perlite culture with *Fomes annosus* and *Boletus bovinus* added. The fungi grown on barley kernels.

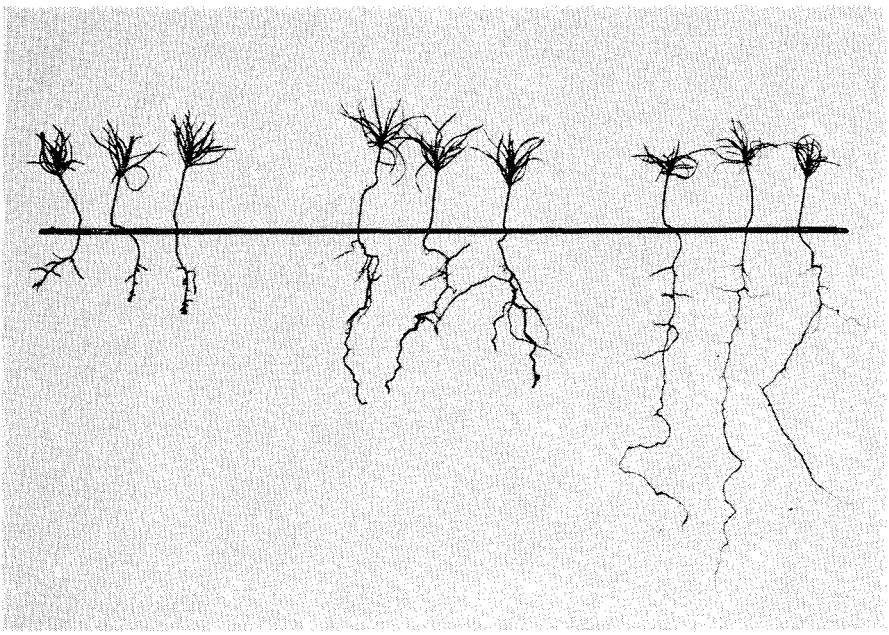

Fig. 6. The influence of *Boletus bovinus* (left), and another mycorrhiza-forming fungus (middle) on the root development of spruce seedlings. Right: controls.

It even appeared that an early contact of the seedling root with the mycorrhiza-forming fungus damaged the plant. This harmful effect was compounded by exposure to *B. bovinus* as shown in figure 6. However when the roots of the seedlings were able to grow for some distance before coming in contact with *B. bovinus* and a long adaptation time was allowed, then a protective effect against the pathogen could be obtained. This finding led to further study on the influence of time lapse between contact of the seedling roots with *B. bovinus* and exposure to *F. annosus*.

In the experiment where the roots of the seedlings were allowed to remain in contact with the *B. bovinus* inoculants for several weeks before exposure to *F. annosus*, three combinations were used; one layer of *B. bovinus* inoculant at the bottom of the seed box; one bottom layer of plain autoclaved barley; controls with only perlite. Seven weeks after planting, the seedlings showed 84, 61 and 82 % survival respectively. *F. annosus* was then introduced in the boxes and figure 7 presents the results observed. All but one seedling pretreated with the mycorrhiza fungus *B. bovinus* escaped damage from *F. annosus*. Pretreatment by exposure of the roots to plain autoclaved barley resulted in an increased mortality, the same situation prevailed in the untreated control seedlings. In the former combination, seedlings began to die out two weeks

