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Effect of diflubenzuron on the development of *Pinus pinaster* seedlings inoculated with ectomycorrhizal fungi

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Dissertação de Mestrado em Contaminação e Toxicologia Ambientais

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Resumo

O Pinheiro bravo (*Pinus pinaster* Ait.) é uma espécie conífera altamente valiosa amplamente distribuída sendo uma espécie florestal importante. Em Portugal a área florestal ocupada por *P. Pinaster* é de 23%. Os fungos ectomicorrízicos formam entre as raízes de plantas lenhosas associações simbióticas de um potencial benefício em condições de alto stress para a planta. O diflubenzuron é um regulador de crescimento de insectos, utilizados no controle de pragas florestais como insecticida. Os objetivos deste estudo foram avaliar o efeito do diflubenzuron sobre a germinação e o desenvolvimento das plântulas de *P. pinaster* e fungos ectomicorrízicos sob condições laboratoriais e estudar a possível protecção por parte de fungos ectomicorrízicos contra os efeitos do diflubenzuron. No teste *in-vitro* sob o fungo ectomicorrízico *Pisolithus tintctorius* foi observado inibição no crescimento das colónias para todas as doses de diflubenzuron – 0.01-100 mg/L. Os resultados de *P. pinaster* expostos a diflubenzuron apresentaram redução do crescimento do sistema radicular, especialmente a 10 e 100 mg/kg. Mesmo baixas concentrações de diflubenzuron (0.01-1 mg/kg) mostraram efeitos inibitórios no comprimento de raízes secundárias. No entanto, nenhum efeito foi observado no comprimento da parte aérea e da taxa de germinação. No teste, com *P. pinaster* inoculado com o fungo ectomicorrízico *P. tinctorius*, os resultados indicaram novamente inibição do crescimento do sistema radicular nas concentrações de 10 e 100 mg/kg. A inoculação não influenciou a maioria dos parâmetros biométricos avaliados. O sistema de defesa antioxidante foi estudado em termos da actividade das enzimas antioxidantes superóxido dismutase e da catalase. A actividade da superóxido dismutase foi aumentada nas doses de 1 e 10 mg/kg em ambas as plântulas de *P. pinaster*, não-inoculadas e inoculadas. A actividade da catalase foi significativamente aumentada nos tratamentos inoculados com doses de 1, 10 e 100 mg/kg. Foram observados efeitos tóxicos, até em baixas concentrações, apesar de não causaram efeitos mortais este composto tem a capacidade de provocar danos sub-letais. A utilização desproporcionada deste insecticida pode levar a maiores quantidades de resíduos de diflubenzuron no solo e na biosfera, pondo em risco as árvores, fungos e sua simbiose.

Abstract

Maritime pine (*Pinus pinaster* Ait.) is a highly valuable coniferous species largely distributed being an important forest species. In Portugal 23% of the national forest area is occupied by *P. pinaster*. Ectomycorrhizal fungi form association between the roots of woody plants incurring in possible benefit under conditions of high stress. Diflubenzuron is an insect growth regulator, used to control forest pests as an insecticide treatment. The objectives of this study were to assess the effect of diflubenzuron on the germination and development of *P. pinaster* seedlings and ectomycorrhizal fungi under laboratory conditions and to study the possible protective role of ectomycorrhizal fungi against the effects of diflubenzuron. The *in-vitro* test of ectomycorrhizal fungus *Pisolithus tinctorius* was observed inhibitory effect on colony growth for all doses of diflubenzuron - from 0.01 to 100 mg/L. The results from *P. pinaster* exposed to diflubenzuron showed reduce growth in the root system, specially at 10 and 100 mg/kg. Lower concentrations of diflubenzuron (0.01 - 1 mg/kg) showed inhibitory effects in secondary root length. However no effect was seen in shoot length and germination rate. In *P. pinaster* inoculated with ectomycorrhizal fungus *P. tinctorius,* results indicated again inhibition of root system growth at concentrations of 10 and 100. Inoculation had no influence on most biometric parameters assessed. The antioxidant defense system was studied in terms of antioxidative enzyme activity of superoxide dismutase and catalase. The activity of superoxide dismutase was increased at 1 and 10 mg/kg doses in both non-inoculated and inoculated *P. pinaster* seedlings and the activity of catalase was significantly increased in the inoculated treatments at 1, 10 and 100 mg/kg. Toxic effects could be seen even at low concentrations, despite causing no lethal effects, this compound has the ability to cause sub-lethal damage. The disproportionate use of this insecticide could lead to increased amounts in soil and biosphere of diflubenzuron residues, endangering trees, fungi and their symbiosis.

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1.1. Pinus pinaster in forest

Maritime pine (*Pinus pinaster* Ait.) is a highly valuable coniferous species (*Pinaceae*), largely distributed in the Mediterranean, Southern Europe, Africa, and the Atlantic coast of Portugal, Spain and France (Alía and Martín, 2003). It is one of the most important forest species, used mainly for carpentry, construction, and pulp and paper production. In Portugal, according to the National Forest Inventory (2005/2006), 23% of the national forest area is occupied by *P. pinaster* (DGRF, 2007), representing 11% of the total of forest product exports in 2000 (Correia *et al*., 2004).

Fig. 1.1. *Pinus pinaster* forest.

P. pinaster (Fig. 1.1) is ecologically versatile occurring on a variety of substrates (i.e., limestone, granite, schist, marly limestone, peridotite) and under a range of Mediterranean climate regimes (semi-arid to humid). It grows most commonly as an open forest (Brosse, 1977) but can also form closed forests (Blanco *et al*., 1997).

Forest pests have considerable impact on the value and role of forest ecosystems, both directly and indirectly. Defoliating insects can cause tree death, thus reducing the vital functions of the forest and its scenic value (Wainhouse, 2005). As forest insects are associated with tree decline and forest succession, forest managers and ecologists disagree on how to act on insect pest control. The main consequences of forest pests are tree growth reduction (Larsson, 1983), aesthetic landscape alteration (Mattson *et al*., 1996), fragile ecosystem disturbance (Simberloff, 2001) and negative human consequences (Vega *et al*., 2000). To control forest pests is potentially attractive the use of temporary suppressive measures such as insecticide treatments (Muzika and Liebhold, 2000).

1.2. Use of diflubenzuron as insect pest control

The use of fluorinated organic compounds has increased this century and they are now ubiquitous environmental contaminants (Giesy and Kannan, 2002; Key *et al*, 1997). Pollution of waters with fluorinated organic chemicals is rising because of the usefulness of fluorination to enhance bioactivity and lifespan of pharmaceuticals and pesticides (Reinhold, 2007). Diflubenzuron (DFB) is an aromatic fluorinated compound used for insect pest control.

Although generally viewed as recalcitrant because of their lack of chemical reactivity, many fluorinated organics, particularly perfluorinated compounds, are globally distributed, environmentally persistent, bioaccumulative, and potentially harmful (Key *et al.*, 1997; Lewandowski *et al.*, 2006). Although many studies report the degradation of fluorinated organic compounds by bacteria and fungi (Amadio and Murphy, 2010; Carvalho, 2009; Kramer *et al.*, 2004; Murphy, 2010) little is known about the occurrence, transport, biodegradation, and toxicity of these compounds in the environment (Lewandowski *et al.*, 2006). Over the past 15 years, the number of fluorine-containing agricultural chemicals has grown from 4% to approximately 9% of all agrochemicals and has increased in number faster than non-fluorinated agrochemicals (Key *et al.*, 1997, 1997). These compounds are primarily used as herbicides (48%), insecticides (23%) and fungicides (18%) (Key *et al.*, 1997).

DFB (Fig. 1.2) is a benzoylphenylurea derivative [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea], which acts as a potent broad-spectrum insect growth regulator interfering with chitin synthesis disrupting normal growth and development processes in insects (Jarvis and Zisman, 1966; Moody and Field, 2000). It is a highly effective chemical used to control numerous forest and agricultural pests (Key *et al*., 1997; Giesy and

Kannan, 2002). This pesticide has been frequently utilised in agricultural areas against insect pests, and also in fish farming due to its efficacy in controlling fish ectoparasites (Martins, 2004; Schalch *et al.*, 2005), among others.

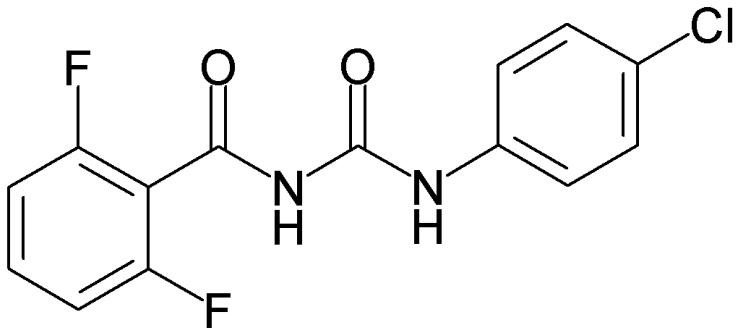

Fig. 1.2. Molecular structure of diflubenzuron.

DFB is degraded in the environment mainly by photodegradation and hydrolysis (Halasz and Millauer, 1976; Dams and Vanneste, 1997) producing as major metabolites 2,6-diflurobenzamide (2,6-DFBA), 4-chlorophenylurea (4-CPU), 4-chloroacetanilide (4-CAA), 4-chloroaniline (4-CA), and N-methyl-4-chloroaniline (NM-4-CA). The National Cancer Institute and the Cancer Assessment Group of the U. S. Environmental Protection Agency classified 4-CA and NM-4-CA as mutagens (Ehrl, 1998). DFB is normally used to control insects in conifer forests by aerial application at a rate usually no greater than 140 g of active ingredient (AI)/ha (Meissner *et al*., 2003). Its persistence in forest ecosystems is not well-known due to its dependence on several factors, such as the applied dose and formulation, aerial application parameters, the number of applications, climatic conditions, and the forest characteristics (Figueiredo and Sabadini, 2003; Schnierer, 1976). Mobility and leachability of DFB in soils is low and increased concentrations of DFB in soils and waters are associated with increased application frequency, wind drift, and excessive rainfall (Cunningham, 1986).

DFB is persistent in postharvest soils during winter and spring months, especially if associated with plant litter (Ivie and Bull, 1978; Bull, 1980). The particle size and soil flora may be important in the soil degradation process. As adsorbed to small particles it can persist for 3-7 days and adsorbed to larger particles may persist for 8-16 weeks (Cunningham, 1986). When used properly in forest management, DFB is unlikely to be leached into ground water from a site of application (Sundaram and Nott, 1989).

Studies indicate that DFB persist for 10-12 weeks on the foliage of a conifer forest. In the 22-30 days following treatment DFB concentrations higher than 370 mg/ kg were found in the foliage (Rodriguez, 2001). Due to its stability and low volatility, DFB residues adhering to plant surfaces are removed primarily through physical effects such as wind

abrasion, rain washing, or the loss of dead leaves (Bull, 1980). Gartrell (1981) showed no significant degradation of DFB residues in leaves for up to 16 weeks after treatment. Aerial application at 56.3 g of AI/ha resulted in deposition levels ranging from 867.5 to 1824.4 mg/kg, depending upon forest characteristics (Rodriguez, 2001). However, soil concentrations are much lower. Residues of DFB were identified 1 and 29 days post application at respective concentrations of 0.35 and 0.14 ppm and 2.68 and 1.07 ppm on soil samples from 2 sites (Nigg, 1986). These residues have been proven to affect terrestrial microorganisms (Martinez-Toledo, 1988).

1.3. Ectomycorrhizal fungi and forest

Ectomycorrhizal (ECM) fungi form association between the roots of woody plants and belong to the Phyla Basidiomycota, Ascomycota and Zygomycota. The ectomycorrhizas consist of a sheath or mantle of hyphae covering the root tip and form a network around the root cortical cells (Wang and Qiu, 2006).

Many tree species in forest ecosystems live in symbiosis with ECM fungi (Fig. 1.3) (Smith and Read, 2008). For the ECM symbiosis the number of plant and fungal species involved is currently estimated to be ca. 6,000 and 20,000–25,000, respectively (Rinaldi *et al.*, 2008; Brundrett, 2009). Based on taxonomic and ecological studies an estimated 86% of terrestrial plant species acquire mineral nutrients via mycorrhizal root symbionts (Brundrett, 2009) and therefore ECM fungi play an important role in seedling establishment and tree growth in habitats across the globe.

