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Mycorrhizal fungi in ecotoxicological studies: soil impact of fungicides, insecticides and herbicides

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Abstract

Biological soil monitoring involves the assessment of soil quality by monitoring living organisms in their natural environment or by toxicity laboratory tests. Soil biomonitoring allows the assessment of the biological effect linked to the bioavailable fraction of polluting substances and, as such, it plays a major role in defining quality criteria for the bioremediation of contaminated sites (Ministerial Decree 471/99) or, more generally, in the assessment of the quality of agricultural and natural soils.

Due to their key role in preserving soil fertility, arbuscular mycorrhizal fungi can be considered as the main non-target microorganisms to be monitored in environmental impact assessments of pesticides used in agriculture. Experimentation was chiefly aimed at validating a model system that provides for the use of the arbuscular mycorrhizal fungus *Glomus mosseae* as a biological indicator of chemical substances applied to the soil, and consequently, of the toxicological risk associated with the man-made pollution of soil ecosystems.

The experimental tests demonstrated that spore germination and/or mycelial growth of G. mosseae are adversely affected by most of the substances tested and, in some cases, at much lower concentrations than those indicated for use (hormesis). The results of the research suggest that *G. mosseae* can be a valuable indicator both for assessing the environmental impact of pesticides and other pollutants and for providing useful indications for the development of new active principles with a low environmental impact.

(Key words: *soil bioassessment, mycorrhizal fungi, pesticides, soil quality, beneficial soil microorganisms*)

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INTRODUCTION

The development of techniques that are useful for carrying out ecotoxicological and biological studies on soil is a relatively recent research activity. It has only been in the last few years that the importance of biodiversity in the ecology of the soil ecosystem has been recognized by worldwide studies, and it was not until the mid-90s that researchers at international level proposed characterization of soils based on biomonitoring.
Biological monitoring (also referred to as biomonitoring) of soil involves assessing the quality of soil by means of living organisms that can be used in a laboratory in toxicity tests or observed in their natural environment, thus acting as indicators of environmental conditions *(bioassessment)* [1].
On its own, chemical characterization of soil does not allow assessments concerning dangers for living organisms. Thus, in order to perform a full-scale assessment, it is necessary to make use of biological and ecotoxicological tools. The biological effect is linked to the bioavailable fraction of polluting substances which, in its turn, depends on the chemical substances that are present and on environmental conditions. This fact makes it necessary to use biological monitoring for a correct assessment of the risks deriving from soil contamination.
In particular, it soon became clear that it was necessary to define some indicators which could set quality criteria for the soil matrix to be used as standards in abatement operations [2] or, more generally, in the assessment of the quality of soils at risk of contamination [3].
The organisms used as biomonitoring investigation tools must show a specific sensitivity towards a number of environmental factors and are generally referred to as "biosensors". Depending on its specific characteristics, a biosensor can be used as a bioindicator or as a bioaccumulator.
More organisms together can be used as bioindicators, particularly when polluting phenomena determine changes that can be measured at ecosystem or community level. It is already a well-established procedure to assess the toxicity of complex matrices such as environmental matrices using a set of bioindicators, with the aim of analysing the broadest spectrum of effects on organisms with different responses to the various compounds in the matrices in different ways.

Application potential of arbuscular mycorrhizal fungi in ecotoxicological studies

Microorganisms play a key role in preserving soil fertility in agroecosystems. The most important biofertilizing microorganisms are arbuscular mycorrhizal (AM) fungi, which form mutualistic symbioses with the roots of most agricultural plants [4,5].
AM fungi are considered to be vital elements for plant nutrition as their hyphae can extend for many metres in the ground and they can absorb and transfer both macro and micro-nutrients present in the soil to the roots [6]. It has been demonstrated recently that symbiotic fungi, apart from absorbing and transferring mineral nutrients to the host plant, also have an important role in the redistribution of energy resources inside vegetal communities, through the development of fungal networks which extend tridimensionally in the ground and connect different plants [7]. The formation mechanism of the networks is represented by the ability of hyphae to form anastomoses with hyphae that have originated from other compatible fungal individuals, thus creating networks of indefinite length [8].
The reduction or even the disappearance of mycorrhizal fungal propagules following some cultural practices of conventional agricultural systems, such as the use of chemicals on the soil (fertilizers, herbicides and fungicides), is an indicator of the decreased stability of the plant-soil system [9,10]. As a consequence, AM fungi can be considered as the most important non-target microorganisms to be monitored in the environmental impact assessments of chemicals used in the agricultural sector.
The general objective of the experimentation was to assess the possibility of using the mycorrhizal symbiont

Glomus mosseae as an impact indicator of chemicals applied to the soil, and, consequently, of the toxicological risk linked to the man-made pollution of soil ecosystems. The research was organized as follows:

- preparation of an experimental model to assess the sensitivity of *Glomus mosseae* to pesticides that are present in the growth medium;
- use of the experimental model for pesticide screening. The percentage of spore germination and the length of the fungal mycelium during the pre-symbiotic growth phase of *Glomus mosseae* were assessed.

