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In vitro interaction studies between *Glomus intraradices* and *Armillaria mellea* in vines

A. Nogales[1], A. Camprubí[1], V. Estaún[1], V. Marfà[2] and C. Calvet[1]

[1] *Protecció Vegetal. Institut de Recerca i Tecnologia Agroalimentàries (IRTA). Ctra. de Cabrils, km 2. 08348 Cabrils (Barcelona). Spain*

[2] *Genètica Vegetal. Institut de Recerca i Tecnologia Agroalimentàries (IRTA). Ctra. de Cabrils, km 2. 08348 Cabrils (Barcelona). Spain*

Abstract

An interaction study was performed with mycorrhizal plants of the grapevine rootstock Richter 110 (*Vitis berlandieri* Planch × *Vitis rupestris* Scheele) and the root pathogenic fungus *Armillaria mellea* (Vahl:Fr.) P. Kumm using an autotrophic *in vitro* culture system. Micropropagated plantlets were transferred to Petri plates with MSR medium lacking sugar and vitamins. Inocula of *Glomus intraradices* (BEG 72) and of *Armillaria mellea* obtained from a root organ culture and from a mycelium colony grown in malt agar respectively, were added to the plates according to each treatment: non-inoculated, inoculated with *G. intraradices*, inoculated with *A. mellea*, and dual-inoculated plants. There were ten replicates per treatment. Fourteen weeks later, the pathogen's mycelium occupied most of the surface/volume of the plate and had produced rhizomorphs. In dual inoculated plates, *A. mellea*'s growth was not affected by the presence of *G. intraradices*, but the latter produced a lower number of spores and its extraradical phase showed granulation, vacuolation and tip swelling. The pathogen induced necrosis and growth decrease in the root. *Glomus intraradices* alleviated these symptoms and there were no differences in root biomass between non-inoculated plants and plants inoculated with both fungi. There were no symptoms of the disease in shoots and *G. intraradices* stimulated shoot growth both, although mycorrhizal colonization was lower when *A. mellea* was present. No direct antagonism or antibiosis against the pathogen was observed, thus the protective effect exerted by the symbiotic fungus in grapevines must be indirect, mediated through the host plant physiology.

Additional key words: control, disease symptoms, grapevine, *in vitro* culture, root pathogenic fungi, tolerance.

Resumen

Estudios de interacción *in vitro* entre el hongo formador de micorrizas arbusculares *Glomus intraradices* y el hongo patógeno *Armillaria mellea* en vid

Se estudió la interacción entre plantas micorrizadas del portainjerto de vid 110 Richter (*Vitis berlandieri* Planch × *Vitis rupestris* Scheele) y *Armillaria mellea* (Vahl:Fr.) P. Kumm en cultivo autotrófico *in vitro*. Plantas micropropagadas fueron transferidas a placas de Petri con medio MSR sin azúcar ni vitaminas. Inóculo de *Glomus intraradices* Schenck and Smith (BEG 72) obtenido a partir de un cultivo axénico de raíces transformadas micorrizadas, e inóculo de *A. mellea* obtenido en medio agar-malta, se añadieron a las placas según tratamiento: no inoculado, inoculación con *G. intraradices*, inoculación con *A. mellea* e inoculación combinada, estableciéndose 10 réplicas por tratamiento. Catorce semanas después, el micelio del hongo patógeno ocupaba casi la totalidad de la superficie/volumen de la placa y había producido rizomorfos. En placas con inoculación mixta, el desarrollo de *A. mellea* no se vio afectado por la presencia de *G. intraradices,* mientras que éste produjo menos esporas, y su fase extraradical presentaba granulaciones, vacuolaciones y engrosamientos en las terminaciones. El patógeno indujo necrosis y menor desarrollo radical. *Glomus intraradices* alivió estos síntomas, y no se observaron diferencias entre la biomasa radical de plantas no inoculadas y de plantas inoculadas con ambos hongos. No aparecieron síntomas de la enfermedad en la parte aérea y *G. intraradices* estimuló en cualquier caso el crecimiento, pero la colonización micorrícica fue menor en presencia del patógeno. No se observó antagonismo directo o antibiosis, el efecto de protección de la simbiosis micorrícica en vid frente a *A. mellea* debe ser indirecto, a través de la fisiología del hospedador.

Palabras clave adicionales: control, cultivo *in vitro*, hongos patógenos de raíz, sintomatología, tolerancia, vid.