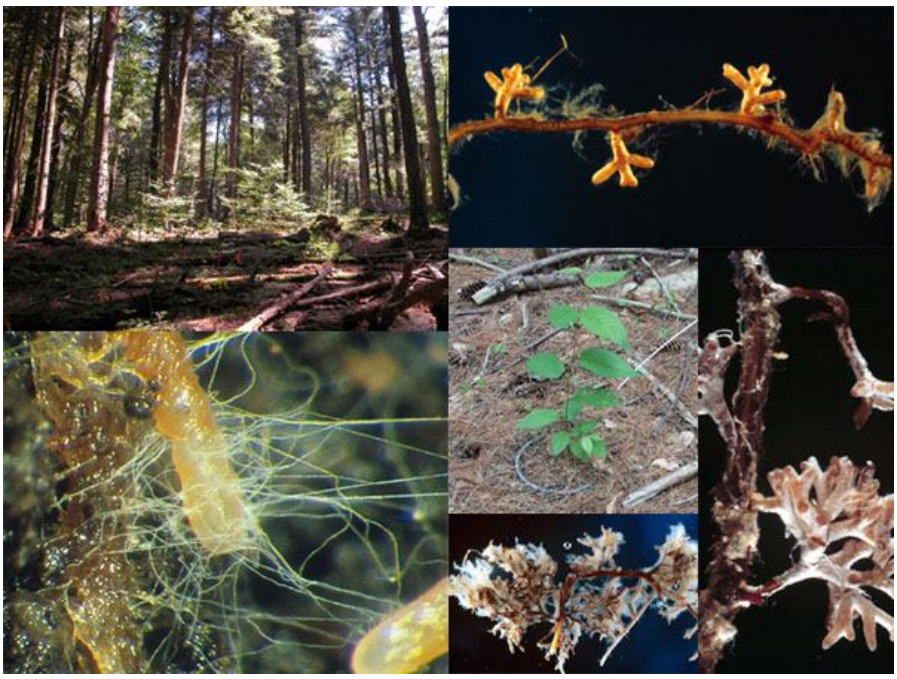

Fig. 1.3. Mycorrhizal forms in association to roots of higher trees.

They are also important for the delivery of carbon to soil and are responsible for a substantial component of forest-soil carbon fluxes (Söderström, 1992; Högberg *et al.*, 2001; Högberg & Högberg, 2002; Godbold *et al.*, 2006; Hobbie, 2006). ECM fungi are key elements of forest nutrient cycles and as strong drivers of forest ecosystem processes (Read *et al.*, 2004).

Mycorrhizas usually incur the greatest benefit under conditions of high stress (Dickie *et al.,* 2002; Dickie and Reich, 2005), principally those where soil nutrients are limiting (Treseder and Allen, 2002; Schwartz and Hoeksema, 2003; Jones and Smith, 2004; Johnson *et al.*, 2006), depending upon the plant and fungal species involved (Johnson *et al.*, 1997; Klironomos, 2003; Jones and Smith, 2004). Mycorrhizas also occur under conditions of toxic stress, including air pollution (Ruotsalainen and Kozlov, in press) and heavy metal contaminated habitats (Van Duin *et al.*, 1991; Wilkinson and Dickinson, 1995).

Pesticide effects on soil microbes have been tested mostly in agricultural soils (Atlas *et al.* 1977, Schüepp and Bodmer, 1991; Tu, 1992, 1993) and only few studies have examined their effects on forest soils (Ingham *et al.*, 1986, Colinas *et al.*, 1994). The effect of pesticides in ECM development is controversial. The development of ectomycorrhizas of pine seedlings in forest nurseries is affected by the herbicides, 2,4-D and trifuralin (Iloba, 1980) and the fungicide triadimefon (Marx *et al.*, 1986). Manninen *et al.* (1998) showed in a field trial with pine seedlings that the fungicides copper oxychloride and propiconazole could both reduce soil microbial activity and impair the development of ectomycorrhizas. On the other hand, pesticide stimulation on the formation of ectomycorrhizas has been reported (Kais *et al.*, 1981; Marx and Rowan, 1981; Pawuk and Barnett, 1981; Trappe *et al.*, 1984).

1.4. Scope of the work and objectives

Despite all information regarding DFB persistence in soil, to our knowledge no terrestrial plant toxicity studies and no bioassays for herbicidal activity of DFB were encountered in either the published literature or in the more recent U.S. EPA reports. (USDA, 2004).

Due to its potential toxicity, commercial companies of mycorrhizal inoculant products classify DFB as an incompatible insecticide (Biocult, 2010).

Despite the controversy regarding persistence of DFB in soil, its effect on higher trees has not been studied. Since DFB is widely used in forest ecosystems it is fundamental to know its toxic potential to trees, mycorrhizal fungi and their symbiosis.

The objectives of this study were to assess the effect of DFB on the germination and development of *P. pinaster* seedlings and ECM fungi under laboratory conditions and to study the possible protective role of ECM fungi against the effects of DFB.

2. *Materials and Methods*

2.1. Fungal inhibition by diflubenzuron

2.1.1. Fungal growth

Fungal strains were maintained 15 days at 25 ºC on Melin-Norkrans (MMN) agar medium modified by Marx (1969). After optimal growth, new MMN Petri dishes were inoculated (5 replicates) with 5 mm diameter pellets cut from the leading edge of colonies of *Pisolithus tinctorius* PT-00 fungal strain. The tested concentrations of diflubenzuron were 0, 0.01, 0.1, 1, 10 and 100 mg/L. The size of the colonies was measured at the 9th, 15th and 21st day. Fungal growth was expressed as the mean of the colony radius of four points as shown in Fig. 2.1.

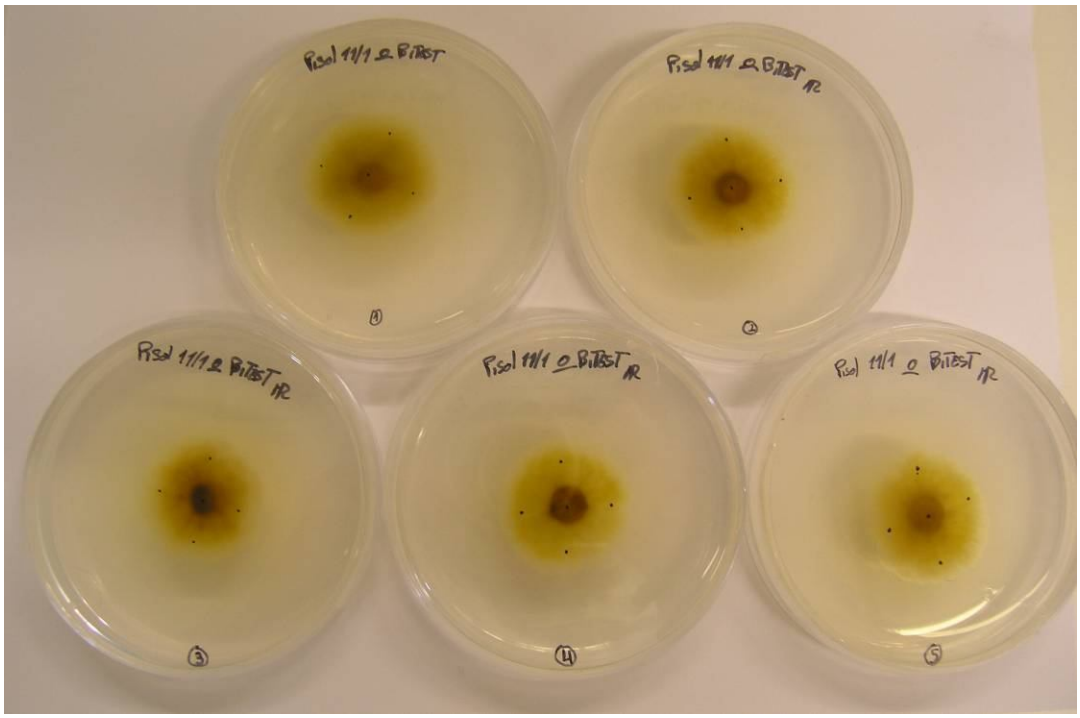

Fig. 2.1. Control group of *Pisolithus tinctorius* agar cultures with black dots showing colony radius measurements.

2.2. Germination and growth of *Pinus pinaster* exposed to diflubenzuron in sand

2.2.3. Plant and soil

The soil used was composed of a mixture of sand (95%) with potting soil (5%) (Table 2.1) containing approximately 3% of organic matter. It was autoclaved twice on consecutive days. DFB solutions were spiked in soil with 200 ml of acetone as solvent (10% of soil volume) which was afterward extracted by evaporation in a fume hood for 30 minutes. The soil was then distributed to pots of 300 ml. The tested concentrations of DFB were 0, 0.01, 0.1, 1, 10 and 100 mg/L.

P. pinaster seeds (Table 2.1) were rinsed overnight in running tap water, surface disinfected by shaking for 60 min in 30% hydrogen peroxide, and finally washed in 2 L of sterile deionised water with agitation.

Table 2.1. Chemical and physical characteristic of sand, potting soil, fertiliser and *Pinus pinaster* seeds used in all the studies.

Characteristics	Sand (Areipor, Portugal)	Commercial potting soil (empresa, Pais)	Fertilizer (BASF, Germany)	*Pinus pinaster* seeds (Cenasef)
Physical	- Apparent density: 1.52 g / mL - Real density: 2.62 g / ml - Hardness: 7 mohs - pH: 7 - Average diameter: 750 micron	- pH: 6-6.5	-	- Lot no. 2 / 08 - Major certificate No: 077/0708 - CNMB PnB 1001 - Place: Melgaço - Year: 2007
Chemical	- Silica: 99.18% - Alumina: 0.527% - Iron Oxide: 0.036% - Titanium Oxide: 0.080% - Calcium Oxide: 0.010% - Magnesium oxide: 0.010% - Oxide Sodium: 0.020%	- Organic matter: 62-74% - Nitrogen: 1.3% - Phosphorus: 0.26% - Magnesium: 0.14% - Iron: 0.6% - Calcium: 2.69% - H_20: 40%	- Nitrogen: 12% - Phosphorus oxide: 12% - Potassium oxide: 17% - Magnesium oxide 2% - Sulphite: 15% - Boron: 0.02% - Iron: 0.1% Zinc: 0.01%	-

Seeds were kept in a controlled room (20 ºC, 70% humidity, photoperiod of 16 hours of light and light intensity of 450 µmol m^{-2} s^{-1}). Seeds were placed in contact with soil treated with DFB and monitored for 21 days after 50% emergence of the seedlings in the control group following the OCDE guidelines for testing chemicals in plants (OCDE, 2006). Each treatment had six replicates with four seeds (Fig. 2.2). The pots were watered twice per week with 25 ml of deionised water.

Fig. 2.2. Germination test of *Pinus pinaster* seed resulting in seedling formation.

This experiment began on the 10th of November of 2009, 50% germination of the control group was obtained on the 31st of November of 2009, and it ended on the 20th of December of 2009. Germination rate was calculated as number of seeds germinated divided by 4, the number of initial seeds. Shoot, root and primary root length were measured with a ruler and secondary root length was measured using the linear intercept method (Brundrett, 1996) to evaluate the toxic potential of DFB on different parts of the seedlings.

2.3. Effect of diflubenzuron on the development of *Pinus pinaster* seedlings inoculated with *Pisolithus tinctorius*

2.3.1. Fungus

Liquid inoculum of MMN was prepared by inoculating with agar pellets of *Pisolithus tinctorius*. These cultures were maintained for 1 month until optimum growth was reached. Fungal biomass was then grinded and transferred to 1 L Erlenmeyer flasks containing 200 ml of MMN. These new cultures were maintained in 1 L Erlenmeyer flasks for 15 days at 25 ^{0}C on a dark growth chamber (Fig. 2.3) The inoculum was homogenised in a grinder immediately before being applied to the seedlings. Inoculation was performed by adding 5 ml of inoculum to the base of each seedling.

Fig. 2.3. *Pisolithus tinctorius* inoculum for inoculation in *Pinus pinaster* seedlings.