1. Materials and methods

1.1 Fungal material

The experiments were conducted on the AM fungus, *Glomus mosseae* (IMA1), from cultures preserved by the Department of Crop Plant Biology at the University of Pisa. *G. mosseae* proved to be the most suitable fungal species for environmental impact tests as it is widespread throughout the world and present in abundance in agricultural ecosystems [4]. "Pot culture" soil (pot cultivation of the mycorrhizal fungus with a host plant) was suspended in the water, decanted and filtered through a series of sieves at least 5 times (mesh: 100 to 500 microns). Sporocarps to be used for plate assays were then isolated from the residual material obtained from the sieves.

1.2 Tested pesticides

The commercial products, their corresponding active principles and the concentrations used in this research are shown in table 1.

Table 1 - Commercial products and active principles used in this research

Commercial product	Percentage and name of the active principle		Doses (mg/l) of the active principle^			
Crittox MZ 80	80.0 Mancozeb		3 200	1 600	800	400
Pomarsol Z Wg	81.0 Ziram	146	73	36	18	9
Ramid 30 Pb	30.0 Copper Hydroxide	108	54	27	14	7
Plantvax 20 E	20.0 Oxycarboxin		300	150	75	38
Rovral	50.0 Iprodione		750	375	188	94
Aliette	80.0 Fosetyl Al		1 600	800	400	200
Ridomil 5 G	5.0 Metalaxyl		123	62	31	15
Biofox C	* *Fusarium oxysporum* 251/2RB		92 320	46 160	23 080	11 540
Basel FL	45.0 Terbuthylazine	81	41	20	10	5
Risolutiv	30.4 Glyphosate	73	36	18	9	5
Agritox Dry 800	88.8 MCPA	21	11	5	3	1
Disetalin L	31.7 Pendimethalin	76	38	19	10	5
Aric 480 L.S.	40.3 Dicamba	10	5	2	1	1
Delfin	6.4 *Bacillus thuringiensis* Sa 11	4	2	1	0.5	0