* Corresponding author: cinta.calvet@irta.cat
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Introduction

Armillaria mellea (Vahl:fr) P. Kumm is a root pathogenic fungus causing white root rot in several crop species such as vines and fruit trees. Control measures are scarce and unefficient, as *A. mellea* has a high infection capacity and a long-term survival in the soil (Aguín *et al.*, 2006). Alternative biological and cultural control methods are under study (Baumgartner and Rizzo, 2006; Nogales *et al*., 2009a) and among them, the use of arbuscular mycorrhizal fungi (AMF) has demonstrated to contribute to increase plant tolerance to *A. mellea* both in the greenhouse (Nogales *et al.,* 2009b) and in the field (Camprubí *et al*., 2008; Nogales *et al*., 2009a).

To our knowledge, *in vitro* interaction studies between AMF and *A. mellea* have never been reported. *In vitro* experiments allow to maintain microorganisms, tissues or cells out of their natural environment, limiting the influence of external, non controllable factors, providing a useful experimental tool. Very often, similar results cannot be expected in field conditions, but *in vitro* studies are a useful experimental approach in research.

The development of a monoxenic culture system for AMF by Bécard and Fortin (1988) and Déclerck *et al.* (1998) in carrot roots, opened up the possibility to study *in vitro* interactions between the AMF *Glomus intraradices* Schenck and Smith and plant pathogenic fungi (Benhamou *et al.*, 1994). Despite its many advantages, the monoxenic culture system has however a severe limitation due to the lack of the whole host plant system. The lack of photosynthetic tissues generates an abnormal hormonal balance and an abnormal physiological relationship between both symbiotic partners (St-Arnaud and Elsen, 2005) that can distort the results of the interaction studies.

Voets *et al*. (2005) developed an autotrophic culture system for the *in vitro* mycorrhization of micropropagated plantlets, where the roots of these plantlets were inoculated with AMF spores under *in vitro* conditions. In this system, the shoots grew in the open air under high light intensity allowing plant photosynthesis (Kozai *et al.,* 1988) while roots developed in the dark. Roots were inoculated with individual AMF spores, and the emerging hyphae were capable to colonize the whole root system, to develop extraradical mycelium and to produce new spores.

The adaptation of the autotrophic culture system to woody species such as vine plants, could be useful for studying the arbuscular mycorrhizal (AM) symbiosis development as well as the short term interactions between the mycorrhizal fungus and root pathogens affecting grapevines. *Armillaria mellea* is a slow-growing pathogen that takes several years to kill grapevines in the field (Baumgartner and Rizzo, 2002), thus, long term studies are needed to assess disease control measures. The development of an *in vitro* mycorrhizal inoculation system for woody species would enable to observe an early response of mycorrhizal vines to *A. mellea*.

The aim of this study was to evaluate the interaction between *G. intraradices* and *A. mellea* in an autotrophic *in vitro* culture system using micropropagated Richter 110 grapevines as the host plant.

Material and methods

Plant material

In vitro micropropagated plantlets of the grapevine rootstock Richter 110 (*Vitis berlandieri* Planch × *Vitis rupestris* Scheele) were first propagated from woody cuttings, and the newly elongated shoots were cut and surface-sterilized. Nodal segments of the disinfected shoots were cultured on a modified Murashigue and Skoog (MMS) medium (Murashigue and Skoog, 1962) adapted to vine by Torregrosa and Bouquet (1996) and kept in a growth chamber set at 25 ± 2°C with 50 µmol m^{-2} s^{-1} photon flux density (PFD), provided by fluorescent lights (Sylvania cool-white) for a 16 hours-photoperiod. New shoots were formed from each segment and grapevine plantlets were sub-cultured by nodal cuttings every five weeks on MMS medium (Torregrosa *et al.*, 2001). Shoots of 4.5-5.5 cm long with uniform root systems were used in the experiments.

Fungal material

The inoculum of *G. intraradices* BEG 72 was obtained from mycorrhizal root organ cultures using transformed carrot roots (*Daucus carota* L.).

The *A. mellea* strain used in the experiment was isolated from a replant vineyard in Vimbodí (Tarragona) in 2004, and subcultured on malt agar medium.

Abbreviations used: AM (arbuscular mycorrhiza), AMF (arbuscular mycorrhizal fungus), MMS (Modified Murashigue and Skoog medium), MSR (Strullu-Romand medium), PFD (photon flux density).

Experiment set up

Four treatments were considered, with ten replicates each: non-inoculated plants, inoculation with *G. intraradices*, inoculation with *A. mellea* and inoculation with both the AMF and the pathogenic fungus.