2.3.2. Plant and soil

The soil used was composed of a mixture of sand (95%) with potting soil (5%) (Table 2.1) obtaining approximately 3% of organic matter. It was autoclaved twice on consecutive days. The DFB solutions were spiked in soil with 200 ml of acetone as solvent (10% of soil volume) which was afterwards extracted by evaporation in a fume hood for 30 minutes. The soil was then distributed in pots of 300 ml. The tested concentrations of DFB were 0, 0.01, 0.1, 1, 10 and 100 mg/L.

P. pinaster seeds (Table 2.1) were rinsed overnight in running tap water, surface disinfected by shaking for 60 min in 30% hydrogen peroxide and washed in 2 L of sterile deionised water with agitation. Seeds were germinated in water agar medium (10 g/L) during 15 days (Fig. 2.4).

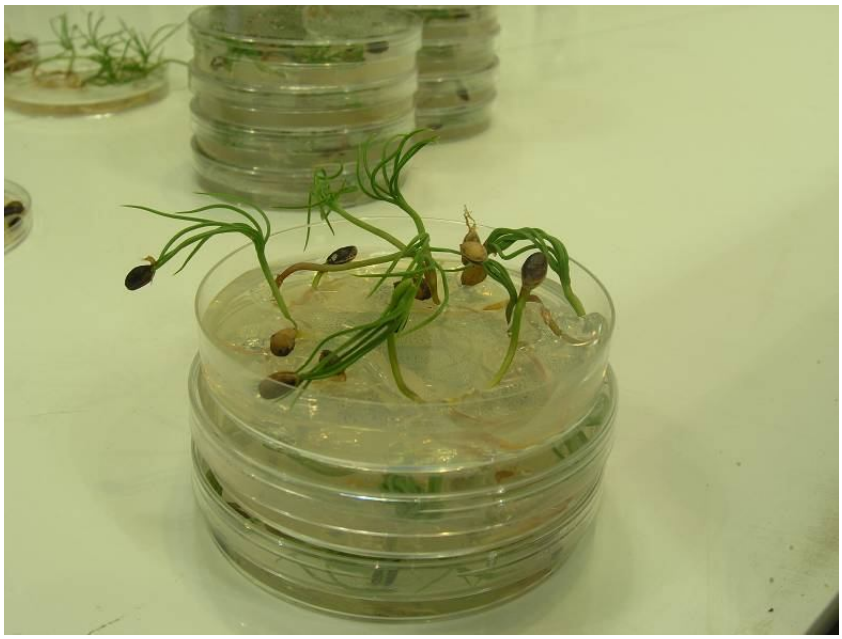

Fig. 2.4. Germination of *Pinus pinaster* in water agar medium.

After germination, 140 *P. pinaster* seedlings with approximately the same size were chosen and placed in contact with soil containing DFB.

The planting was done between the 26th and the 29th of October of 2009, the inoculation on the 6th of November 2009 and the placement of 200 mg of fertiliser (Table 2.1) on the 20th November of 2009 and it ended on the 4th of March of 2010.

The seedlings grew for 5 months in the previously described plant growth room (Fig. 2.5).

Shoot, root and primary root length were measured with a ruler and the secondary root length was measured through the linear intercept method (Brundrett, 1996). Roots were removed for assessing the ECM colonisation rate and the number of ectomycorrhizas per cm of root (mycorrhizal expansion rate) by the linear intercept method (Brundrett, 1996). Shoot and root biomass were determined after drying the plant material at 60 ºC to obtain the dry weight parameters.

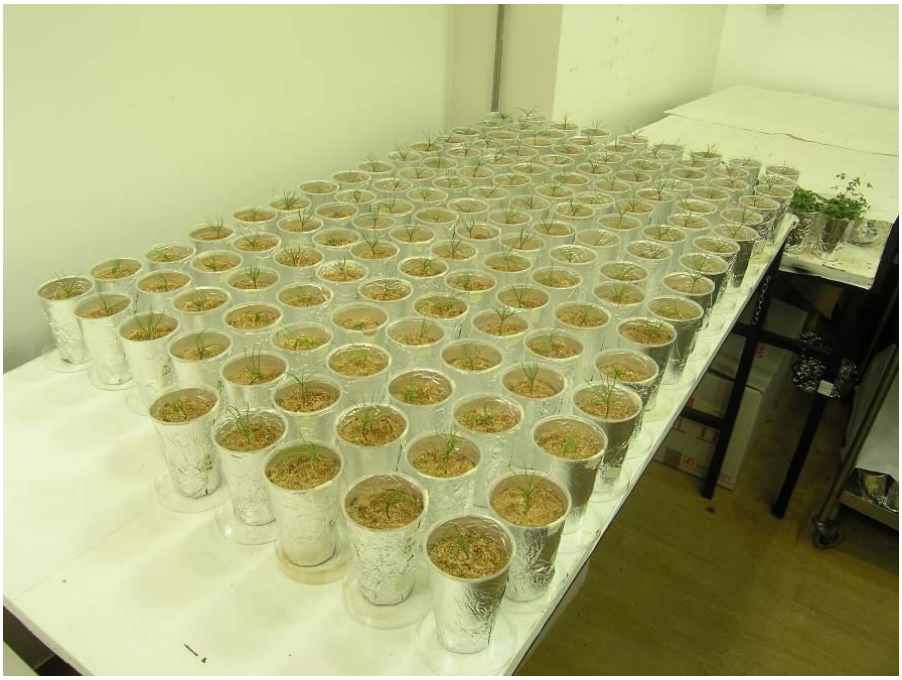

Fig. 2.5. *Pinus pinaster* seedlings in the plant growth room.

2.3.3. Activity of SOD and CAT enzymes

To isolate fractions of solute proteins, 0.5 g of root tissue were thoroughly ground with a cold mortar and pestle in an ice bath until no fibrous residue could be seen. To 1 g of root tissue 1 ml of grinding medium was added. The grinding medium (1.5 ml/g fresh weight) consisted of 0.05 M phosphate buffer (pH 7.2), 0.1 mM ethylenediamine tetraacetic acid (EDTA), 5% (w/v) polyvinylpolypyrrolidone (PVPP) and 0.3% (v/v) Triton X-100. The homogenate was centrifuged at 12000g for 1 min to remove all non-soluble material.

The protein content of all extracts was determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used as a standard. To visualise the activity of superoxide dismutase (SOD) and catalase (CAT) natives, polyacrylamide gel (PAGE) was performed on 13% gels for SOD, or 8% gels for CAT, using the Laemmli (1970) buffer system without sodium dodecyl sulfate (SDS). Each extract was applied to the gels at concentration of 60 μg of protein. Electrophoresis was carried out at 4 ºC and 100 V for approximately 2 hours. Enzyme activity was visualised according to Beauchamp and Fridovich (1971) for SOD, and according to Claire *et al.* (1984) for CAT. Identification of different SOD forms was performed using the procedure of gel incubation in a staining buffer containing 2 mM KCN. Four replicates were used for each treatment. Quantification

of the activity of SOD and CAT was performed by densitometry using Quantity One software (Bio-Rad).

2.4. Statistical Analysis

Data were statistically analysed by SPSS 17.0 software package for Windows: Two–way and one-way ANOVA with Duncan test ($P<0.05$), for multiple sample comparison were applied after normality and homogeneity of variance were verified.

3. *Results*

3.1. Fungal inhibition by diflubenzuron

The inhibitory effect of DFB on fungal cultures of *P. tinctorius* was assessed at 5 different concentrations - from 0.01 to 100 mg/L - of the compound, through measurements of colony growth in MMN media (Fig. 3.1.1). The inhibitory effect was in the same order of magnitude for the range of concentrations tested (Fig. 3.1.2). Inhibition rates were 14-20%, 27-50% and 22-41% at the 9th, 15th and 21st days, respectively.

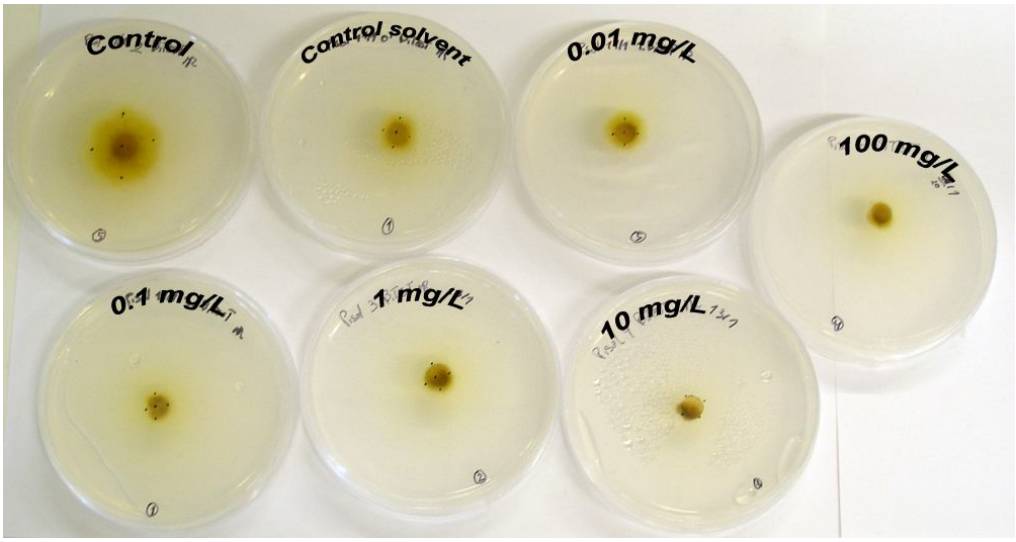

Fig. 3.1.1. Agar plates of 2 control groups (with and without solvent) and 5 different concentrations – from 0.01 to 100 mg/L - of diflubenzuron.

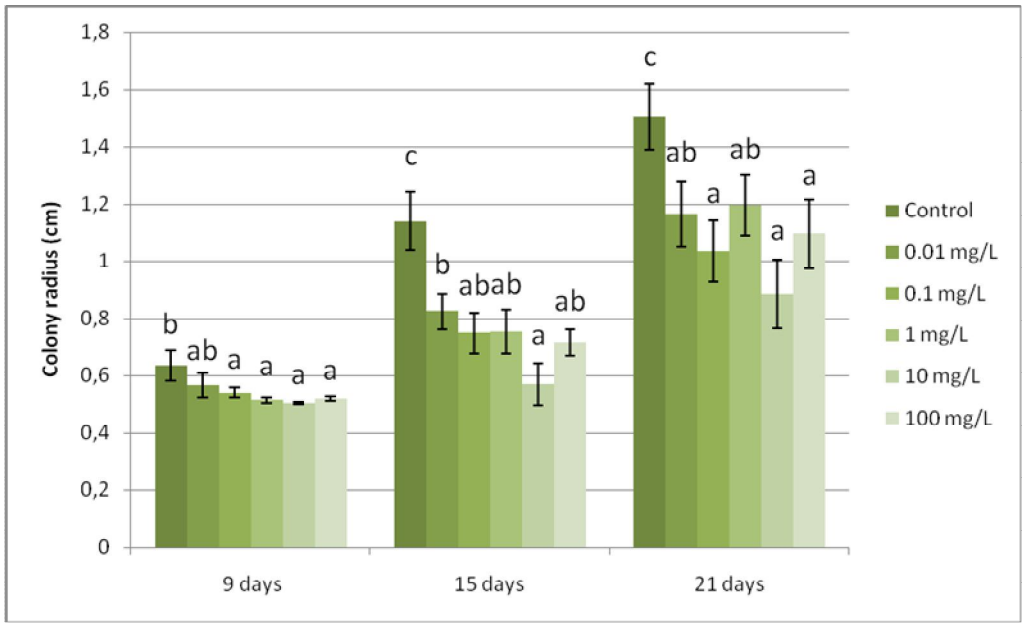

Fig. 3.1.2 Mean colony radius (cm) of *Pisolithus tinctorius* measured at the end of the experiment in the presence of 0, 0.01, 0.1, 1, 10 and 100 mg/L of diflubenzuron. Values are means of five replicates. Error bars indicate standard error of mean. Columns marked with different letters are significantly different according to Duncan's multiple range test (P<0.05).

3.2 Germination and growth of *Pinus pinaster* exposed to diflubenzuron in sand

The effect on DFB on seedling emergence and early growth of *P. pinaster* upon exposure to soil spiked with the compound was assessed. Germination rate, shoot growth, root length and root architecture (primary and secondary root length) were assessed to evaluate the toxic potential of DFB on different parts of the plant.