* 10^5 propagules /mg

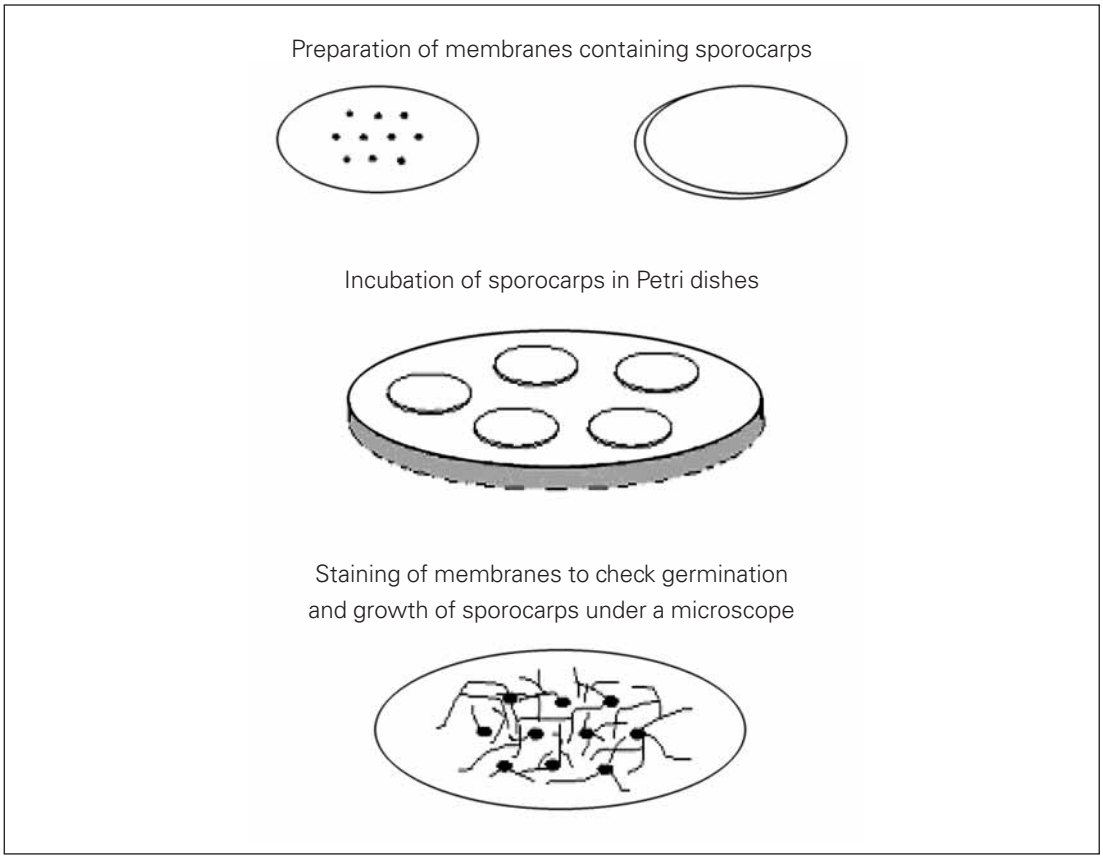
^ propagules /ml per Biofox C

2. Results

2.1 Experimental model

In order to analyse the impact of pesticides on (non-target) beneficial symbiotic fungi, an experimental model was devised, based on the "sandwich system" used to study the first stages of the life cycle of AM fungi [11,12]. The sporocarps isolated from the soil were collected and placed on membranes of cellulose esters (Millipores). A membrane was laid on each membrane containing 14 sporocarps; the sandwiches thus obtained were placed in Petri dishes containing sterile quartz, and incubated in the dark at 25°C, in the presence of the chemical substance to be tested (figure 1). After 10 days, the membranes were removed from the quartz, opened and checked for spore germination and the growth of the mycelium using Trypan blue staining (0.05 % in lactic acid).
The results of the research showed that the symbiont fungus *Glomus mosseae* responds in a differential way to the different chemical substances tested and in the two different stages of its life cycle studied, i.e. the germination of the quiescent spores and the growth of the mycelium.

Figure 1 - Experimental system to assess the impact of pesticides on arbuscular mycorrhizal fungi



2.2 Effects on spore germination

As far as the germination of quiescent spores is concerned, the active principles (AP) of the fungicides Copper Hydroxide and Mancozeb showed effects of complete inhibition, even at the minimum doses tested (table 2).

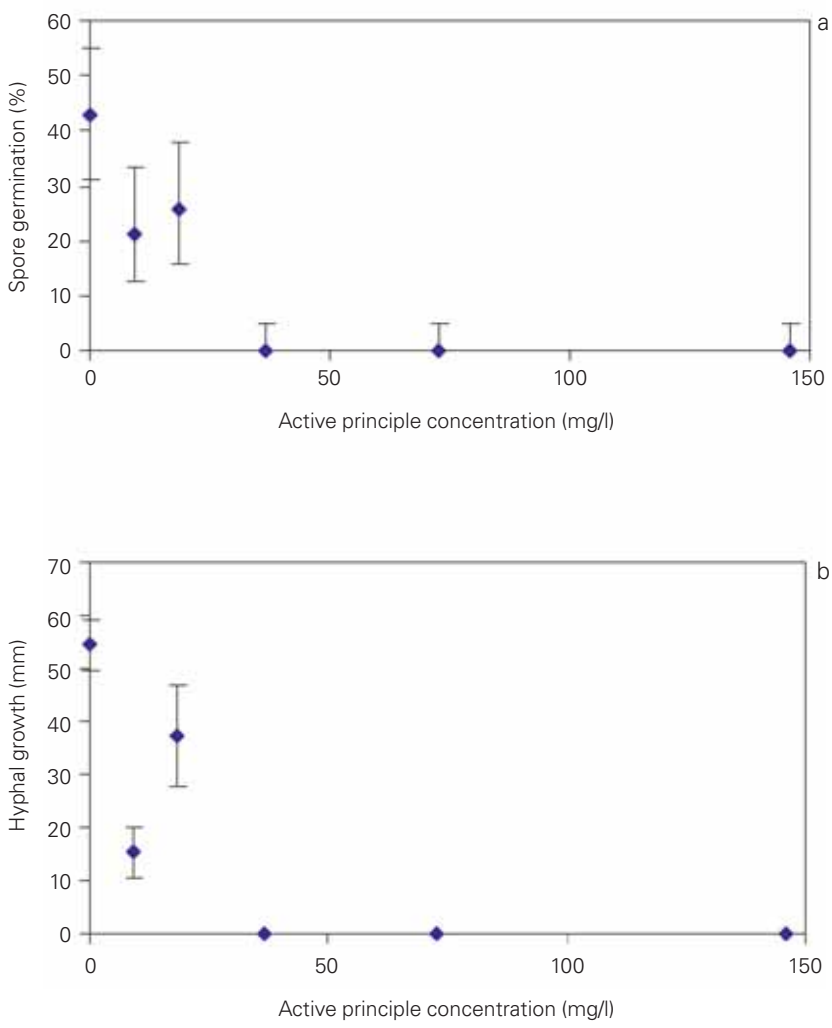
The *G. mosseae* fungus showed a dose-effect response to the AP of the fungicide Ziram (figure 2a). The fungus sensitivity to Ziram proved fairly high: even minimum doses of the active principle were able to halve the spore germinative capacity, which was completely inhibited from a 36 mg/l dose upwards.