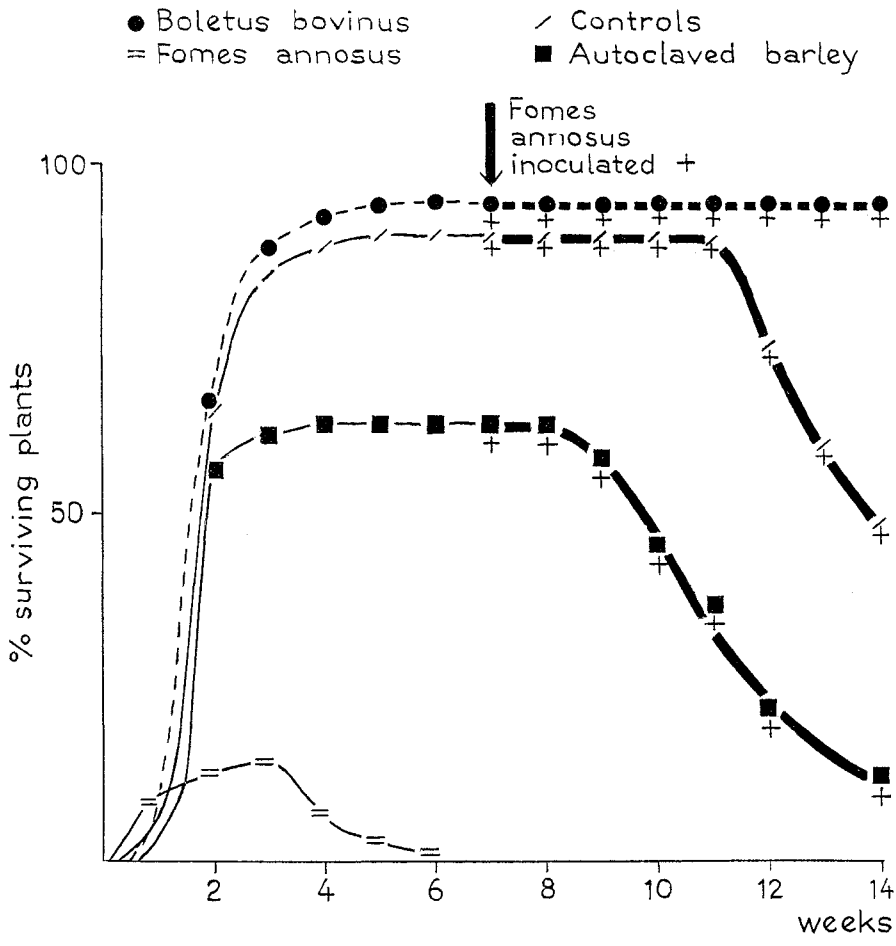

Fig. 7. Effect of *Fomes annosus* inoculated after 7 weeks on spruce seedlings pretreated with *Boletus bovinus* grown on barley kernels, and with autoclaved barley kernels only. Controls without fungi and barley.

after inoculation and after 14 weeks, when the experiment was terminated, only 8 % of the seedlings were still live. In the controls, seedlings showed damage six weeks after inoculation and 40 % live seedlings remained by the end of the experiment.

The major difference in mortality between the pretreated seedlings and the others suggested that the antagonistic action of *B. bovinus* observed earlier *in vitro* (HYPPEL 1968) existed also under greenhouse conditions. This antagonism can result in a protective effect for a host plant.

The plain autoclaved barley, used as a substrate for the preparation of

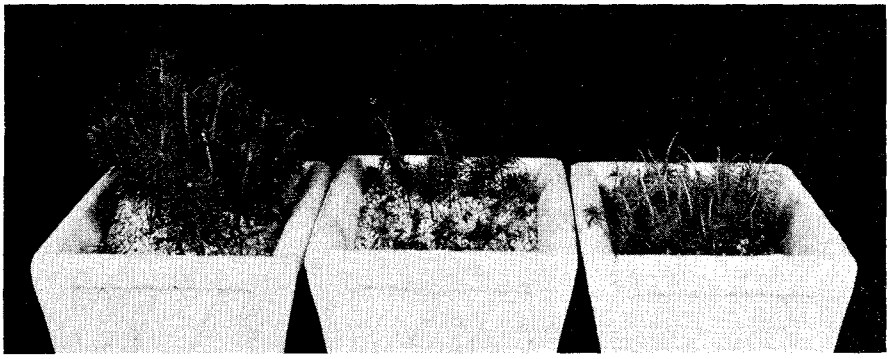

Fig. 8. *Boletus bovinus* (one layer in the bottom) | Barley kernels (one layer in the bottom) | Control (no pretreatment)
Fomes annosus inoculated after 7 weeks in spruce seedlings in perlite culture with *Boletus bovinus* grown on kernels of barley and in control cultures. The seedlings 6 months old.