P. pinaster seeds were placed in contact with soil spiked with DFB and evaluated for effects following 21 days after 50% emergence of the seedlings in the control group. The used solvent did not affect any test being suitable the for plant experiments.

3.2.1 Germination rate of *Pinus pinaster*

After 21 days of exposure, seedling emergence showed a homogeneous germination in all treatments, from 0 to 100 mg DFB/kg soil, with no interference of DFB revealed, leading to 80-100% of rate of germination (Fig. 3.2).

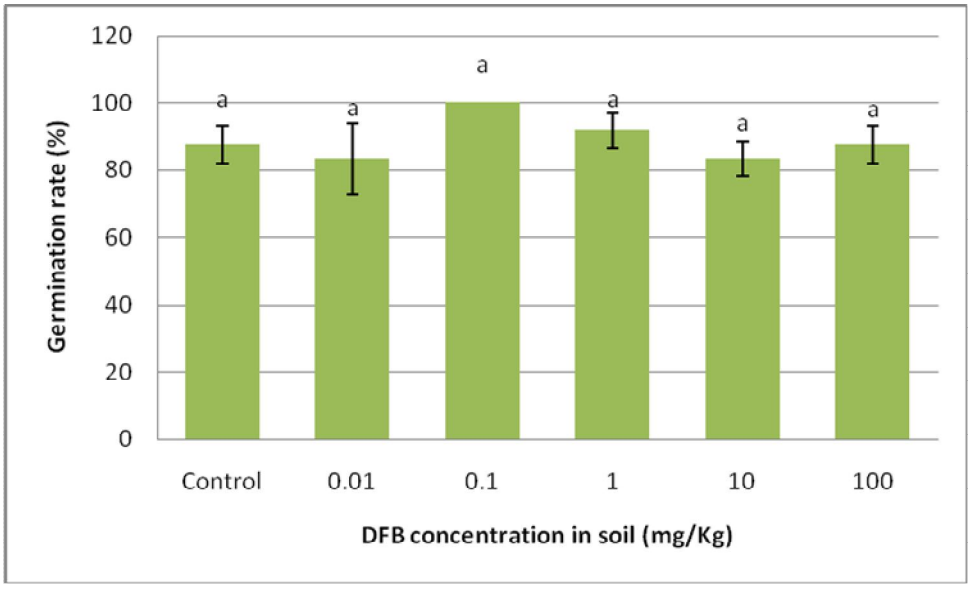

Fig. 3.2. Mean germination rate (%) determined at the end of the experiment on *Pinus pinaster* in the presence of 0, 0.01, 0.1, 1, 10 and 100 mg/Kg of diflubenzuron. Values are the mean of 24 replicates. Error bars indicate standard error of mean. Columns marked with different letters are significantly different according to Duncan's multiple range test ($P<0.05$).

3.2.2. Pinus pinaster root and shoot measurements

The shoot length was measured after 21 days. DBF did not affect the growth of *P. pinaster* (Fig. 3.3). No significant growth differences were observed between seedlings exposed to DFB concentrations and the control. However, a reduced growth in root length of plants treated with DFB was found (Fig. 3.3). The inhibitory effect was observed at 1, 10 and at 100 mg DFB/kg soil with inhibitions rates of 25, 44 and 59% respectively.

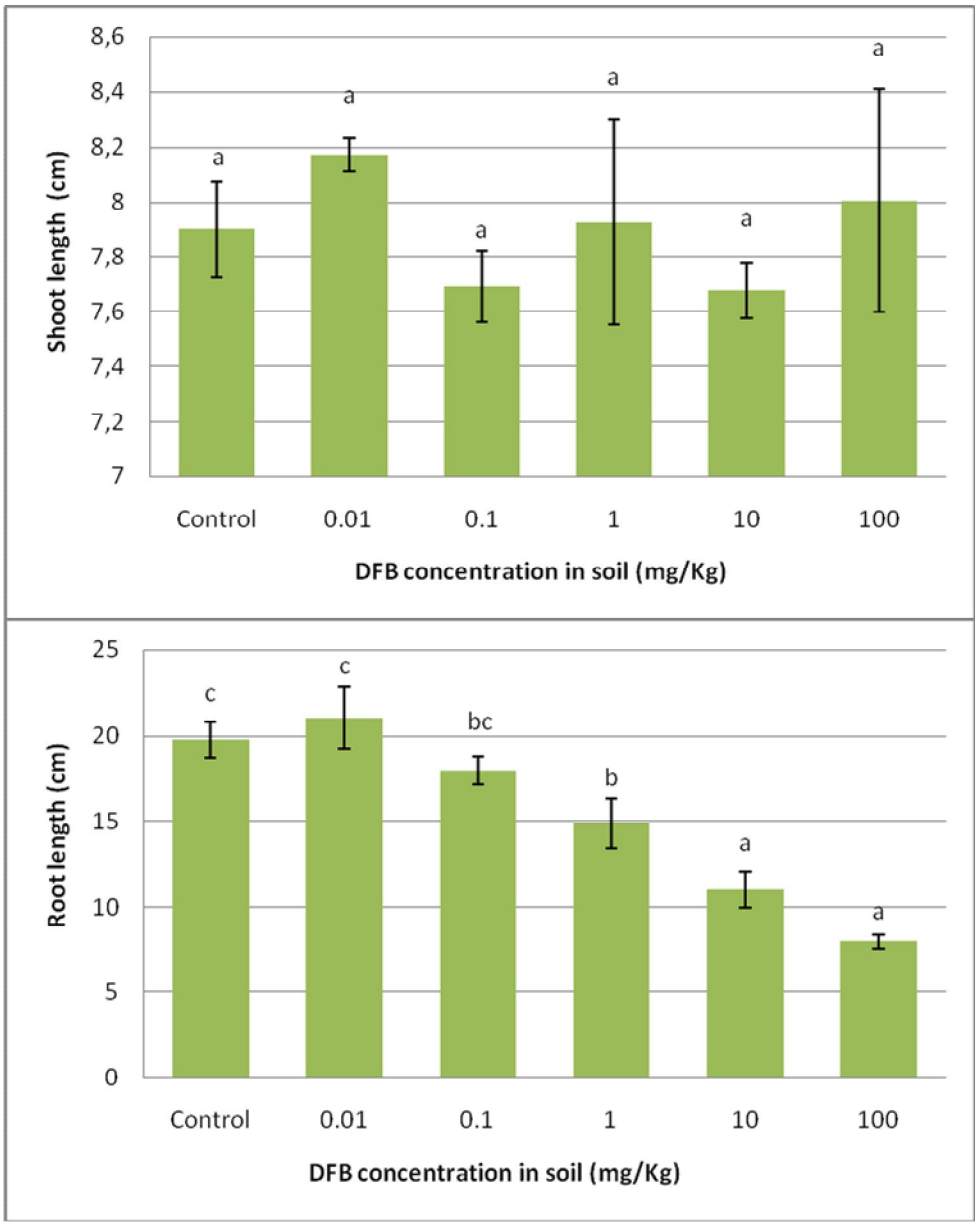

Fig. 3.3. Mean shoot and root length (cm) measured at the end of the experiment on *Pinus pinaster* in the presence of 0, 0.01, 0.1, 1, 10 and 100 mg/Kg of diflubenzuron. Values are the mean of 24 replicates. Error bars indicate standard error of mean. Columns marked with different letters are significantly different according to Duncan's multiple range test ($P<0.05$).

3.2.3 Root architecture analysis

Noting the previously root inhibition, a more detailed analysis on root growth was performed to evaluate the extent of the toxic effects on the root architecture. The length of the primary and secondary roots was measured separately.

Measurements of the length of primary roots showed almost the same inhibition effect as seen on total root length. The toxic doses which induced the growth inhibitory effects on the primary roots were 1, 10 and 100 mg DFB/kg soil, with inhibitions rates of 19, 47 and 57%, respectively (Fig. 3.4).

In the case of secondary root length, lower doses of DFB could reduce their growth in *P. pinaster*. Even at the lowest concentration tested, 0.1 mg DFB/kg soil, significant differences were found when compared to the control treatment, showing that small doses of DFB could affect the root architecture by decreasing the secondary root length. The secondary root inhibition rates at 0.1, 1, 10 and 100 mg DFB/kg soil were 39, 59, 60 and 72%, respectively (Fig. 3.4).

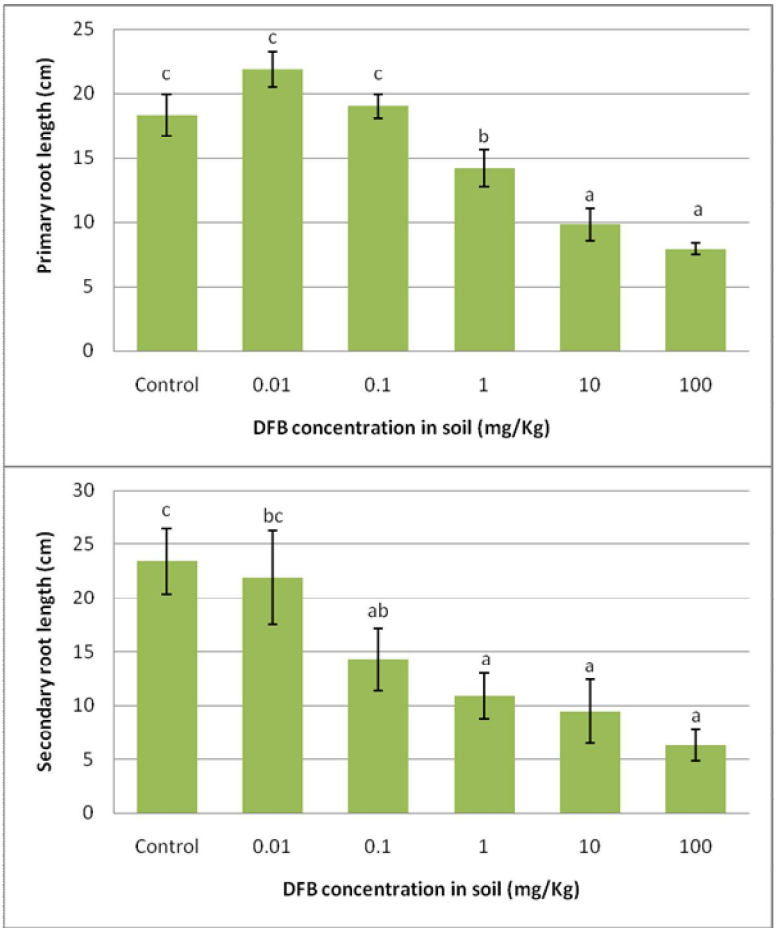

Fig. 3.4. Mean primary and secondary root length (cm) measured at the end of the experiment on *Pinus pinaster* in the presence of 0, 0.01, 0.1, 1, 10 and 100 mg/Kg of diflubenzuron. Values are the mean of 24 replicates. Error bars indicate standard error of mean. Columns marked with different letters are significantly different according to Duncan's multiple range test ($P<0.05$).

3.3 Effect of diflubenzuron on the development of *Pinus pinaster* seedlings inoculated with *Pisolithus tinctorius*

In order to evaluate how the fungus-plant symbiosis would respond to the exposure to a toxic, an experiment combining the toxic doses and the state of inoculation of *P. pinaster* trees was carried out. Inoculated and non-inoculated plants were exposed to different concentrations of toxic during five months. Shoot growth, root architecture (primary and secondary root lengths), fresh and dry weight of shoot and root, ECM colonisation rate, mycorrhizal expansion rate and activities of SOD and CAT were assessed. The used solvent did not affect any test being suited for plant experiments.

