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ORIGINAL ARTICLE

The arbuscular mycorrhiza fungus *Rhizophagus irregularis* MUCL 41833 decreases disease severity of Black Sigatoka on banana c.v. Grande naine, under *in vitro* culture conditions

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Abstract – Introduction. *Mycosphaerella fijiensis*, the fungal pathogen causing Black Sigatoka disease, attacks almost all cultivars of bananas and plantains. Currently, the repeated application of fungicides is the most widespread control measure, while the use of bio-control agents remains almost ignored. Here we investigated, under *in vitro* culture conditions, whether an arbuscular mycorrhizal fungus (AMF – *Rhizophagus irregularis* MUCL 41833) could reduce the severity of disease caused by *M. fijiensis* MUCL 47740 on banana. **Materials and methods.** Prior to their transfer to autotrophic *in vitro* culture systems and subsequent inoculation by the pathogen, the banana plantlets cultivar Grande Naine (AAA genome Cavendish group) were grown in the extra-radical mycelium network of the AMF, arising from *Medicago truncatula* plantlets, for fungal root colonization. **Results and discussion.** At the time of infection with *M. fijiensis*, the AMF colonization of the banana plantlets was 12%, 56% and 10% for hyphae, arbuscules and spores/vesicles, respectively, and the number of spores produced in the medium was above 200. At day 21 and day 35 following infection by the pathogen, the number of extra-radical spores as well as the abundance of arbuscules in roots, were significantly higher than in the non-infected plantlets. Conversely, the severity index measured in the mycorrhizal plantlets decreased by more than 80% and 60%, respectively, 21 and 35 days after infection by the pathogen. Similarly, the area under disease progress curve, estimated at day 35, was reduced by 59% in the mycorrhizal banana plantlets as compared to the control plantlets. Whatever the treatment (mycorrhizal or non mycorrhizal plantlets in presence/absence of *M. fijiensis*), no significant differences were observed in plantlet dry weight, pseudostem height and diameter, and leaf area at both observation times. **Conclusions.** These results emphasize the complex multitrophic interactions between above and below-ground microorganisms mediated via the plant. They further suggest that AMF may decrease, at least at the early stage of infection, *M. fijiensis* symptoms possibly *via* the induction of a systemic resistance pathway. The important decrease in symptoms observed in the mycorrhizal banana plantlets suggest that these microorganisms may represent a potentially attractive option to consider in the context of an integrated control of this important disease.

Keywords: banana / *Musa accuminata* / vesicular arbuscular mycorrhizae / *Mycosphaerella fijiensis* / *Rhizophagus irregularis* / disease control method / *in vitro* culture

Résumé – Le champignon mycorhizien à arbuscules *Rhizophagus irregularis* MUCL 41833 décroît la sévérité de la maladie des raies noires chez le bananier c.v. Grande naine en conditions de culture *in vitro*. Introduction. *Mycosphaerella fijiensis*, le champignon responsable de la maladie des raies noires, s'attaque à la grande majorité des cultivars de bananiers et plantains. À l'heure actuelle, l'application répétée de fongicides est la principale mesure de contrôle, alors que l'utilisation d'agents de bio-contrôle reste largement ignorée. Dans cette étude, menée *in vitro*, nous avons évalué le rôle d'un champignon mycorhizien à arbuscules (CMA – *Rhizophagus irregularis* MUCL 41833) dans le contrôle de la maladie des raies noires causée par *M. fijiensis* MUCL 47740, chez le bananier. **Matériel et méthodes.** Avant leur transfert en système *in vitro* autotrophe et leur inoculation consécutive par le pathogène, les plantules de bananier du cultivar Grande Naine (génome AAA, groupe Cavendish) ont été placées dans le mycélium extra-racinaire du CMA issu de plantules de *Medicago truncatula,* pour colonisation racinaire. **Résultats et discussion.** Au moment de l'infection avec *M. fijiensis*, la colonisation racinaire par le CMA était de 12 %, 56 % et 10 % pour les hyphes, arbuscules et spores/vésicules, respectivement et le nombre de spores produites dans le milieu était supérieur à 200. Aux jours 21 et 35 suivant l'infection par le pathogène, le nombre de spores extra-racinaires ainsi que l'abondance des arbuscules dans les racines étaient significativement supérieurs comparativement aux plantules non infectées. Par ailleurs, l'index