Petri plates 15 cm in diameter were filled with MSR medium lacking sugar and vitamins (Voets *et al.,* 2005) and two plugs of inoculum isolated from an AM fungal root organ culture of *G. intraradices* containing approximately 50 spores were placed in the centre of the plate. Simultaneously three plugs of 0.5 cm diameter from an *A. mellea* mycelium colony grown on malt agar were placed in the edge of the plates to ensure an homogeneous growth of the fungal mycelium in the plate.

This inoculation method was aimed at achieving a simultaneous exposure to both, the AMF and the pathogen, as it occurs in the field.

Approximately 5 cm long *in vitro* micropropagated plantlets of Richter 110, were then transferred to the Petri plates and an autotrophic culture system was established. The roots remained in the Petri plate on the culture medium, devoid of sucrose and vitamins, while the shoots grew in open air conditions as described for potato plants (Voets *et al.,* 2005). Petri dishes were covered with opaque plastic strips in order to keep the root system in the dark, and plants were kept inside a plastic box at 100% of relative humidity in a growth chamber set at 25°C with 16h photoperiod and a PFD of 200 μmol m^{-2} s^{-1}. Ten days later their acclimatization was induced by progressively opening the box. Sterilized (121°C for 15 min) MSR medium lacking sucrose and vitamins was periodically added to the Petri plates to maintain an adequate level of nutrients and liquid in the plates.

After 13 weeks, Petri plates were observed under the microscope (Zeiss, West Germany) for detecting possible interactions between two fungi at hyphal level. The observations were done placing the plates directly under the microscope.

At harvest, 13 weeks after the set up of the experiment, root and shoot biomass, the number of *G. intraradices* spores and the number of rhizomorphs produced by *A. mellea* were recorded. Mycelial growth of *A. mellea* was estimated as the percentage of the total medium surface occupied by the pathogen, and the percentage of mycorrhizal intraradical colonization was estimated by the grid-line intersect method (Giovannetti and Mosse, 1980) on a 1 g portion of each root system which was cleared and stained following the method of Phillips and Hayman (1970), modified by Koske and Gemma (1989).

Statistical analysis

Biomass data were analyzed by a two way ANOVA followed by a Duncan's test ($P \leq 0.05$). The number of spores and rhizomorphs, and the percentage of the surface covered by *A. mellea*'s mycelium and the intraradical mycorrhizal colonization percentage were analyzed by a student t test.

Results

The autotrophic culture system established for vine plants was a useful tool to study the interaction between *G. intraradices* and *A. mellea*. The system allowed to observe and quantify the mycelial development of both fungi and the growth of the vine plants. It was also possible to observe non-destructively under the microscope the morphology of AMF spores and the fungal mycelia.

At harvest, 14 weeks after the establishment of the systems, *A. mellea* had grown around the roots of Richter 110 plantlets and had infected them. The infection was set directly from the *A. mellea* mycelium and also from the newly formed rhizomorphs. Necrosis and rot symptoms were observed in the roots (Fig. 1) but there were no disease symptoms in the shoots.

In root biomass, a significant effect of the pathogen was detected. Plants inoculated with *A. mellea* had a

Figure 1. Roots of a Richter 110 grapevine rootstock plant infected by *Armillaria mellea* after 13 weeks growth in an autotrophic culture system.

Table 1. Response of Richter 110 grapevine rootstock plants after 13 weeks growth in autotrophic culture systems inoculated or not with *Glomus intraradices* and *Armillaria mellea*

Treatment	Shoot dry weight (g)	Root dry weight (g)
Control	0.547[b]	0.188[a]
G. intraradices	0.908[a]	0.218[a]
A. mellea	0.608[b]	0.125[b]
G. intraradices + *A. mellea*	0.822[a]	0.172[ab]
Mycorrhiza	*	ns
Pathogen	ns	*
Interaction	ns	ns

Data are means of ten replicates per treatment. Different letters indicate statistically significant differences according to Duncan's test ($P \leq 0.05$). The effect of the mycorrhizal fungus, the effect of the pathogen and the interaction between both was determined by a two way ANOVA. The asterisk indicates significant differences at a probability level of $P \leq 0.05$.

lower root weight than non-inoculated plants. The decrease in root growth was alleviated by the mycorrhizal symbiosis, and plants inoculated with both *G. intraradices* and *A. mellea* did not differ significantly in root dry weight from healthy plants (Table 1).