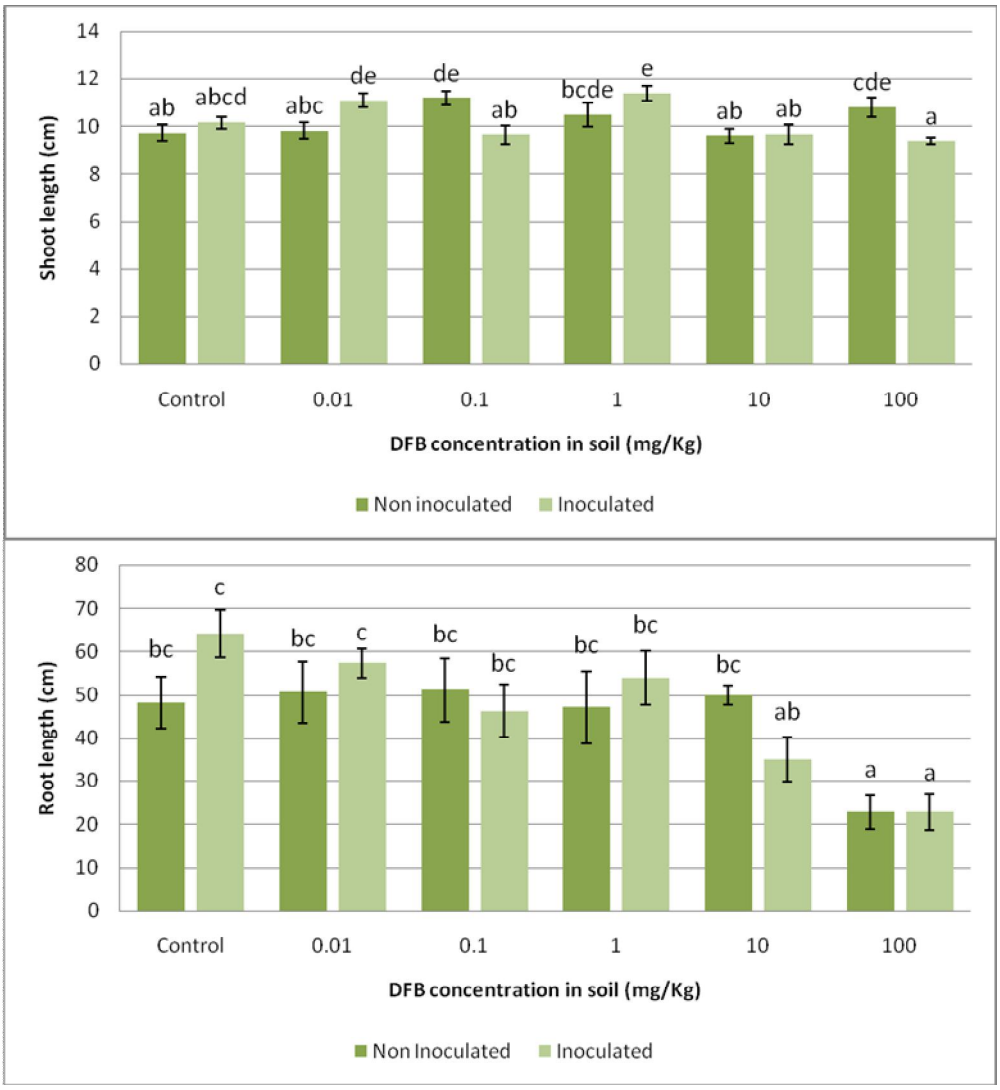

Fig. 3.5. Mean shoot and root length (cm) measured at the end of the experiment on *Pinus pinaster* in the presence of 0, 0.01, 0.1, 1, 10 and 100 mg/Kg of diflubenzuron, with and without the inoculation with *Pisolithus tinctorius*. Values are the mean of 10 replicates. Error bars indicate standard error of mean. Columns marked with different letters are significantly different according to Duncan's multiple range test ($P<0.05$).

3.3.1 Shoot and root analysis of *Pinus Pinaster* inoculated with *Pisolithus tinctorius*

The shoot length measurements showed no inhibition effect of DFB. In the inoculated treatments of 0.01 and 1 mg DFB/kg soil, shoot length was significant higher when compared with non-inoculated control plants (Fig. 3.5). The same was observed in non-inoculated plants challenged with 0.1 mg DFB/kg soil. The inoculation influenced the growth of the shoot at 0.01, 0.1 and 100 mg DFB/kg soil. Inoculated plants had lower growth than non-inoculated plants when exposed to the same dose, except at 0.01 mg DFB/kg soil.

Regarding root length, at 100 mg DFB/kg root inhibition was observed (Fig. 3.5). The inoculation did not affect the growth of *P. pinaster* roots (Fig. 3.5).

The analysis of root architecture showed differences between treatments. The primary root length measurements showed again that 100 mg DFB/kg is a root inhibition dose and the inoculation factor did not cause any difference in growth (Fig. 3.6). The analysis of the length of the secondary roots showed that at 10 and 100 mg DFB/kg inoculated plants were significantly lower comparing with non-inoculated control treatment (Fig. 3.6).

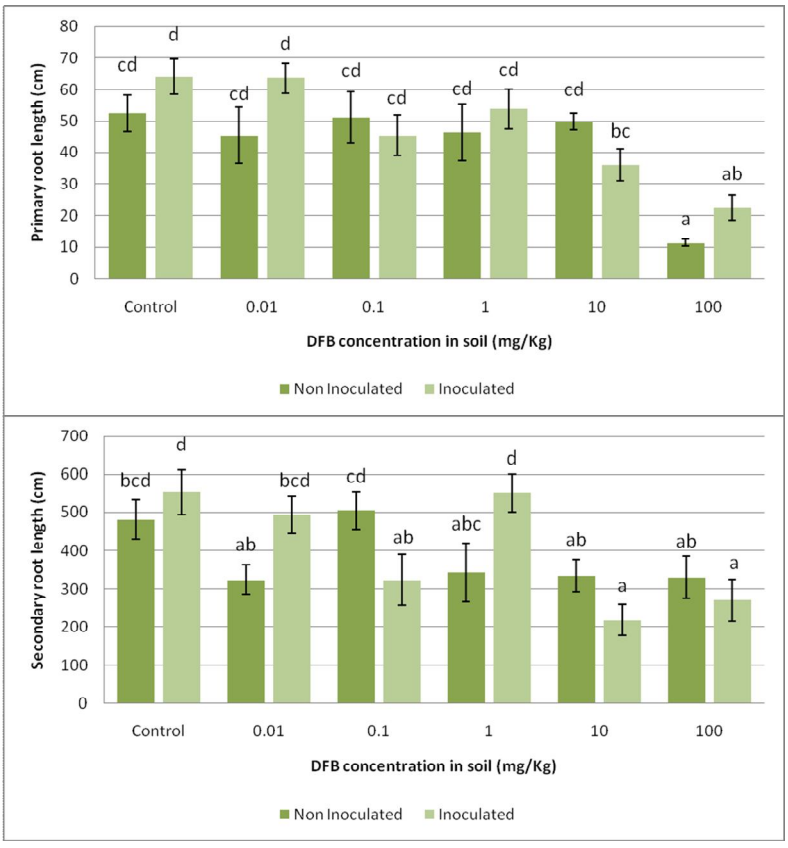

Fig. 3.6. Mean primary and secondary root length (cm) measured at the end of the experiment on *Pinus pinaster* in the presence of 0, 0.01, 0.1, 1, 10 and 100 mg/Kg of diflubenzuron, with and without the inoculation of *Pisolithus tinctorius*. Values are the mean of 10 replicates. Error bars indicate standard error of mean. Columns marked with different letters are significantly different according to Duncan's multiple range test ($P<0.05$).

3.3.2 Dry weight from shoot and root of *Pinus pinaster* inoculated with *Pisolithus tinctorius*

Inoculated plants exposed to 10 mg DFB/kg and 100 mg DFB/kg had significantly lower shoot dry weight than those from non-inoculated control group (Fig. 3.8). The same happened for all non-inoculated treatments except for 0.1 mg DFB/kg soil.

Root dry weight from 100 mg DFB/kg inoculated plants was significantly lower when compared with the control plants. The inoculation factor did not affect the dry weight of shoot and root of *P. pinaster* seedlings except at 0.01 mg DFB/kg in shoot dry weight.

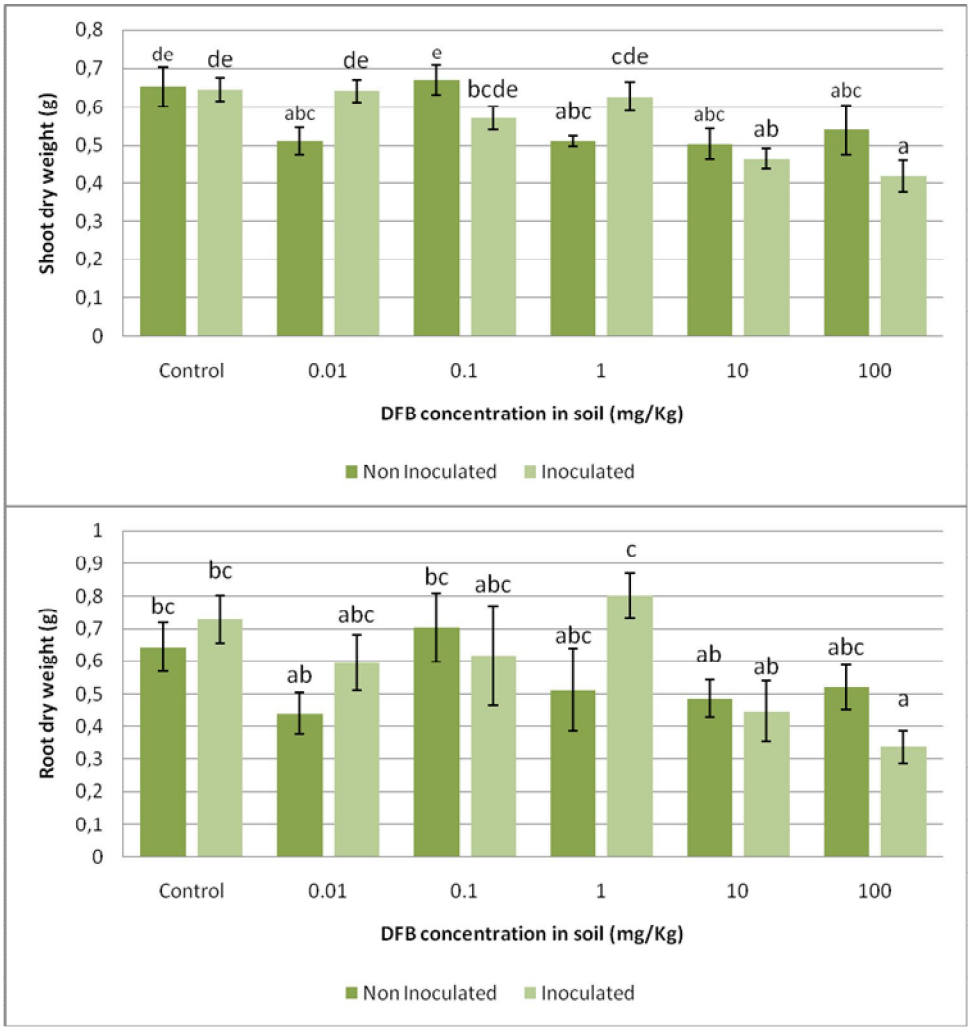

Fig. 3.8. Mean shoot and root dry weight (g) at the end of the experiment on *Pinus pinaster* in the presence of 0, 0.01, 0.1, 1, 10 and 100 mg/Kg of diflubenzuron, with and without the inoculation of *Pisolithus tinctorius*. Values are the mean of 10 replicates. Error bars indicate standard error of mean. Columns marked with different letters are significantly different according to Duncan's multiple range test ($P<0.05$).

3.3.3 Ectomycorrhizal colonisation analysis of *Pinus pinaster* seedlings inoculated with *Pisolithus tinctorius*

For the ECM colonisation rate and mycorrhizal expansion rate a significantly higher rate in all inoculated treatments was observed. However the ECM colonisation rate was not significantly different between inoculated treatments (Fig. 3.9). The mycorrhizal expansion rate revealed that 100 mg DFB/kg dose could enhance even more mycorrhizal expansion than those with lower doses of DFB (Fig. 3.9).

