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Microbiological Research

Effects of the antimycotic molecule Iturin A2, secreted by *Bacillus subtilis* strain M51, on arbuscular mycorrhizal fungi

Anna Silvia Citernesi[1], Carlo Filippi[2], Giovanna Bagnoli[3] and Manuela Giovannetti[2]

[1]Scuola Superiore di Studi Universitari e Perfezionamento S. Anna, Pisa, Italy;
[2]Istituto di Microbiologia Agraria, Università di Pisa;
[3]Centro di Studio per la Microbiologia del Suolo, CNR, Pisa, Italy.

Università di Pisa, Istituto di Microbiologia Agraria
Via del Borghetto, 80, 56124 Pisa, Italy

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Abstract

A new system, devised for the study of early stages of arbuscular mycorrhizal infection, was used to test the effect of the biological control agent Iturin A2, secreted by the strain M51 of *Bacillus subtilis*, on the development of arbuscular mycorrhizal fungi.

The saprophytic growth of the fungus *Glomus mosseae* was inhibited by Iturin A2 concentrations higher than 100 µg/g of sand; whereas, in the presence of the tomato host plant, both, pre-infection events and intraradical growth were not negatively influenced by the antifungal compound; furthermore, the development of arbuscular mycorrhizal symbiosis was not impeded by the biocontrol agent in field conditions, while *Fusarium oxysporum* f. sp. *lycopersici* infection was hindered.

Keywords: Biocontrol agent – Iturin A2 – *Glomus mosseae* – Arbuscular mycorrhizal fungi – *Fusarium oxysporum* f. sp. *lycopersici*

Introduction

Iturin A is an antifungal and hemolytic antibiotic, produced by some strains of *Bacillus subtilis*, which has a wide suppressive spectrum on many phytopathogenic fungi and bacteria (Peypoux *et al.* 1978; Gueldner *et al.* 1988; Phae *et al.* 1990b). The iturin-producing strains can be utilized as biological control agents with the aim of reducing the use of chemical pesticides in agriculture (Phae and Shoda 1990; Phae *et al.* 1990b; Filippi and Bagnoli 1991).

The strain M51 of *Bacillus subtilis*, isolated by Filippi and co-workers (Filippi *et al.* 1984), produces an antimycotic molecule identified as Iturin A2 (Filippi and Bagnoli 1991), previously described by other authors (Winkelmann *et al.* 1983). Bioprotection by means of *Bacillus subtilis* strain M51 has been obtained in both *in vitro* and *in vivo* tests (Filippi *et al.* 1984), though the protection persisted only for a limited period in respect to the pathogenic microorganism virulence (Bagnoli *et al.* 1985; Filippi *et al.* 1987).

Nevertheless further studies aimed at investigating the possible application of Iturin A2 in agriculture, have shown a strong and stable activity of the molecule against different phytopathogenic and saprophytic fungi (Phae *et al.* 1990a; Filippi *et al.* 1992).

Biological control agents, when utilized against soilborne fungal pathogens, may have effects on non-target soil and rhizosphere organisms, and may affect arbuscular mycorrhizal (AM) formation. It is well known that arbuscular mycorrhizae improve the growth of many agricultural plants by increasing their photosynthetic and transpiration rates, their nutrient uptake, and by improving their tolerance to biotic and abiotic stresses (Gianinazzi *et al.* 1990). Inhibition of mycorrhizal infection and changes in population composition of arbuscular mycorrhizal fungal species, induced by chemicals, have been frequently overlooked, though they may have an

Corresponding author: A. S. Citernesi

important ecological significance for soil fertility and plant production (Bethlenfalvay and Linderman 1992; Sieverding 1991). The evaluation of biological control agents should therefore include tests of their effects on mycorrhizal activity.

In this paper the new system devised for the study of early stages of arbuscular mycorrhizal infection (Giovannetti *et al.* 1993 a) was utilized with the aim of testing the effect of the molecule Iturin A2 on germination, hyphal growth and infectivity of the arbuscular mycorrhizal fungus *Glomus mosseae*.

Material and methods

Fungal cultures. The arbuscular mycorrhizal fungus used was *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (Rothamsted isolate), maintained in alfalfa (*Medicago sativa* L.) pot cultures. Pure culture of a phytopathogenic strain *Fusarium oxysporum* f. sp. *lycopersici,* kindly provided by Prof. Zazzerini, Plant Pathology Institute, University of Perugia, was grown in PDA medium.

Plant material. Solanum lycopersicum L. (cv. Early pack 7, Petoseed Italiana, P. O. Box 173. 34100 Parma, Italy) was used.