The development of *A. mellea* was not significantly different in plants inoculated only with the pathogen and in plants inoculated with both *A. mellea* and *G. intraradices* (Table 2). Moreover, *A. mellea* produced dicotomically branched rhizomorphs in both treatments (Fig. 2). Concerning the development of the mycorrhizal fungus, both the number of newly produced spores and the percentage of mycorrhizal colonization were lower in mycorrhizal plants inoculated with *A. mellea* (Table 3). However, the shoot dry weight was not significantly different from that recorded in mycorrhizal plants non-inoculated with *A. mellea*, and it was higher

Table 2. Growth development of *Armillaria mellea* in autotrophic *in vitro* culture systems of Richter 110 plants inoculated or not with *Glomus intraradices*

Treatment	Plate surface covered by *Armillaria mellea* (%)	No. of rhizomorphs
A. mellea	78.9[a]	22[a]
G. intraradices + *A. mellea*	80.1[a]	22[a]

Data are means of 10 plants per treatment. Data followed by a different letter differ significantly at $P \leq 0.05$ (Student t test).

Figure 2. Rhizomorphs of *Armillaria mellea* after 13 weeks growth in an autotrophic culture system with a Richter 110 plant.

than the shoot dry weight of non-mycorrhizal plants (Table 1).

In the extraradical phase of dual inoculated plates, mycelia of both fungi grew overlapped. At microscopic level, whereas *A. mellea* development was not affected by the presence of *G. intraradices* mycelium, the AM fungus growth was affected by the pathogen. Hyphal vacuolation (Fig. 3a), tip swelling (Fig. 3b) and granulation inside the *G. intraradices* hyphae (Fig. 3c) were clearly observed. There was also a decrease in spore production, and the spore shape was occasionally abnormal (Fig. 3d).

Discussion

In vitro experiments are research tools that allow the control of many parameters, thus enabling detailed interaction studies. The extrapolation of these results to field conditions should be done with caution, as

Table 3. Growth development of *Glomus intraradices* in autotrophic *in vitro* culture systems of Richter 110 plants inoculated or not with *Armillaria mellea*

Treatment	Number of spores	Intraradical colonization (%)
G. intraradices	4,758[a]	26[a]
G. intraradices + *A. mellea*	1,422[b]	10[b]

Data are means of 10 plants per treatment. Data followed by a different letter differ significantly at $P \leq 0.05$ (Student t test).

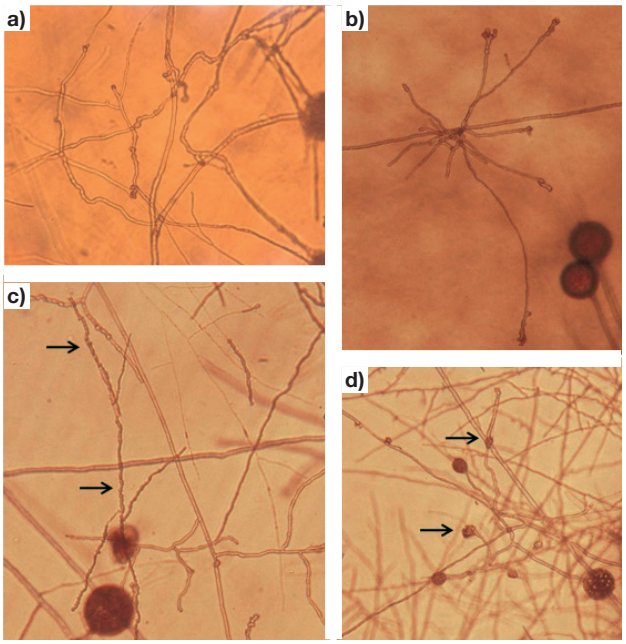

Figure 3. Microscopic images (magnification 100×) of *Glomus intraradices* mycelium in the presence of *Armillaria mellea* showing hyphal vacuolation (a), tip bursting (a and b), granulation inside hyphae (c) and abnormal spore formation (d).

environmental factors such as the soil/substrate physicochemical parameters and the rhizospheric microbial populations can have a strong influence in the expected outcome.

In our experimental system, *A. mellea* development was not affected by the extraradical phase of *G. intraradices*. The number of rhizomorphs and the growth of the mycelium were not different in both *A. mellea* inoculated treatments. Several studies have demonstrated that AM fungi have an influence on rhizosphere microorganisms by affecting the host plant, due in part to modifications in root exudates (Schwab *et al.,* 1983; McAllister *et al.,* 1995). It has been suggested that exudates from mycorrhizal roots may be implicated in an altered susceptibility of mycorrhizal plants against soil borne pathogens (Vierheilig and Piché, 2002; Jones *et al.,* 2004; Vierheilig, 2004a).