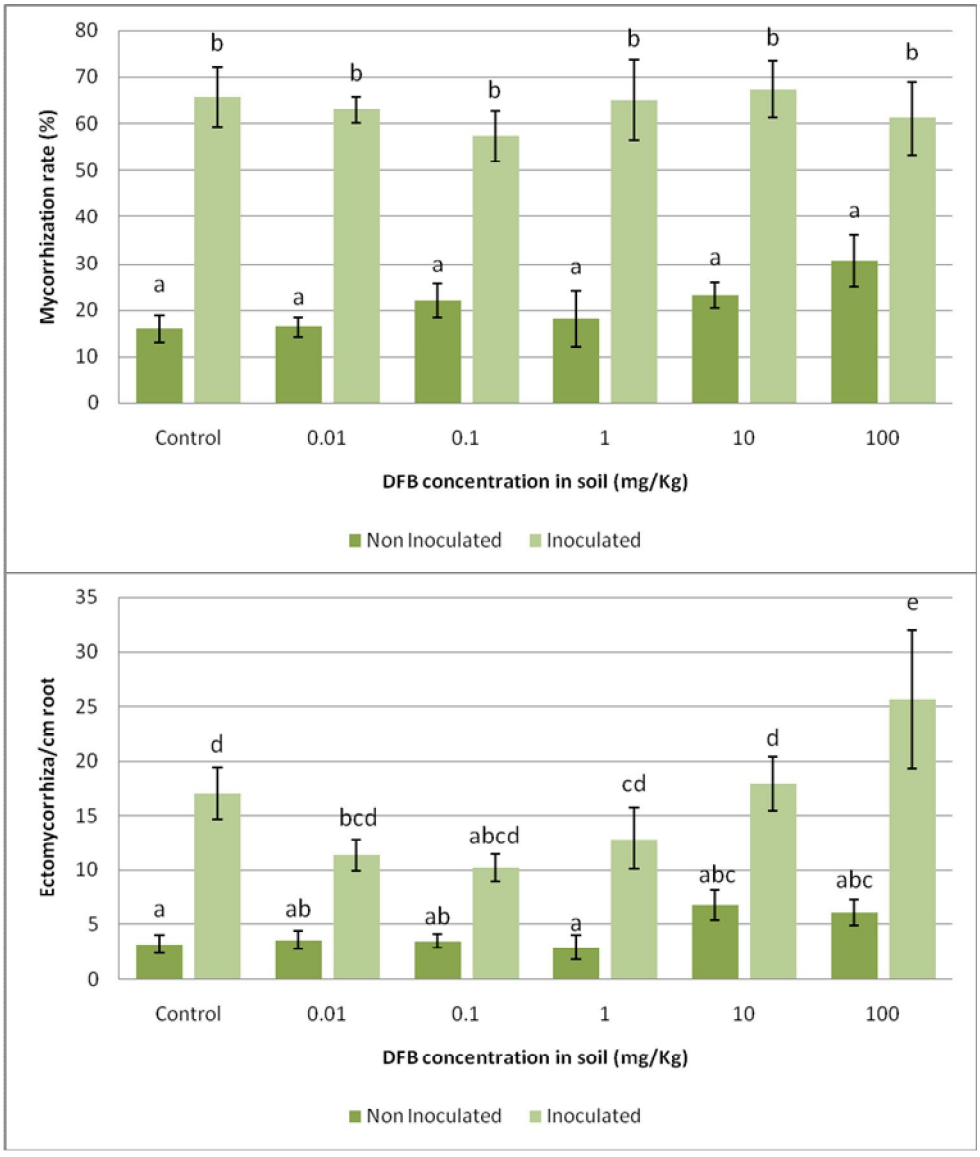

Fig 3.9. Mean ectomycorrhizal colonisation rate (%) and mycorrhizal expansion rate (ectomycorrhiza/cm of root) at the end of the experiment on *Pinus pinaster* in the presence of 0, 0.01, 0.1, 1, 10 and 100 mg/Kg of diflubenzuron, with and without the inoculation of *Pisolithus tinctorius*. Values are the mean of 10 replicates. Error bars indicate standard error of mean. Columns marked with different letters are significantly different according to Duncan's multiple range test ($P<0.05$).

3.3.4 Activity of SOD and CAT enzymes

The activities of SOD and CAT in the root were very similar in inoculated and non-inoculated treatments except for SOD activity at 10 mg DFB/kg (Fig. 3.10). At 10 mg DFB/kg the enzime activities were higher than in the non-inoculated control group. For SOD activity, non-inoculated 10 mg DFB/kg was the treatment that stood out, in contrast to the CAT activity, where the inoculated 10 mg DFB/kg treatment was the one that was significantly higher.

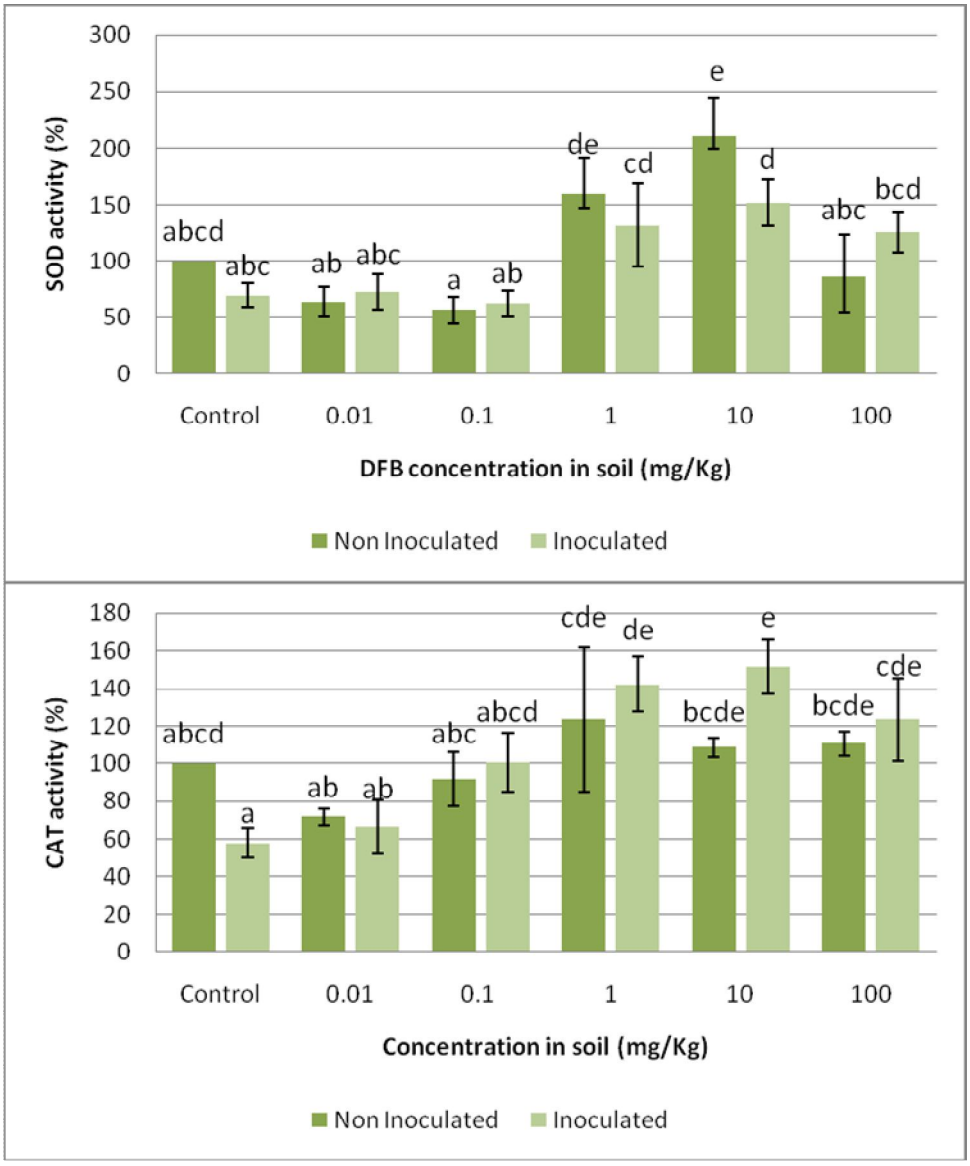

Fig. 3.10. Mean activities of SOD and CAT at the end of the experiment on *Pinus pinaster* in the presence of 0, 0.01, 0.1, 1, 10 and 100 mg/Kg of diflubenzuron, with and without the inoculation of *Pisolithus tinctorius*. Values are the mean of 4 replicates. Error bars indicate standard error of mean. Columns marked with different letters are significantly different according to Duncan's multiple range test ($P<0.05$).

Grouping the plants by their state of inoculation showed that 1 and 10 mg DFB/kg doses increased the activity of SOD in both non-inoculated and inoculated *P. pinaster* seedlings (Table 3.1). The activity of CAT was significantly affected in the inoculated treatments at 1, 10 and 100 mg DFB/kg soil (Table 3.1).

Table 3.1. Mean activities of SOD and CAT at the end of the experiment on *Pinus pinaster* in the presence of 0, 0.01, 0.1, 1, 10 and 100 mg/Kg of diflubenzuron, with and without the inoculation of *Pisolithus tinctorius*. Values are the mean of 4 replicates.

Treatments		Control	0.01 mg DFB/kg soil	0.1 mg DFB/kg soil	1 mg DFB/kg soil	10 mg DFB/kg soil	100 mg DFB/kg soil
SOD	Non inoculated	100±0a	64±13a	57±11a	160±32b	210±34b	86±37a
	Inoculated	69±10xy	72±16xy	62±11x	132±37z	152±21z	126±18yz
CAT	Non inoculated	100±0ab	72±4a	92±15ab	123±38b	109±5ab	111±7ab
	Inoculated	58±8x	67±14x	100±16xy	142±15yz	152±14z	123±22yz

4. *Discussion*

4.1 Fungal inhibition by diflubenzuron

The results showed differences between control group and DFB treatments in *P. tinctorius* growth. The ECM fungus suffered inhibition in all treatments with DFB.

Concerning toxicity of DFB upon fungi, Booth (1978) found no inhibition of fungal growth in several genera of fungi (*Aspergillus*, *Fusarium*, *Rhizopus*, *Trichoderma*) at concentrations of up to 100 ppm in growth media. Inhibition of *Rhizoctonia solani*, another terrestrial fungus, has been noted at 300 ppm (Townshend *et al*., 1983). The degradation of DFB by soil microorganisms suggests that this compound is not toxic to them (EPA, 1997). DFB products are used as insect growth regulators and act by blocking the synthesis of chitin (EPA, 1997). Fungi do contain chitin in their cell walls and thus could be a potential target for this fluorinated compound. (USDA, 2004). This blocking effect could be the responsible for the fungal inhibition. Other chitin-synthase inhibitors compounds were already demonstrated to decrease or totally inhibit the growth of mycorrhizal fungi (Bago *et al.*, 1996).

4.2 Germination and growth of *Pinus pinaster* exposed to diflubenzuron in sand

4.2.1. Germination rate of *Pinus pinaster*

Plant toxicity studies and bioassays for herbicidal activity of DFB were encountered previous studies or reports of environmental agencies (USDA, 2004).

The exposure to DFB did not affect the germination of *P. pinaster*. This is consistent with similar studies carried out with trifluoroacetic acid (TFA), another fluorinated organic compound, in which no inhibitory effect on the germination of plants even at the maximum concentration tested (1000 mg DFB/kg soil) was found (Thompson and Windeatt, 1994; Boutonnet *et al*., 1999). This may be explained by the thick protective shell of coniferous seeds.

4.2.2 Pinus pinaster root and shoot

The shoot length parameter indicated no inhibition of DFB towards *P. pinaster* growth. Davison and Pearson (1997) verified no effect of TFA on the height and leaf number of seven species of terrestrial plant at the maximum concentration tested of 100

mg DFB/kg after 3 weeks of exposure. However, more severe symptoms were reported when the TFA solution was able to reach the roots.

Despite the fact that DFB showed no inhibition effect on shoot length, reduced growth in total and primary root length treated with 100 mg DFB/kg soil DFB doses were found. This is consistent with the study of Emerich (1997) which showed TFA toxic effects on the development of plants at 10 and 100 mg TFA/kg concentrations.

4.3. Effect of diflubenzuron on the development of *Pinus pinaster* seedlings inoculated with *Pisolithus tinctorius*

4.3.1. Shoot length and root architecture analysis

Shoot length of inoculated seedlings was stimulated at 0.01 and 1 mg DFB/kg soil exposure. At 0.1 and 100 mg DFB/kg, non-inoculated seedlings were also stimulated. Similar results were previously observed (Smit *et al*., 2009), when fluorinated organic compound doses of 0.625 and 2.5 ppm had the same effect in *Phaseolus vulgaris* and *Zea mays*. The inoculated treatments of 0.01 mg DFB/kg had higher shoot length than those non-inoculated, probably receiving more nutrients and water providing better growth as evidenced in other studies (Wingler *et al*., 1996; Prima-Putra *et al.*, 1999).