F. annosus and *B. bovinus* inoculant, had no protective effect on the seedlings. If anything, it enhanced the detrimental action of the root rot fungus.

The results of the experiments on the mode of action of *B. bovinus* are shown in figure 9. It appeared that the water extract of *B. bovinus* mycelium had an inhibiting effect on the pathogenic action of *F. annosus*. This inhibition decreased however as time went on. Yet the effect of the water extract was not exclusively beneficial as 52 % only of the seedlings in plain perlite survived when irrigated with water extract from the mycelium of the mycorrhiza-forming fungus. In the controls, 76 % of the seedlings survived.

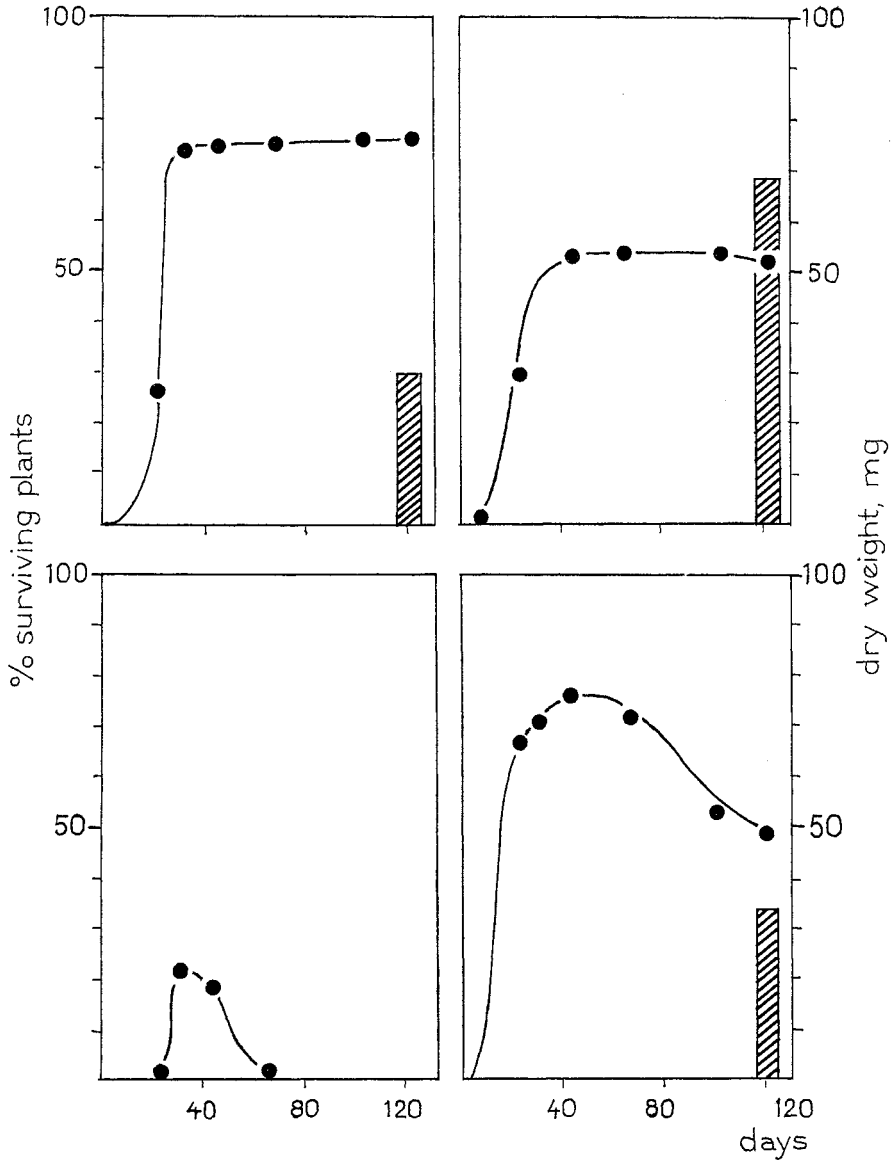

Fig. 9. Survival and average dry weight of spruce seedlings exposed to *Fomes annosus* added in kernels of barley and irrigated with extracts of *Boletus bovinus*.
Upper left: Controls (no *Fomes annosus*, no barley)
Upper right: Irrigation with extract (no *Fomes annosus*, no barley)
Down left: *Fomes annosus* added, no extract.
Down right: *Fomes annosus* added, irrigation with extract.

Discussion

The results observed were unexpected to a certain extent; since preliminary experiments had indicated that *B. bovinus* could provide protection to Norway spruce seedlings against subsequent exposure to *F. annosus*. This was found to occur in these experiments also, but only for the first two or three weeks. Later, damage to the seedlings would develop extensively. It seemed however that a carefully timed pretreatment of the seedling roots would provide protection against the root rot fungus, *F. annosus*. The mode of action for this preventive effect was not clear, but it appeared that a water-soluble metabolite of *B. bovinus* had an inhibiting effect on *F. annosus*. This finding was consistent with previous observations by HYPPEL (1968) on the inhibiting effect on *F. annosus* of toxic substances present in nutrient solutions used to grow *B. bovinus* and also an isolate from *Monotropa hypopitys* L. MARX (1967) found also that diatretyne derivatives active on damping off fungi could be extracted from culture broth of the mycorrhiza fungus *Leucopaxillus cerealis* var. *piceina*.