Hyphal interference is a form of antagonism where growth inhibition and a subsequent vacuolation, granulation and lysis of the cells occur as the hyphae of both species come into close proximity (Ikediugwu and Webster, 1970; Skidmore and Dickinson, 1976; Shankar *et al.,* 1994). These interactions are mediated by non-enzimatic diffusible metabolites (antibiotics) that alter the permeability of cell membranes leading to plasmolysis and hyphal death (Ikediugu and Webster, 1970; Skidmore and Dickinson, 1976; Boddy, 2000). The negative interactions observed in the extraradical mycelium of *G. intraradices* in the presence of *A. mellea* may indicate the potential production of antibiotics or toxic compounds by the pathogen.

Hepper (1979) observed that the spore germination and the growth of AM fungal mycelium could be stimulated or inhibited by different compounds, and the production of toxic secondary metabolites by *Armillaria* sp. has been reported by several authors (Peipp and Sonnenbichler, 1992; Sonnenbichler *et al.,* 1994). The release of these compounds, induced by the presence of antagonistic fungi and plant cells, can inhibit the growth of other microorganisms and even induce cell death before any contact occurs (Peipp and Sonnenbichler, 1992). Soluble or volatile compounds produced by the pathogenic fungus could therefore have inhibited the sporulation and the development of the extraradical mycelium of *G. intraradices*.

The lower root AMF colonization found in plants inoculated with both fungi, *G. intraradices* and *A. mellea*, might be a consequence of the reduced development of the extraradical mycelium of *G. intraradices* observed in these systems. Although the pathogen had a negative effect on plant roots, where early symptoms of the disease such as necrosis and decreased growth were observed, the presence of the AMF in co-inoculated plants reduced the symptoms, and root biomass was not different from that recorded in plants non-inoculated with the pathogen. This could be due to an improvement in the root development caused by the AMF colonization, especially in the absorption zone, which can compensate the loss in root biomass caused by the pathogen (Pozo *et al.,* 2002).

The development of pathogenic infections has been inversely correlated with the intraradical AMF colonization (Caron *et al.,* 1986; Cordier *et al.,* 1998; Vierheilig, 2004b; Scheffknecht *et al.,* 2006), but in our results no direct relationship between the intraradical colonization and the decrease in disease symptoms was determined.

The pathogen did not have a significant effect in shoot growth although *G. intraradices* had a lower intraradical development in the presence of *A. mellea,* while the mycorrhizal fungus had a stimulatory effect on shoot development. These results indicate that the AM symbiosis has a protective effect at plant level, reducing disease symptoms. A direct action of AMF against pathogens through antagonism, antibiosis or degradation has never been shown, therefore, the bioprotection effect of the AM symbiosis against diseases must be

indirect, through changes in the host physiology, inducing resistance mechanisms, improving plant nutrition, through competition for infection sites or for space or nutrients with pathogens, or by producing changes in the soil microbiota that can negatively affect the pathogen (Rodríguez-Kábana and Calvet, 1994; St-Arnaud *et al.,* 1995).

The *in vitro* grapevine culture system provides a method where factors as improved nutrition and changes in the rhizospheric microbiota can be discarded. In the particular case of *A. mellea* there is neither competition for infection *loci*, nor for host photosyntates, as both fungi colonise different host tissues, and have different trophic requirements. The increased tolerance observed in the plant roots could also be accounted for by an accumulation of newly formed products in the AMF infection site (Rosendahl and Rosendahl, 1990), including symbiosis related proteins, phenolic compounds, hydrolases like quitinases with antimicrobial potential and structural polymers, such as lignin. Although these changes are still controversial, and many have been shown to be transient, from our results the effect of AMF on pathogen tolerance seems to be exerted through the host plant physiology.

The system allowed the quantification of plant and fungal development and non destructive observations of the interaction of both fungi under the microscope. Although the pathogen negatively influenced the symbiont's extraradical and intraradical development, symbiotic plants had a higher root and shoot biomass than healthy non symbiotic plants, despite the presence of the pathogen. The results show an indirect bioprotection effect of *G. intraradices* against *A. mellea* in the early stages of the disease development, under our experimental conditions.

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