Biological control chemical. The antimycotic molecule Iturin A2 was isolated from *Bacillus subtilis* strain M51, grown in liquid culture for 7 days at 28 °C. It was extracted and purified as previously described (Filippi and Bagnoli 1991; Filippi *et al.* 1992).

Experimental design. Experiment 1. This experiment investigated the influence of Iturin A2 on germination and hyphal growth of *G. mosseae. Ten sporocarps of G. mosseae* were placed between two millipore membranes (0.45 µm diameter pores) 0, 60, 90, 100, 125 and 150 µg/g of the antimycotic agent Iturin A2 had previously been added. Petri dishes were incubated in the dark at 24 °C. Five replicate sandwiches for each concentration were used.

The activity of the molecule in this system was tested utilizing *F. oxysporum* f. sp. *lycopersici* as control organism. Millipore membranes were inoculated with conidia of *F. oxysporum* f. sp. *lycopersici* and then placed into the same dishes of each treatment.

After 10 days the sandwiches were removed from the sand, the millipore membranes containing sporocarps or conidia were opened and examined under a dissecting microscope, after staining with a few drops of 0.05% trypan blue in lactic acid. Sporocarp germination was checked and hyphal growth was assessed by the gridline intersect method after positioning a gridline on the millipore membrane (Gionannetti and Mosse, 1980). The percentage of concidia germination was assessed, after mounting cut pieces of millipore membranes on microscope slides, under a Polyvar Reichert-Jung microscope.

A similar experiment was carried out using sterile sand with Iturin A2 added at the active concentrations (0, 100, 125, 150 µg/g sand), maintained for 40 days at 24 °C. After this time, millipore membranes containing *G. mosseae* sporocarps were covered by the sand with Iturin A2. This test was aimed at evaluating the persistence of molecule activity.

Experiment 2. This experiment investigated the effect of Iturin A2 on the infective ability of *G. mosseae.* Ten sporocarps pf *G. mosseae* were sandwiched between two millipore membranes (0.45 µm diameter pores); the sandwiches were transferred into Petri dishes containing moistened sterile sand and incubated in the dark at 24 °C. The seeds of *s. lycopersicum* were surface-sterilized for 10 min in 1% W/V sodium hypochlorite and germinated in sterile sand. After 6 days they were removed from the sand using water, the root systems gently washed, then placed between the two millipore membranes containing the pregerminated sporocarps of *G. mosseae* (Giovannetti and Citernesi 1993). The seedlings with the "root sandwiches" were then transferred into Petri dishes containing 40 g of sterile sand with 0, 60, 120 µg/g of the antimycotic agent. The experiment was conducted in a climate chamber with a day temperature of 24 °C, a night temperature of 20 °C, a 12 h photoperiod and 60% of relative humidity. The seedlings were fertilized every two days with 5 ml/Petri dishes of half-strength Hoagland solution without phosphorus, through a small hole on the surface of the Petri dishes. Five replicate sandwiches for each concentration of Iturin A2 were used.

Plants were harvested 10 days after the beginning of the mycorrhizal fungus-host plant-Iturin A2 interaction; the sandwiches were carefully opened, the roots were cleared in 10% (W/V) KOH for 20 min, stained with 0.05% trypan blue (Phillips and Hayman, 1970), and the percentage of infected root length was assessed by the gridline intersect method.

Experiment 3. This experiment investigated the effects of Iturin A2 on intraradical growth of *G. mosseae.* The experiment was performed using *S. lycopersicum* seedlings, pre-infected by *G. mosseae:* the seedling root systems were sandwiched between two millipore membranes containing pregerminated sporocarps of *G. mosseae* and they were placed in Petri dishes with sterile sand without Iturin A2. After 10 days, the time required to obtain a successful mycorrhizal infection, the plants were

transplanted in sterile sand containing different concentrations of Iturin A2, as described in Exp. 2. Five replicate sandwiches for each concentration were used. The mycorrhizal plants were harvested 10 days after the contact with the antimycotic compound and the percentage of infected root length was assessed.

Experiment 4. The effect of Iturin A2 on mycorrhizal and *F. oxysporum* f. sp. *lycopersici* infection in a non-sterile soil system was investigated. Surface-sterilized seeds of *Solanum lycopersicum* were germinated in sterile sand. After 6 days the seedlings were removed from the sand and transplanted in pots containing 400 g sandy soil. Some seedlings were inoculated with *G. mosseae;* some were inoculated with G. mosseae and tranplanted in pots added with 350 µg/g soil of Iturin A2 (Filippi *et al.* 1992). After 10 days all the plants were inoculated with *F. oxysporum* f. sp. *lycopersici* conidia according to the methodology suggested by Anchisi *et al.* (1985).