The dose-effect response was particularly evident in the presence of the AP of the fungicides Oxycarboxin and Iprodione. Even in the presence of high AP concentrations, corresponding to the doses generally used in the field, spore germination was never completely inhibited.

Table 2 - Effect of the substances with fungicide action on sporocarp germination of *Glomus mosseae*

Active Principle	mg/l	Germination (%)	Confidence limits of the mean (95%)	
Copper Hydroxide	0	42.9	31.1	55.2
	6.8	0	0	5.1
	13.6	0	0	5.1
	27.2	0	0	5.1
	54	0	0	5.1
	108	0	0	5.1
Mancozeb	0	88.0	75.7	95.5
	400	0	0	7.1
	800	0	0	7.1
	1 600	0	0	7.1
	3 200	0	0	7.1
Oxycarboxin	0	56.0	41.3	70.0
	37.5	46.0	31.8	60.7
	75.0	33.0	19.5	46.7
	150.0	9.0	2.2	19.2
	300.0	4.0	0.5	13.7
Iprodione	0	79.0	69.7	86.5
	93.8	79.0	69.7	86.5
	187.5	60.0	49.7	69.7
	375.0	68.0	58.0	77.0
	750.0	45.0	35.1	55.3

FIGURE 2 - Percentage of germination (a) and growth of the mycelium (b) of the sporocarps of *Glomus mosseae* in the presence of increasing doses of the PA of the fungicide Ziram



The active principles of the fungicides Fosetyl Al and Metalaxyl did not show any effects on the percentage of the spore germination, which did not differ from that of the control (table 3).
The antifungal product for biological control based on *Fusarium oxysporum* did not show any effects on spore germination. In fact, the germination percentage ranged from 82% to 88% even at the highest doses, and from a statistical viewpoint it did not differ from the control (81%) (table 4).
Similarly, the other product for biological control with insecticide action based on *Bacillus thuringiensis* did not show any inhibitory action on spore germination of *G. mosseae* (table 4).

Table 3 - Effect of substances with a fungicide action on sporocarp germination of *Glomus mossae* and on mycelial growth

Active principle	mg/l	Germination (%)	Confidence limits of the mean (95%)		Hyphal growth (mm)
Fosetyl Al	0	79.0	64.0	88.5	68.9 ± 4.9
	200.0	82.0	68.6	91.4	n.d.
	400.0	86.0	73.3	94.2	n.d.
	800.0	80.0	66.3	90.0	71.2 ± 3.5
	1 600.0	70.0	55.3	80.5	30.0 ± 7.7
Metalaxyl	0	79.0	64.0	88.5	68.9 ± 4.9
	15.5	90.0	78.2	96.7	n.d.
	31.0	89.0	75.7	95.5	n.d.
	62.0	79.0	66.3	90.0	n.d.
	124.0	83.0	68.6	91.4	120.6 ± 14.2

Table 4 - Effect of the products used in the biological control, based on *Fusarium oxysporum* and *Bacillus thuringiensis*, on the sporocarps germination of *Glomus mosseae* and on mycelial growth

Active principle	mg or propagules/l	Germination (%)	Confidence limits of the mean (95%)		Hyphal growth (mm)
Bacillus thuringiensis	0	42.9	31.1	55.3	54.3 ± 4.8
	0.2	38.1	32.1	56.7	70.5 ± 10.5
	0.4	59.2	46.2	70.6	93.7 ± 9.4
	0.8	54.2	41.2	65.7	45.0 ± 8.3
	2	61.4	49.0	72.8	80.5 ± 9.8
	4	84.3	73.6	91.9	98.0 ± 2.5
Fusarium oxysporum	0	81.0	66.3	90.0	68.9 ± 4.9
	1.1E+08	82.0	68.6	91.4	n.d.
	2.3E+08	86.0	73.3	94.2	n.d.
	4.6E+08	88.0	75.7	95.5	n.d.
	9.2E+08	86.0	73.3	94.2	95.9 ± 7.9

The active principles of the herbicides Terbuthylazine, Glyphosate, MCPA, Pendimethalin and Gluphosynate did not seem to affect the spore germination of the fungus, even at the highest doses (table 5).