A reduced growth in total and primary root length treated with 100 mg DFB/kg was found, similar to that reported by Smit *et al*. (2009) with other fluorinated organic compounds in concentrations that reached up to 160 ppm.

4.3.2. Dry weight of shoot and root of *Pinus pinaster*

Shoot dry weight of inoculated seedlings exposed to 10 and 100 mg DFB/kg soil was significantly lower than that from non-inoculated control group. The same effect was observed for the non-inoculated treatments at 0.01, 1, 10, 100 mg DFB/kg soil. Ahemad and Khan (2010) showed general decreases in pea plant dry weight when exposed to insecticides. However, the inoculation with symbiotic microorganisms reduced the toxic effect of insecticides. Root dry weight from inoculated 100 mg DFB/kg treatment was significant lower than the control group. Decreases in root dry weight have been previously reported at concentrations of 40 and 160 ppm of fluorinated organic compounds in *Zea mays* (Smit *et al*., 2009).

4.3.3. Ectomycorrhizal colonisation analysis of *Pinus pinaster* seedlings inoculated with *Pisolithus tinctorius*

As noted in Fig. 3.9, inoculation resulted in a significant increase in the rate of ECM colonisation and mycorrhizal expansion rate. Within inoculated treatments, the rate of ECM colonisation showed no differences as the DFB concentration increased. Similar results were obtained by O'Neill and Mitchell (2000) which reported no influence of pesticides on mycorrhization.

However, there was a tendency for the expansion rate to increase with increasing DFB concentrations, especially at 100 mg DFB/kg soil in relation to the inoculated control group. The previous increase was also reported in studies where fungicides and pesticides were used (Kais *et al*., 1981; Marx and Rowan, 1981; Pawuk and Barnett, 1981; Trappe *et al*., 1984).

4.3.4. Activity of SOD and CAT enzymes

The DFB metabolite 4-chloroaniline via cytochrome P450, can generate reactive oxygen species. The increased activity of detoxifying enzymes such as glutathione S-transferase and antioxidant enzymes such as SOD and CAT might result from oxidative stress induced by DFB (Maduenho and Martinez, 2008).

Activity of SOD was significantly increased at 1 and 10 mg DFB/kg and CAT activity was significantly affected in the inoculated treatments exposed to 1, 10 and 100 mg DFB/kg, having higher activity levels than inoculated control group (Table 3.1). In studies with *Zea mays* exposed to diesel, the activities of SOD and CAT increased at low diesel concentration (Tang *et al.*, 2009). In the present study the same was observed. These effects may be due to SOD and CAT being activated by lower DFB stress, as a sign of increased defense against the presence of ROS. In this study the activity of CAT in plants inoculated with mycorrhizal fungi was higher than those non-inoculated (Table 3.1). However, SOD activity was lower in the plants inoculated with mycorrhizal fungi than those without inoculation. This was described in previous studies in water stress experiences in soybean and *Poncirus trifoliate* (Porcel and Ruiz-Lozano, 2004; Maduenho and Martinez, 2008).

4.4. General effects of fungal inoculation and presence of diflubenzuron on plant and fungus parameters

The effect of mycorrhizal inoculation and presence of DFB can be seen in Table 3.2, where a two-way ANOVA was applied to the results. In all plant biometric parameters there were significant effects when DFB was present. The parameters most affected were the total, primary and secondary root length and shoot dry weight. Another fact observed was the influence of interaction of DFB and inoculation, causing more significant effects on shoot length and light effects on the shoot dry weight and secondary root length. Regarding ECM colonisation parameters, inoculation factor had a significant effect on rate and expansion of mycorrhizal root tips. The same was observed in the activity of SOD and CAT enzymes.

Table 3.2. Two-way ANOVA of all parameters analysed in the development of *Pinus pinaster* seedlings inoculated with *Pisolithus tinctorius* exposed to diflubenzuron.

	Shoot length	Root length	Primary root length	Secondary root length	Shoot dry weight	Root dry weight	ECM colonisation rate	Mycorrhizal expansion	Activity of SOD	Activity of CAT
Doses of toxic (C)	3.158 (**)	6.533 (***)	7.024 (***)	4.639 (***)	6.489 (***)	2.648 (*)	0.422(NS)	3.476 (**)	0.905 (NS)	0.585 (NS)
Inoculation (I)	0.106 (NS)	0.226 (NS)	1.003 (NS)	0.960 (NS)	0.009 (NS)	0.785 (NS)	211.364 (***)	74.275 (***)	11.383 (***)	8.369 (***)
C x I	5.199 (***)	1.198 (NS)	1.255 (NS)	3.313 (**)	2.996 (*)	1.311 (NS)	0.836 (NS)	1.574 (NS)	1.570 (NS)	2.980 (*)

5. *Conclusion*

This study showed that despite DFB being an insecticide, with the potential to protect forest trees such as pine trees, can cause adverse effects on plants and fungi. There was inhibition of growth in *P. pinaster* root system and growth of colonies of the ECM fungus *P. tinctorius*. The DFB doses that caused significant differences were mostly 10 and 100 mg DFB/kg soil. Even in antioxidant parameters showed potential toxic effects in these same concentrations. However lower concentrations of DFB (0.01 - 1 mg DFB/kg soil) showed inhibitory effects in secondary root length.

There were no toxic effects in the most visible parameters of health of the *P. pinaster* as the germination rate and shoot length, assuming that would not have much influence in the early stages as in normal growth. Another factor observed was the absence of mortality of trees exposed by the DFB. Inoculation had no influence on most biometric parameters assessed.

Concentrations of DFB after aerial application may be variable, between 0.35 and 2.68 mg/kg reported in more optimistic studies or more alarmist studies reaching 1824 mg/kg. Since some non-lethal toxic effects could be seen even at low doses of DFB (0.1 - 1 mg DFB/kg soil), this compound has the ability to cause sub-lethal effects. The use of this insecticide should be mediated as appropriate, not exaggerating in the doses and applications, running the risk of causing harmful effects on the trees, fungi and their symbiosis.

6. *Bibliography*

Ahemad, M. and Khan, M.S., 2010. Comparative toxicity of selected insecticides to pea plants and growth promotion in response to insecticide-tolerant and plant growth promoting Rhizobium leguminosarum. Crop Protection 29, 325–329.

Alía, R. and Martín, S., 2003. Euforgen. In: Technical Guidelines for Genetic Conservation and Use for Maritime Pine (*Pinus pinaster*), International Plant Genetic Resources Institute, Rome, p. 6.

Altas, R. M., Pramer, D., Bartha, R., 1977. Assessment of pesticides on non-target soil microorganisms. Soil Biology & Biochemistry 10, 231–239.

Amadio, J. and Murphy, C.D., 2010. Biotransformation of fluorobiphenyl by *Cunninghamella elegans*. Applied Microbiology Biotechnology 86, 345–351.

Bago, B., Chamberland, H., Goulet, A., Vierheilig, H., Lafontaine J.G., Piché Y., 1996. Effect of Nikkomycin Z, a chitin-synthase inhibitor, on hyphal growth and cell wall structure of two arbuscular-mycorrhizal fungi. Protoplasma 192, 80-92.

Beauchamp, C.H., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry 44, 276–87.

Biocult. http://www.biocult.co.za/faq.html, consultado a 30 de Julho de 2010.

Blanco, E., Casado, M.A., Costa, M., Escribano, R., García, M., Génova, M., Gómez, A., Gómez, F., Moreno, J.C., Morla, C., Regato, P. and Sainz, H., 1997**.** In:Los bosques ibéricos. Una interpretación geobotánica. Barcelona: Planeta.

Boutonnet, J.C., Bingham, P., Calamari, D., de Rooij, C., Franklin, J., Kawano, T., Libre, J., McCul-loch, A., Malinverno, G., Odom, J. M., Rusch, G.M., Smythe, K., Sobolev, I., Thompson, R. and Tiedje, J. M., 1999. Environmental Risk Assessment of Trifluoroacetic Acid, Human and Ecological Risk Assessment: An International Journal 5:, 1,59 — 124

Brosse, J., 1977. In: Atlas de arbres de France et d'Europe occidentale. Paris: Bordas

Brundrett, M.C., 2009. Mycorrhizal associations and other means of nutrition of vascular plants: understanding global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant Soil 320, 37–77.

Brundrett, N., Bougher, N., Dell, B., Grove, T., Malajczuk, N., 1996. Working with mycorrhizas in forestry and agriculture. ACIAR Monograph. 32, p. 374.

Bull, D. L., 1980. Fate of diflubenzuron after application to cotton and the boll weevil. The Southwestern Entomologist, Supplement 1, 2-7.
Bull, D. L., and G. W. Ivie., 1978. Fate of diflubenzuron in cotton, soil, and rotational crops. Journal of Agricultural and Food Chemistry 26, 513-520.

Carvalho, M.F., de Marco, P., Duque, A.F., Pacheco, C.C., Janssen, D.B., Castro, P.M.L., 2009. Labrys potucalensis, a bacterial strain with the capacity to degrade fluorobenzene New Biotechnology 25, S68.

Claire, D.A., Ngoc D.M., Darr, D., Archibald, F., Fridovich, I., 1984. Effects of molecular oxygen on detection of molecular oxygen and detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. Analytical Biochemistry 140,532–7.

Colinas, C., Ingham, E., Molina, R., 1994. Population responses of target and non-target forest soil organisms to selected biocides. Soil Biology & Biochemistry 26, 41–47.

Correia, I., Almeida, M.H., Aguiar, A., 2004. Variabilidade do Crescimento e da Forma de Proveniências de Pinus pinaster Aiton aos 8 anos, na Mata Nacional do Escaroupim. Silva Lusitana 12, 151–182.

Cunningham, P. A., 1986. A review of toxicity testing and degradation studies used to predict the effects of diflubenzuron (dimilin) on estuarine crustaceans. Environmental Pollution 40A, 63-86.

Dams, R.J. and Vanneste, W.J., 1997. Fire-fighting agents containing polysaccharides and fluorochemical oligomeric surfactants, WO Patent 97/45167.

Davison, A.W. and Pearson, S., 1997. Toxicity of TFA to plants. Final Report to AFEAS by University of Newcastle (Project SP91–18.23/BP96–31).

DGRF, 2007. Inventário Florestal Nacional 2005/2006. Direcção Geral das Florestas.

Dickie, I.A., Reich, P.B., 2005. Ectomycorrhizal fungal communities at forest edges. Journal of Ecology 93, 244–255.

Dickie, I.A., Xu, B., Koide, R.T., 2002. Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. New Phytologist 156, 527–535.

Ehrl, W., 1998. Fluorine-containing alkylsulfobetaines, a process for their preparation and their use, DE Patent 3104766.

Emerich, D., 1997. Effects of TFA on symbiotic nitrogen fixation by soybean. Final Report to AFEAS by University of Missouri (Project SP91–18.24/BP95–32).

EPA (U.S. Environmental Protection Agency), 1997. Reregistration Eligibility Decision (RED): Diflubenzuron. EPA 738-R-97-008 dated August 1997.

Figueredo, R.C.R. and Sabadini, E., 2003. Fire-fighting foam stability: the affect of the drag reducer poly(ethylene)oxide, Colloids Surf. A 215, 77–86.

Gartrell, M., 1981. Diflubenzuron. U.S. Food and Drug Administration, Bureau of Foods, HFF-420.8 pp.

Giesy, J.P. and Kannan, K., 2002. Perfluorochemicals surfactants in the environment, Environmental Science Technology, 147A.

Godbold, D.L., Hoosbeek, M.R., Lukac, M., 2006. Mycorrhizal hyphal turnover as a dominant process for carbon input into soil organic matter. Plant Soil 281, 15–24.

Halasz, S.P. and Millauer, H., 1976. Fluoroverbindungen organische Ullmans Encyklopädie der Technischen Chemie, vol. 11, Verlag Chemie, Weinheim, 1976, p. 632.

Hobbie, E.A., 2006. Carbon allocation to ectomycorrhizal fungi correlates with belowground allocation in culture studies. Ecology 87, 563–569.