One month after transplanting the plants were removed from the pots and mycorrhizal infection was assessed as described in Exp. 2. The pathogenic agent was detected in the plant tissue: the stem was disinfected by immersion in ethanol 96%, sectioned in 1 cm sections and the pieces were placed on potato dextrose agar (PDA) (Difco) in Petri dishes. They were incubated at 27 °C for 72–96 h according to Dimock (1948), and the percentage of the sections showing pathogen growth was assessed. Total phosphorus content of the plants was determined using the Watanabe and Olsen method (1985).

Results

Experiment 1

10 days after inoculation, germination and total hyphal length of *G. mosseae* in the sand containing 60 and 90 µg/g Iturin A2, were similar to the control, whereas their values were significantly lower than the controls at 100, 125 and 150 µg/g of Iturin A2 (Fig. 1 and 2).

Sporocarp germination was partially inhibited by Iturin A2 concentrations higher than 100 µg/g, whereas hyphal length was strongly inhibited by the same concentration of antifungal substance. The perdentage of conidia germination of *F. oxysporum* f. sp. *lycopersici*, at different concentrations of Iturin A2 in the sand, shows the activity of the antifungal compound in the experimental system (Fig. 3).

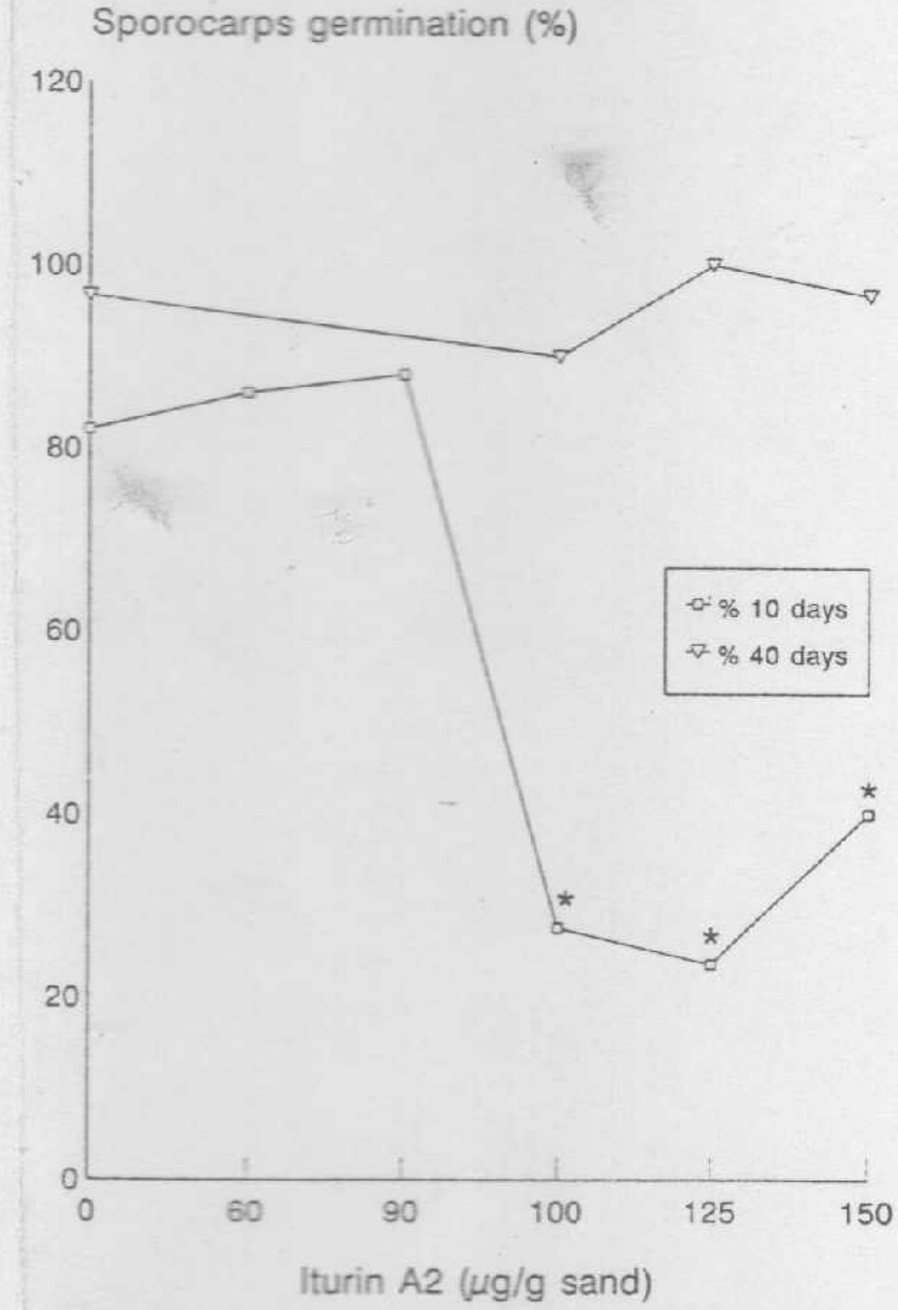

Fig. 1. Sporocarp germination of *Glomus mosseae* in the presence of different concentrations of Iturin A2, 10 and 40 days after the addition of the antifungal compound to the sand. Asterisks represent values significantly different from the control at $P < 0.01$.

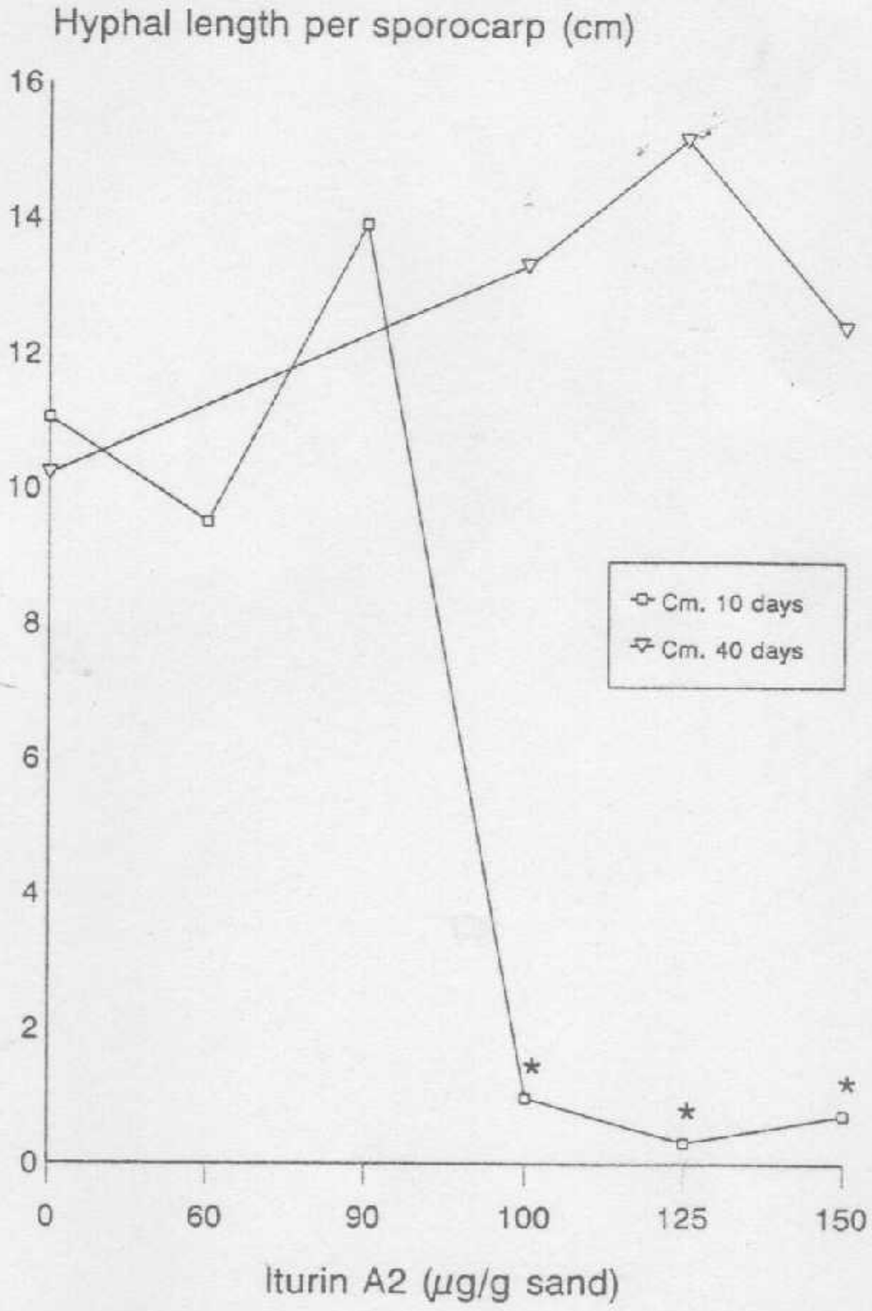

Fig. 2. Hyphal growth of *Glomus mosseae* in the presence of different concentrations of Iturin A2, 10 and 40 days after the addition of the antifungal compound to the sand. Asterisks represent values significantly different from the control at $P < 0.01$.