Table 5 - Effect of the substances with herbicide action on the germination of *Glomus mosseae* sporocarps

Active Principle	Concentration mg/l	Germination (%)	Confidence limits of the mean (95%)	
Terbuthylazine	0	74.3	62.4	84.0
	5.2	50.0	37.8	62.2
	10	58.6	46.2	70.2
	20.4	52.9	40.6	64.9
	40.4	76.8	65.1	86.1
	81.2	55.7	43.3	67.6
Glyphosate	0	74.3	62.4	84.0
	4.4	80.0	68.7	88.6
	9.2	95.7	88.0	99.1
	18.4	66.7	55.0	78.8
	36.4	84.3	73.6	91.9
	72.8	82.9	72.0	90.8
MCPA	0	74.3	62.4	84.0
	1.2	87.0	76.7	93.9
	2.8	70.0	57.9	80.4
	5.2	92.7	82.4	98.0
	10.8	64.3	51.9	75.4
	21.2	50.8	37.5	64.1
Pendimethalin	0	75.7	63.99	85.17
	4.8	68.6	56.37	79.15
	9.6	43.0	31.74	56.70
	19.2	42.3	30.61	54.56
	38	58.6	46.17	70.23
	76	47.1	35.09	59.45
Gluphosynate	0	75.7	63.99	85.17
	8.8	50.0	37.80	62.20
	17.6	58.6	46.17	70.23
	35.2	37.1	25.89	49.52
	70	27.1	17.20	39.10
	140	47.1	35.09	59.45

2.3 Effects on the growth of the mycelium

As for the growth of the mycelium, the following active principles with fungicide action were tested: Ziram, Iprodione, Fosetyl Al.

G. mosseae showed a dose-effect response to the fungicide Ziram, similar to the one relating to spore germination (figure 2 b). The sensitivity of the fungus to Ziram proved fairly high: even minimum doses of the active principle could drastically reduce mycelial growth, which was completely inhibited from a 36 mg/l dose upwards.

The active principle Iprodione induced a clear dose-effect response, consistent with the results of the germination (figure 3). The active principle Fosetyl Al induced a less evident dose-effect response (table 3).

The product for biological control with a fungicide action based on *Fusarium oxysporum* did not show effects on the growth of the mycelium. Similarly, the biological product with insecticide activity based on *Bacillus thuringiensis* did not show any inhibitory action on the mycelial growth of *G. mosseae* (table 4).

The active principles of the herbicides Terbuthylazine, Glyphosate and MCPA showed a considerable inhibitory action on the growth of *G. mosseae*. The mycelial growth of the fungus showed a clear dose-effect response exclusively in the presence of the active principle of the herbicide Pendimethalin (figure 4).

FIGURE 3 - Mycelial growth of the arbuscular fungus *Glomus mosseae* in the presence of increasing doses of the active principle of the fungicide Iprodione