Högberg, M.N. and Högberg, P., 2002. Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces, together with associated roots, half the dissolved organic carbon in a forest soil. New Phytologist 154, 791–795.

Högberg, P., Nordgren, A., Buchmann, N., Taylor, A.F.S., Ekblad, A., Högberg, M.N., Nyberg, G., Ottosson-Löfvenius, M., Read, D.J., 2001. Large-scale forest girdling shows that current photosynthesis drives soil respiration. Nature 411, 789–792.

Iloba, C., 1980. The use of chemicals for weed control in forest tree nurseries. In: Tropical mycorrhiza research (Ed: Mikola, P.). Oxford, Clarendon Press, 121–123.

Ingham, E. R., Cambardella, C., Coleman, D. C., 1986. Manipulation of bacteria, fungi and protozoa by biocides in Lodgepole pine forest soil microcosms : effects on organism interactions and nitrogen mineralization. Canadian Journal of Soil Science 66, 261–272.

Jarvis, N.L. and Zisman, W.A., 1966. Surface chemistry of fluorochemicals Kirk-Othmer Encyclopedia of Chemical Technology, vol. 9, John Wiley, New York, p. 707.

Johnson, N.C., Graham, J.H., Smith, F.A., 1997. Functioning of mycorrhizal associations along the mutualism–parasitism continuum. New Phytologist, 135, 575–586.

Johnson, N.C., Hoeksema, J.D., Bever, J.D., Chaudhary, V.B., Gehring, C., Klironomos, J., Koide, R., Miller, R.M., Moore, J., Moutoglis, P., Schwartz, M., Simard, S., Swenson, M., Umbanhowar, J., Wilson, G., Zabinski, C., 2006. From Lilliput to Brobdingnag: extending models of mycorrhizal function across scales. Bioscience 56, 889–900.

Jones, M.D., Smith, S.E., 2004. Exploring functional definitions of mycorrhizas: are mycorrhizas always mutualisms? Canadian Journal of Botany 82, 1089–1109.

Kais, A. G., Snow, G. A., Marx, D. H., 1981. The effects of benomyl and *Pisolithus tinctorius* ectomycorrhizae on survival and growth of longleaf pine seedlings. Southern Journal of Applied Forestry 5, 189–194.

Key, B.D., Howell, R.D., Criddle, C.S., 1997. Fluorinated organics in the biosphere, Environmental Science Technology. 31, 2445.

Klironomos, J.N., 2003. Variation in plant response to native and exotic mycorrhizal fungi. Ecology 84, 2292–2301.

Kramer, C., Kreisel, G., Fahr, K., 2004. Degradation of 2-fluorophenol by the brown-rot fungus *Gloeophyllum striatum*: evidence for the involvement of extracellular Fenton chemistry. Applied Microbiology Biotechnology 64, 387–395.

Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680–5.

Larsson, S., 1983. Effects of artificial defoliation on stem growth in Salix smithiana grown under intensive culture. Acta Oecologica, Oecologia Applicata 4, 343-349.

Lewandowski, G., Meissner, E., Milchert, E., 2006. Special applications of fluorinated organic compounds. Journal of Hazardous Materials A136, 385–391.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265-275.

Maduenho, L.P., Martinez, C.B.R., 2008. Acute effects of diflubenzuron on the freshwater fish Prochilodus lineatus. Comparative Biochemistry and Physiology C148, 265–272

Manninen, A.M., Laatikainen, T., Holopainen, T., 1998. Condition of Scots pine fine roots and mycorrhiza after fungicide application and low-level ozone exposure in a 2-year field experiment. Trees 12, 347–355.

Martinez-Toledo, M. V., Rubia, T., Moreno J., Gonzalez-Lopez, J., 1988. Effect of diflubenzuron on Azotobacter nitrogen fixation in soil. Chemosphere 17, 829-834.

Martins,M.L., 2004. Cuidados básicos e alternativas no tratamento de enfermidades de peixes na aquacultura brasileira. In: Ranzani-Paiva, M.J.T., Takemoto, R.M., Lizama, M.A.P. (Eds.), Sanidade de Organismos aquáticos. Editora Livraria Varela, São Paulo, pp. 357–370.

Marx, D. H., Cordell, C. E., France, R. C., 1986. Effects of triadimefon on growth and ectomycorrhizal development of Loblolly and Slash pines in nurseries. Phytopathology 76, 824–831.

Marx, D. H., Rowan, S. J., 1981. Fungicides influence growth and development of specific ectomycorrhizae on loblolly pine seedlings. Forestry Science 10, 167–176.

Marx, D.H., 1969. The influence of ectotrophic mycorrhyzal fungi on the resistance of pine roots to pathogenic infections: I. Antagonism of mycorrhizal fungi and soil bacteria. Phytopathology 59, 153-163.

Mattson, W.J., Niemela, P., Rousi, M., 1996. Dynamics of Forest Herbivory: Quest for Pattern and Principle. General Technical Report North Central Forest Experiment Station, USDA, Forest Service No. NC-183, p. 286.

Meissner, E., Twardochleb, B., Milchert, E., Wróblewska, A., 2003. Estimation of foam forming properties and surface tension of fine-extinguishing agents, Tenside Surf. Det. 40, 353–362.

Moody, C.A. and Field, J.A., 2000. Perfluorinated surfactants and the environmental implications of their use in fire-fighting foams, Environmental Science Technology 34, 3864–3870.

Murphy, C.D., 2010. Biodegradation and biotransformation of organofluorine compounds. Biotechnology Letters 32, 351–359.

Muzika, R.M. and Liebhold, A.M., 2000. A critique of silvicultural approaches to managing defoliating insects in North America. Forest Entomology 2, 97–105.

Nigg, H.N., 1986. Bull Environmental Contamination Toxicology 36, 833-8.

O'Neill, J. M., Mitchell, D. T., 2000. Effects of benomyl and captan on growth and mycorrrhizal colonization of Stika-spruce (Picea sitchensis) and ash (Fraxinus excelsior) in Irish nursery soil. Forest Pathology 30, 165–174.

OCDE, 2006. Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test. In: OCDE GUIDELINES FOR THE TESTING OF CHEMICALS.

Pawuk, W. H., Barnett, J. P., 1981. Benomyl stimulates ectomycorrhizal development by *Pisolithus tinctorius* on shortleaf pine growth in containers. USDA For. Serv. SO 267, 1–3.

Porcel. R., Ruiz-Lozano, J.M., 2004. Arbuscular mycorrhizal influence on leaf water potential, solute accumulation, and oxidative stress in soybean plants subjected to drought stress. Journal of Experimental Botany 55, 1743–50.

Prima-Putra, D., Berredjem, A., Chalot, M., Dell, B. and Botton, B., 1999 Growth characteristics, nitrogen uptake and enzyme activities of the nitrate-utilising ectomycorrhizal Scleroderma verrucosum. Mycological Research 103, 997–1002.

Read, D.J., Leake, J.R., Perez-Moreno, J., 2004. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. Canadian Journal of Botany 82, 1243–1263.

Reinhold, D.M., 2007. Ph.D. thesis: Fate of fluorinated organic pollutants in aquatic plant systems: Studies with Lemnaceae and Lemnaceae tissue cultures. Georgia Institute of Technology.

Rinaldi, A.C., Comadini, O., Kuyperm T.W., 2008. Ectomycorrhizal fungal diversity: separating the wheat from the chaff. Fungal Divers, 33, 1–45.

Rodríguez, E., Barrio, R. J., Goicolea, A., Peche, R., Balugera, Z. G., Sampedro, C., 2001. Persistence of the Insecticide Dimilin 45 ODC on Conifer Forest Foliage in an Atlantic-Climate Ecosystem. Environmental Science Technology 35, 3804-3808.

Ruotsalainen, A.L., Kozlov, M.V. Fungi and air pollution: is there a general pattern? In: Rhodes, D. (Ed.), New Topics in Environmental Research. Nova Science Publishers, Hauppauge, N.Y., in press.

Schalch, S.H.C., Belo, M.A.A., Soares, V.E., Moraes, J.R.E., Moraes, F.R., 2005. Diflubenzuron effectiveness in *Dolops carvalhoi* (Crustacea: Branchiura) control in juveniles pacus *Piaractus mesopotamicus* (Osteichthyes: Characidade) naturally infected. Acta Sci. 27, 297–302.

Schnierer, E., 1976. Tenside Delerg. 13, 1–5.

Schüepp, H., Bodmer, M., 1991. Complex response of VA-mycorrhizae to xenobiotic substances. Environmental Toxicology & Chemistry 30, 193–199.

Schwartz, M.W., Hoeksema, J.D., 1998. Specialization and resource trade: biological markers as a model of mutualisms. Ecology 79, 1029–1038.

Simberloff, D., 2001. Management of boreal forest biodiversity - a view from the outside. Scandinavian Journal of Forest Research 3, 105-118.

Smit, M. F., van Heerden, P.D.R., Pienaar, J.J., Weissflog, L., Strasser, R.J., Krüger, G.H.J., 2009. Effect of trifluoroacetate, a persistent degradation product of fluorinated hydrocarbons, on Phaseolus vulgaris and Zea mays. Plant Physiology and Biochemistry 47, 623–634.

Smith, S.E. and Read D.J., 2008. Mycorrhizal symbiosis, third ed., Academic Press, London, 787 p.

Söderström, B., 1992. The ecological potential of the ectomycorrhizal mycelium. Mycorrhiza in Ecosystems (Alexander IJ, Fitter AH, Lewis DH & Read DJ, eds), pp. 77–83. CAB International, London.

Sundaram, K. M. S. and R. Nott., 1989. Mobility of diflubenzuron in two types of forest soils. Journal of Environmental Science and Health B24, 65-86.

Tang, M., Chen H., Huang, J.C., Tian, Z.Q., 2009. AM fungi effects on the growth and physiology of Zea mays seedlings under diesel stress. Soil Biology & Biochemistry 41, 936–940.

Thompson, R.S. and Windeatt, A.J., 1994. Sodium trifluoroacetate: Effects on seed germination (ten species, by aqueous exposure). Brixham Environmental Laboratory Report BL5197/B.

Townshend, J.L., Pree, D.J., Broadbent, A.B., 1983. Population development and reproduction of fungus and bacterium feeding nematodes in the presence of insect growth regulators. Journal of Nematology 15(1), 105-110.

Trappe, J. M., Molina, R., Castellano, M., 1984. Reactions of mycorrhizal fungi and mycorrhiza formation to pesticides. Annual Review of Phytopathology 22, 231–259.

Treseder, K.K., Allen, M.F., 2002. Direct nitrogen and phosphorus limitation of arbuscular mycorrhizal fungi: a model and field test, New Phytologist 155, 507–515.

Tu, C. M., 1992. Effects of some herbicides on activities of microorganisms and enzymes in soil. Journal of Environmental Science & Health B27, 695–709.

Tu, C. M., 1993. Effect of fungicides, captafol and chlorothalonil, on microbial and enzymatic activities in mineral soil. Journal of Environmental Science & Health B28, 67–80.

USDA, 2004. Human Health and Ecological Risk Assessment for Diflubenzuron (Dimilin). Forest Health Protection. SERA TR 04-43-05-03b dated July 2004.

Van Duin, W.E., Griffioen, W.A.J., letswaart, J.H., 1991. Occurrence and function of mycorrhiza in environmentally stressed soil. In: Rozema, J., Verkleij, J.A.C. (Eds.), Ecological Responses to Environmental Stresses. Kluwer, Dordrecht, pp. 114-123.

Vega, J.M., Moneo, I., Armentia, A., Vega, J., de la Fuente, R., Fernandez, A., 2000. Pine processionary caterpillar as a new cause of immunologic contact urticaria. Contact Dermatitis 43, 129-132.

Wainhouse, D., 2005. Ecological Methods in Forest Pest Management. Oxford University Press, Oxford.

Wang, B. and Qiu, Y.L., 2006. Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza 16, 299–363.

Wilkinson, D.M., Dickinson, N.M., 1995. Metal resistance in trees: the role of mycorrhizae. Oikos 72, 298-300.

Wingler, A., Wallenda, T. and Hampp, R., 1996. Mycorrhiza formation on Norway spruce (Picea abies) roots affects the pathway of anaplerotic CO2 fixation. Plant Physiologist 96, 699–705.