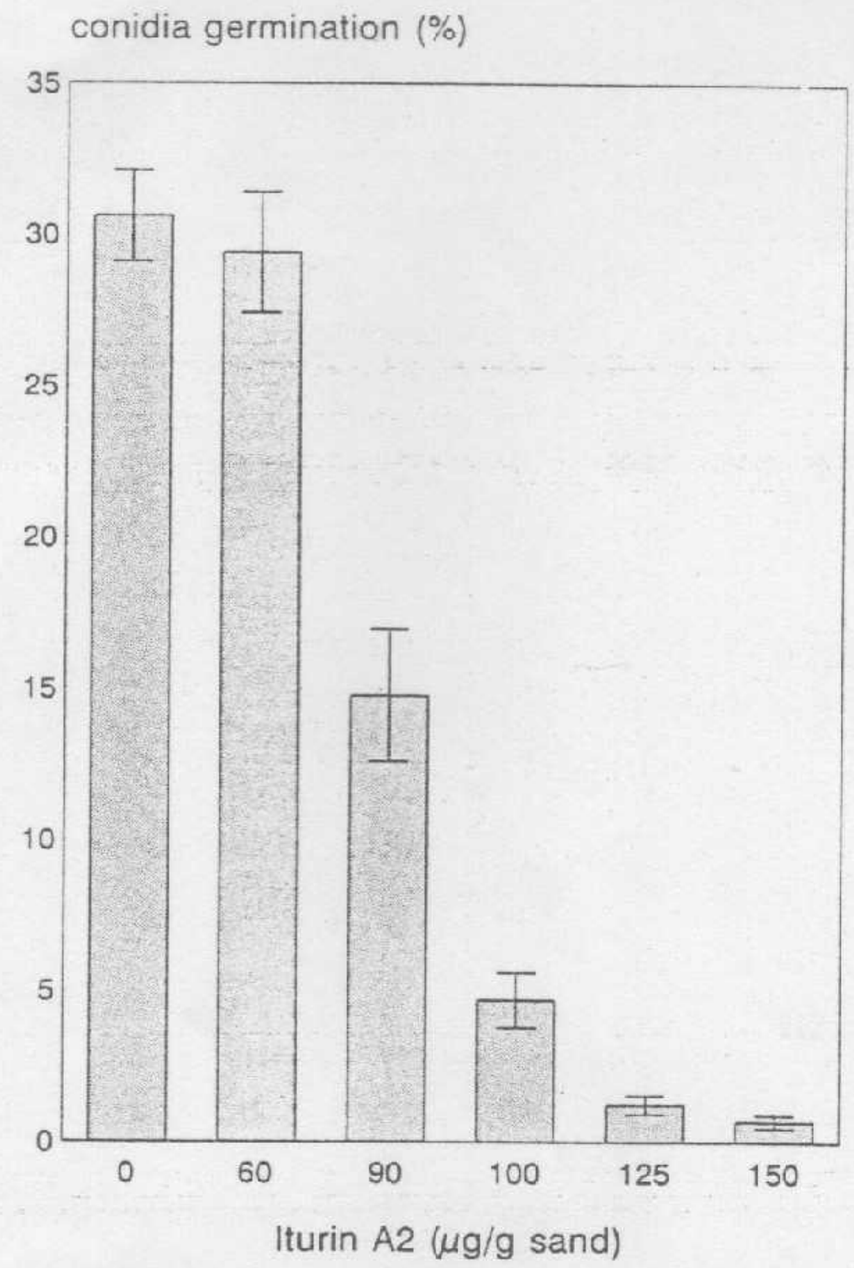

Fig. 3. Conidia germination of *Fusarium oxysporum* f. sp. *lycopersici* in the presence of different concentrations of Iturin A2. Bars indicate the standard errors of the means.