In the investigation reported here, the duration of the experiments was too short to allow formation of true mycorrhizal association, and no evidence of any such development was ever observed. However the appearance of the seedlings was affected as a result of exposure to the mycelium of *B. bovinus*. The root growth was stunted near the layer of fungus inoculant, while the stems were longer and the needles greener than in the controls. Whether this result is due to an increase in uptake and assimilation of growth substances, sugars, nitrogen or other substances released from the fungus layer or to some other factor is not understood at this point.

Summary

The protective effect of *Boletus bovinus* against *F. annosus* was tested in the greenhouse with seedlings of Norway spruce. A new technique was designed for that purpose. It involved seed boxes filled with perlite and planted with the seedlings. Fungal inoculants were added as kernels of barley overgrown with the mycelium of the microorganisms tested.

Upon exposure to *F. annosus*, the seedlings pretreated with *B. bovinus* grew taller and appeared greater than the control plants. The mortality in the

control plants was also quite noticeable; seven weeks after inoculation the controls showed only 48 % plants surviving, while the seedlings treated with *B. bovinus* remained healthy, unaffected by the root-rot fungus.

No mycorrhiza formation took place and the protective effect may be due to a water-soluble substance released by the mycorrhiza fungus.

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Sammanfattning

Fomes annosus' inverkan på groddplantor av *Picea abies* i närvaro av *Boletus bovinus*

I föreliggande arbete beskrivs en metod att uppföröka såväl en antagonistiskt verksam mykorrhizasvamp *Boletus bovinus* som rotrötesvampen *Fomes annosus* på ett substrat baserat på korn. De kornburna kulturerna har kunnat utnyttjas för olika inokuleringsändamål i växthusmiljö. Genom att förbehandla groddplantor av gran med kornkultur av *B. bovinus* förblev de opåverkade av inokulerad rotrötesvamp, under det att obehandlade kontrollplantor påverkades fatalt. Under en sju veckor lång försökstid gick således överlevelseprocenten ner från 82 till 48.

Ingen verklig mykorrhiza bildades under observationstiden. Det skydd som konstaterats härrör sannolikt från ett eller flera vattenlösliga extrakt som visats ha likartad skyddseffekt på groddplantmaterial.