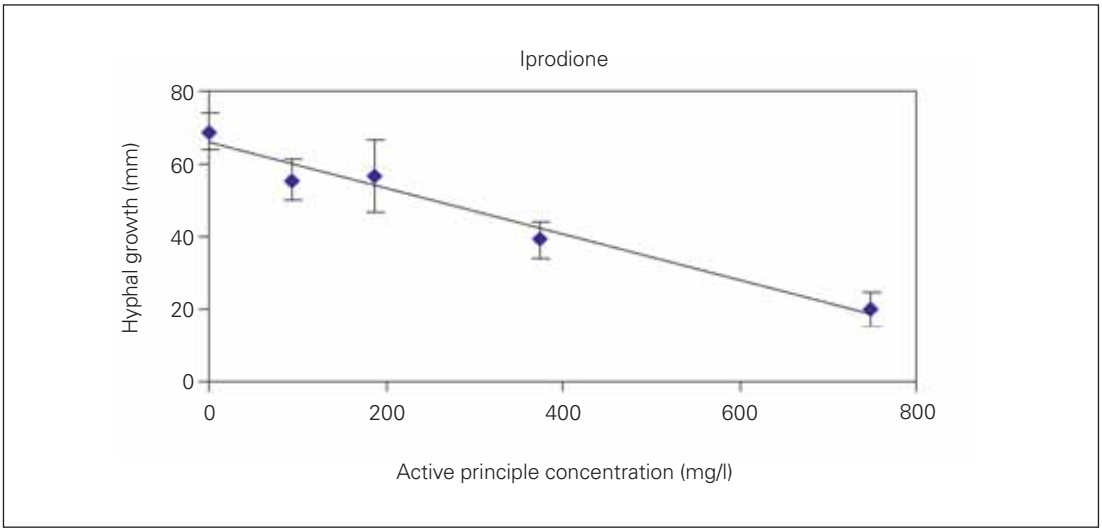
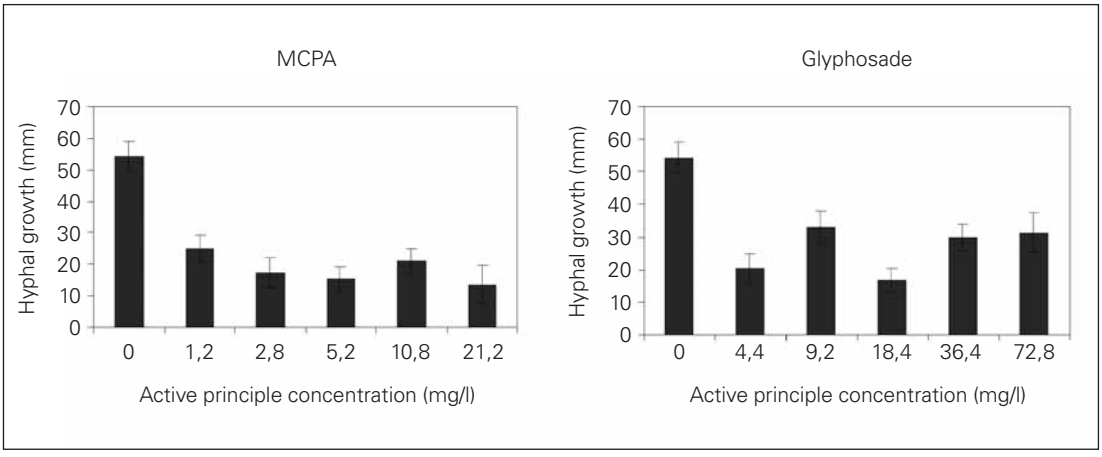
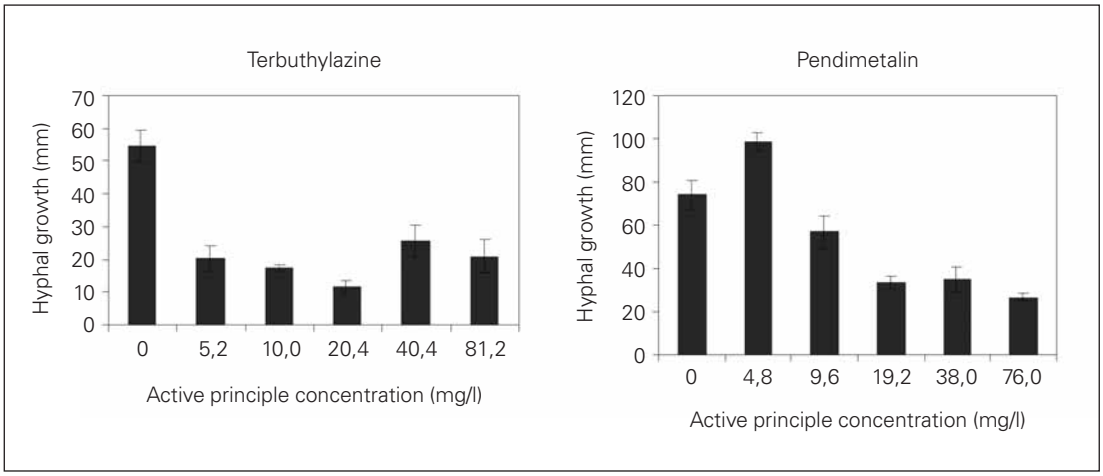


FIGURE 4 - Mycelial growth of the arbuscular fungus *Glomus mosseae* in the presence of increasing doses of different active principles with herbicide action





3. Discussion

This experimental system allowed the assessment of the impact of a large number of the most important chemical products used in agriculture on a non-target beneficial organism - the mycorrhizal fungus *Glomus mosseae*. This organism establishes symbiosis with most crop species and is widespread in agricultural ecosystems all over the world, representing a fundamental element for biological fertility of soils [4,5].

It is important to stress that the system requires AM fungi to be cultivated without host plants, in order to separate the response of the symbiont fungi from that of other components of the soil ecosystem. In fact, in natural conditions, very complex interactions occur between pesticides, host plants and AM fungi which make it difficult to assess the effects on a single organism. According to literature, the alterations induced by pesticides on the health and physiology of plants have significant effects on fungal symbionts [13-15]. It was also observed that the response of mycorrhizal fungi to chemical treatments can be influenced by the different species of host plants [16]. The presence of a functional mycorrhizal symbiosis can sometimes diminish the incidence of iatrogenic diseases, i.e. diseases resulting from the misuse of pesticides [17-19]. AM fungi establish very close relationships not only with the plants but also with the other components of the telluric microflora: some soil microorganisms seem to play an important role in regulating growth and root colonization by AM fungi [20,21]. According to some authors, the presence of pesticides can determine changes in interactions that occur among the various organisms and microorganisms living in the soil, due to differential toxicity for these organisms [22,23].

Assessments in the experimental system were carried out by observing effects at concentration levels that are close to levels at which treatments are carried out in practice and by comparing these data with those available in literature. This is because the field dose is noxious for fungal pathogens and it is therefore possible to assess the degree of sensitivity of the mycorrhizal fungus with respect to the target organisms. Furthermore, it is reasonable to consider that the concentration reached by a plant protection product in microenvironments where AM fungi are present is proportional to the concentration at which treatments are performed. It is extremely difficult, however, to determine the pesticide concentrations that AM fungi are effectively subject to, due to the complexity of the soil ecosystem.