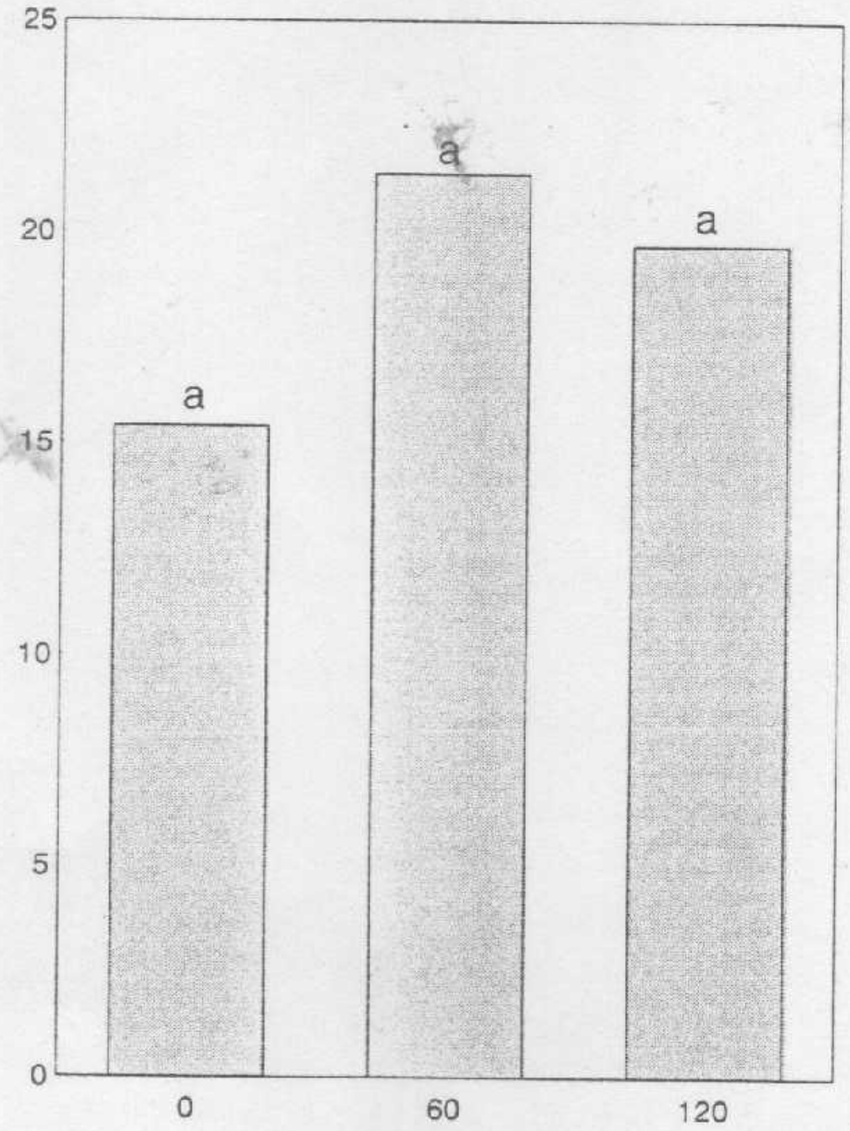

Fig. 4. Percentage of infected root length, in tomato plants inoculated by *Glomus mosseae*, in the presence of different concentrations of Iturin A2 added to the sand during the pre-infection phase. Similar letters indicate values not significantly different at $P < 0.01$.

In the control experiment, performed 40 days after the presence of Iturin A2 in the sand, no inhibition of the germination and hyphal elongation of *G. mosseae* was observed.

Experiment 2

The molecule Iturin A2 did not interfere with the ability of *G. mosseae* to infect roots of *S. lycopersicum*. In fact the percentage of infected root length of plants grown in the presence of Iturin A2 was not significantly different from that of the control (Fig. 4).

Experiment 3

The antimycotic substance did not inhibit intraradical development of *G. mosseae*. In fact at harvest, the percentage of infected root length of the tomato seedlings grown in the sand with Iturin A2, was similar to that of the controls (Fig. 5).

Experiment 4

The presence of Iturin A2 in the soil did not inhibit the development of mycorrhizal symbiosis, while it inhibited vascular wilt disease in the tomato plants. The plants inoculated only with *F. oxysporum* f. sp. *lycopersici* were all infected and 75% of the stem sections showed pathogen infection. All mycorrhizal plants were infected by *F. oxysporum* f. sp. *lycopersi-*

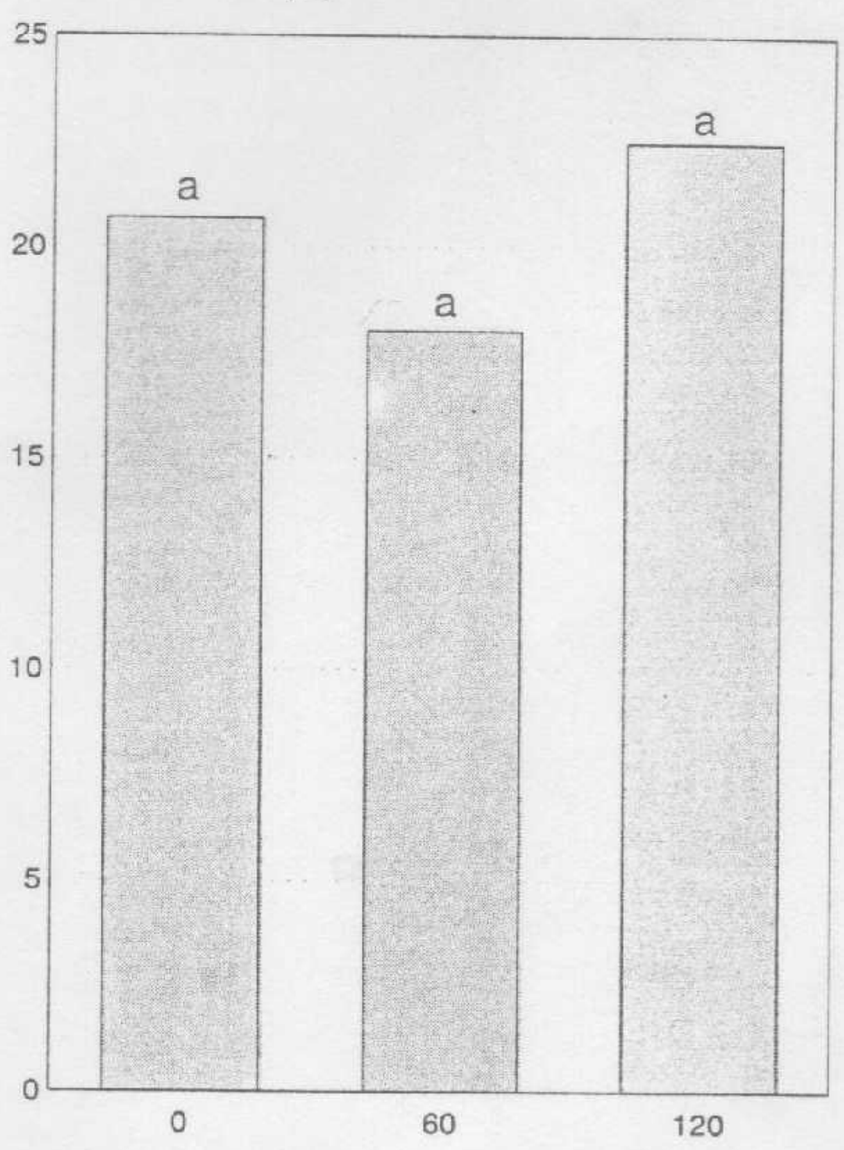

Fig. 5. Percentage of infected root length, in tomato plants inoculated by *Glomus mosseae*, in the presence of different concentrations of Iturin A2 added to the sand during the post-infection phase. Similar letters indicate values not significantly different at $P < 0.01$.

ci, but in this case only 41% of the stem segments supported *F. oxysporum* f. sp. *lycopersici* growth. The plants grown in the soil containing Iturin A2 showed 2.5% stem pieces infected by the pathogen and had the same degree of AM colonization as that without Iturin A2; in fact the plants developed in the presence of the antifungal compound showed $6.3 \pm 0.7\%$ of AM colonization and the ones grown without Iturin A2 had $5.6 \pm 0.8\%$ of AM infection. The percentage of total phosphorus content was the same for all the plant treatments (0.25% of the dry mass).

Discussion

The results of this work show that: i) the antifungal compound Iturin A2 reduces sporocarp germination of *G. mosseae* and inhibits hyphal elongation in experimentally controlled conditions during the saprophytic growth phase of the fungus; ii) both pre-infection events and intraradical growth are not negatively influenced by Iturin A2; iii) arbuscular mycorrhizal symbiosis is not impeded by Iturin A2 in field conditions, while *F. oxysporum* f. sf. *lycopersici* infection is hindered.