The results obtained show that the mycorrhizal fungus *G. mossae* can represent a valuable indicator for assessing the environmental impact of pesticides used in agriculture [24]. The germination of spores and/or the growth of the mycelium of *G. mosseae* are, in fact, negatively influenced by most of the products tested. In some cases, much lower concentrations than those suggested for use in the field

(up to 1/8) proved to be toxic for non-target beneficial fungus. The inhibitory effects of some dithiocarbamates (Ziram and Mancozeb) on root colonization by the fungus and on spore production have been noted in literature [25,26]. Data from literature and from the experiments conducted in this study indicate that AM fungi show different sensitivity to pesticides during the various phases of the biological cycle. In particular, the presymbiotic growth might represent a very delicate phase since it occurs outside the host and thus the fungus comes into direct contact with the residual products which are present in the surrounding solution. This hypothesis could also validate the results obtained by other authors for other pesticides, according to the different periods of application [16-27].

The reduction in germination and growth in the presence of Oxycarboxin that was observed in this study turns out to be consistent with the *in planta* observations, which show a decrease in the phosphorus absorption capacity, as well as in the percentage of colonization by the mycorrhizal fungus [20-28].

The smaller negative effect of Iprodione on AM fungi suggests that the impact of this product, which has specific action mechanisms, is reduced with regard to non-target organisms. In fact, it was observed that Iprodione does not have toxic effects on *Bacillus pumilus* and *Bacillus amyloliquefaciens*, antagonists of *Botrytis cinerea*, but, rather, it develops synergic effects with them in the control of grey mould [29].

As regards Fosetyl Al, previous studies also indicate that the product does not present toxicity for AM fungi. On the contrary, it exerts stimulating action both on the host and on the symbiont, increasing plant growth and mycorrhizal colonization [30,31]. In this study, the highest dose that was used showed a fungistatic effect during the presymbiotic growth phase, which might not reflect on observations conducted on the basis of the infection capacity and symbiotic functionality of the fungus.

According to some authors, Metalaxyl increases radical colonization [32] and the length of external hyphae, even though it provokes a lower phosphorous absorption per length unit of the hyphae caused by the decrease of the development of the plant-fungus interface [31]. With regard to this, it is interesting to note how this product inhibits the formation of haustoria, which can be considered as structures that are similar to arbuscules. The increased root colonization could be determined by indirect effects due to the control that this product exerts on antagonists of AM fungi [20,21] or by a direct action of the pesticide, which, in this study, has proved capable of stimulating germination and hyphal growth in the presymbiotic phase.

As for the products based on *Fusarium oxysporum* and *Bacillus thuringiensis* that are used for biological control, it is interesting to note their complete lack of toxic effects on the mycorrhizal fungus *G. mosseae*, which was not influenced in any phase of its life cycle. The effects of the microorganisms used as biocontrol agents on AM fungi have rarely been researched. Bacterical biocontrol agents, for example *Pseudomonas putida*, did not appear to negatively affect AM fungi; on the contrary, they resulted in an increase in spore production by the symbiotic fungus [33]. The biological product analysed in this research is based on a non-pathogenic saprophyte strain of *Fusarium oxysporum*, referred to as 251/2 RB in the fungi culture collection of the University of Turin. This strain was isolated from a soil which was found to be naturally suppressive to carnation tracheofusariosis. In addition, its activity seems not to be linked to the production of toxins or fungistatical metabolites but, rather, to the capacity to colonize the plant root system, thus establishing a mechanism of competition for infection sites.

These results are quite interesting as antagonist microorganisms represent a valuable alternative to the use of pesticides with a view to sustainable agriculture and, in particular, in organic agriculture and its possible toxicity on non-target beneficial microorganisms as mycorrhizal fungi could result in the loss of biological fertility of soils and in the need to use fertilizers.