The reduced germination and growth inhibition of *G. mosseae* in laboratory experimwents may be greatly influenced by the absence of the host plant, since AM fungi are obligate biotrophs, showing a very limited saprophytic phase (Hepper 1983; Mosse 1988). These results confirm that the span of "independent" growth in these fungi may be greatly influenced by any factor altering the precarious equilibrium which allow them to survive without the host. In fact, not only pesticides but also nutrients have been reported to stop the growth of AM fungi *in vitro* (Hepper 1979; Hepper 1983).

When Iturin A2 was tested in the presence of a host plant, it did not interfere with the recognition events prior to the estabilishment of a functional symbiosis. In the same way, the intraradical development of the fungus did not change when the root system was treated with Iturin A2. These results confirm the importance of not considering the AM fungi as separate from the host plant. In fact, after recognition events have occurred, a different morphogenesis and growth pattern are expressed in the fungus, leading to a penetration phase, which makes the fungus less dependent on environmental conditions (Giovannetti *et al.* 1993 b).

The actual impact of Iturin A2 on symbiosis development was investigated in field conditions, where different edaphic and rhizospheric relationships were established between AM fungi and mycorrhizal plants. In these conditions, Iturin A2 did not have a negative impact on AM fungal growth and infectivity, whereas it showed a strong activity against *F. oxysporum* f. sp. *lycopersici*. these consistent findings show that Iturin A2 could be efficiently utilized as a biological control agent in sustainable agricultural systems.

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Microbiological Research

The effect of some antibiotics on *Enterococcus faecium* SF 68 inoculated in germ-free mice

Luigi Daniele Trovatelli[1], Pier Giacomo Sarra[2], Salvatore Massa[3], Donatella Fraioli[1], Francesco Canganella[1]

[1]Dipartimento di Agrobiologia e Agrochimica, Università della Tuscia, Viterbo, Italy;
[2]Istituto di Microbiologia, Università Cattolica del S. Cuore, Piacenza, Italy;
[3]Dipartimento di Biologia, Difesa e Biotecnologie Forestali, Università della Basilicata, Potenza, Italy

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Abstract

A strain of *Enterococcus faecium* SF 68 (sensitive to penicillin, tetracycline, virginiamicin and tylosin, but resistant to streptomycin) was administered to five groups of germ-free mice. Each group was subsequently given 40 μg/ml and then 80 μg/ml of a single antibiotic. The following determinations were made: a) colonization of the bacterial strain before and after administration of the antibiotic and b) the MICs in the original strain and after administration of the antibiotic (80 μgIml). The results show that in the mice treated with streptomycin, colonization is not influenced by the antibiotic treatment; in mice treated with antibiotics to wich the strain is sensitive, the colonization increases in proportion to the level of the antibiotic resistance (tylosin and tetracycline).

Keywords: Germ-free mice – *E. faecium* SF 68 – antibiotic resistance

Introduction

In animal husbandry, the use of antibiotics has steadily increased each year (Hinton *et al.* 1986). Antibiotics are used in animals both therapeutically and for prophylaxis; in addition, they may be added in low concentration to the animal feed as growth-promoters. During the last 20 years the usage of antibiotics has been often considered to be the cause for the flourishing of antibiotic-resistant bacteria at the gastrointestinal level (Smith *et al.* 1971; Frolich *et al.* 1974; Siegel *et al.* 1974; Gaines *et al.* 1980; Dawson *et al.* 1984). It was suggested that the use of subinhibitory concentrations of antibiotics should be avoided in animal feeds whereas only the therapeutic treatment of animals shoud be allowed (Swann 1969; van Houweling 1972; Anonymous 1988).

A large number of investigations regarding the in vitro sensitivity of enterococci to antibiotics have been carried out concerning the study of "clinical" isolates from human patients (Leclerq *et al.* 1991; Shlaes *et al.* 1991; Spera and Farber 1992; Caron *et al.* 1993) and a few studies were related to the therapeutic effect and the ability of antibiotics to promote the growth of faecal enterococci in healthy animals (Kaukas *et al.* 1987; 1988).

Due to the inherent difficulty of studying antibiotic resistance in microorganisms isolated from an ecological niche as complex as the animal intestine, we carried out an investigation to determine the effect of some antibiotics on a specific bacterial strain that was artificially inoculated into germ-free mice. Methods and results of this work are here reported and discussed.

Materials and methods

Animals. Male and female germ-free OF1 mice with an average weight of 14–18 g were used for this experiment. A commercial diet sterilized by irradiation as well as autoclaved water were both given *ad libitum* to the animals maintained inside plastic isolators.

Corresponding author: L. D. Trovatelli