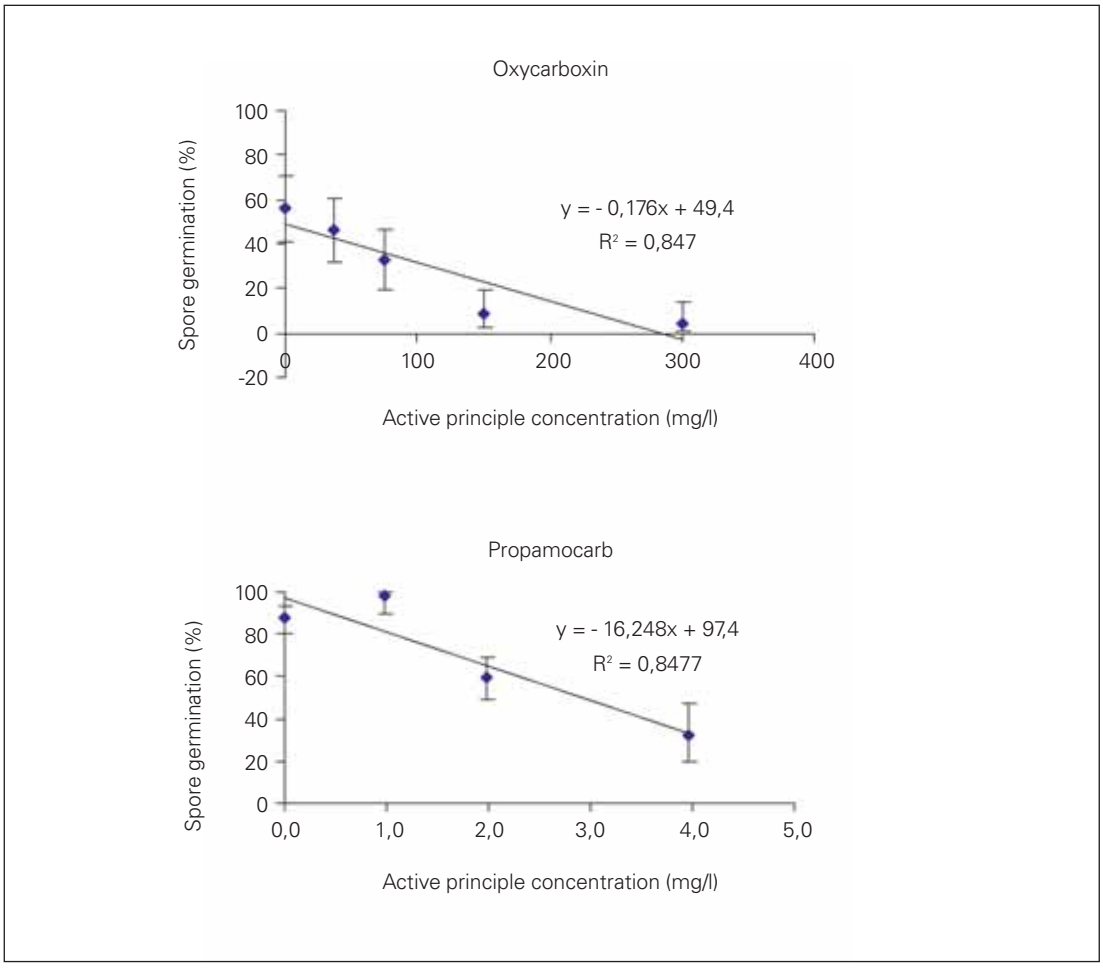
Our experimental research, further extended to the study of other pollutant agents, may allow the identification of valid parameters for the assessment of alterations in the soil ecosystem. This is of great importance to develop useful new methods for biomonitoring, to assess the environmental impact of pesticides and of other pollutants, and to provide information that is useful for the synthesis of new

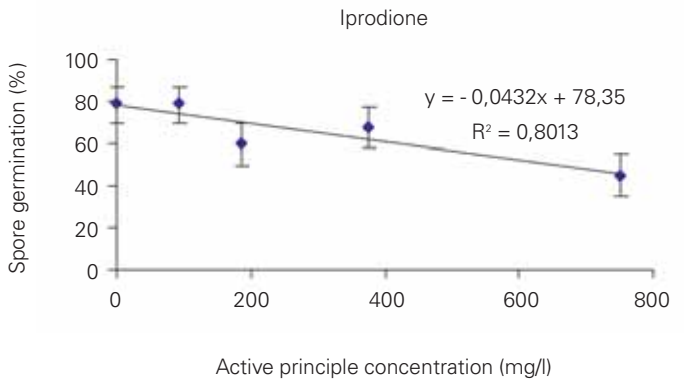
chemicals with a low environmental impact. Consequently, it is necessary to know more about the effects of pesticides on non-target organisms at all possible levels: cells, population, population genetics, interactions between microorganisms.

Thanks to its characteristics, this experimental system could turn out to be adequate for developing dose-effect curves for which a large amount of data is required. Even though the aim of this research was not to determine the pesticide doses to be used in bioassays, i.e. the interval where the dose-effect response is linear, it was however possible to highlight significant dose-effect relationships in the responses induced by the active principles of the fungicides Oxycarboxin, Propamocarb and Iprodione. In fact, the variance analysis for these active principles showed a statistically significant regression (F probability values: p = 0.027, 0.068, 0.04). Furthermore, the equation of the regression line shows a remarkable statistical significance represented by the R^2 values of 0.85, 0.85, 0.8 respectively (figure 5).

On the basis of the results obtained, this experimental study can be considered to be a useful method for providing an assessment of the activity of diverse toxic compounds in relation to *Glomus mosseae*. However, it should be taken into account that in this study only the acute effects were examined. Often, a toxic compound that can cause modest acute effects but high chronic effects can prove as noxious as a compound with a high acute toxicity.

FIGURE 5 - Dose effect relationship between concentration of the active principle and spore germination of *Glomus mosseae*





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