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de sévérité mesuré dans les plantules mycorrhizées a diminué de plus de 80 % et 60 % respectivement 21 et 35 jours après infection par le pathogène. De même, l'aire sous la courbe de progression de la maladie, estimée au jour 35, a été réduite de 29 % chez les plantules mycorrhizées par rapport aux plantules témoins. Quel que soit le traitement (plantules mycorrhizées ou non en présence/absence de *M. Fijiensis*), aucune différence significative n'a été observée dans la biomasse sèche des plantules, la taille et le diamètre du pseudo-tronc et la surface foliaire aux deux dates d'observation. **Conclusions.** Ces résultats soulignent la complexité des relations multi-trophiques entre les microorganismes aériens et souterrains *via* la plante. Ils suggèrent, par ailleurs, que le CMA peut diminuer, dans les stades précoces d'infection, les symptômes causés par *M. fijiensis*, possiblement *via* l'induction d'un mécanisme de résistance systémique. La diminution effective des symptômes observée chez les plantules mycorrhizées suggère que ces microorganismes peuvent représenter une option potentiellement intéressante à considérer dans le contexte d'un contrôle intégré de la maladie des raies noires.

Mots clés : bananier / *Musa accuminata* / mycorrhize à vésicules et à arbuscules / *Mycosphaerella fijiensis* / *Rhizophagus irregularis* / méthode de lutte contre la maladie / culture *in vitro*

1 Introduction

Black Sigatoka, caused by *Mycosphaerella fijiensis*, is the most devastating disease of banana and plantain worldwide [1]. It belongs to the 'Sigatoka disease complex', which includes *M. musicola*, the causal agent of Sigatoka leaf spot disease and *M. eumusae*, which causes Eumusae leaf spot disease [2]. The disease was detected for the first time in the Fiji islands in 1963 [3] and is nowadays present in most production areas [4]. The disease affects the leaves, reducing their photosynthetic activity and carbohydrate production and can also reduce the green life of fruits [5], and impacts pulp color and early ripening [6], impediments to export in case of strong infestation. The fungus is particularly aggressive due to the massive production of ascospores, a short life cycle and prominence for rapid tissue colonization [7]. It is nowadays considered as the most economically important threat to the banana industry [1] affecting the popular dessert banana (AAA – commonly from the Cavendish subgroup, and some AAB genomes) and plantain (AAB genome) cultivars [2].

Conidia from diseased plants are mostly disseminated by water over short distances, while ascospores are disseminated over long distances *via* the wind. In intensive banana cultivation systems oriented toward export, *M. fijiensis* is essentially controlled by repeated application of fungicides. The use of resistant cultivars is another option considered, especially for small farmers unable to afford chemical control products [8]. Breeding plantains and cooking bananas widely used for local markets and as staple food has been successful (*e.g.* FHIA-25, FHIA-91). However, for dessert bananas (*e.g.* Cavendish subgroup) conventional breeding is not possible because of the female sterility of all cultivars in this group. Breeding programs to develop resistant cultivars of dessert and cooking bananas, however, do exist and genetic transformation is being attempted (see [8]). In parallel to these strategies, a number of cultural practices, such as the elimination of infected leaves [9], proper watering and drainage, and control of weeds have been used to decrease sources of inoculum and to circumvent the conditions favorable to the development of the pathogen [10].

Over the last few years, microorganisms such as plant growth-promoting rhizobacteria, arbuscular mycorrhizal fungi (AMF) and Trichoderma spp. [11, 12] have been proposed as promising alternatives to control plant pathogens. In soil, AMF are prevalent microorganisms that form symbiotic associations with 80% of plant species including most agricultural crops [13]. These obligate root symbionts provide the plants with minerals (*e.g.* phosphorus and nitrogen) in exchange for carbohydrates. They also influence the physiology of their host plants by modulating their responses to biotic and abiotic stresses [14, 15]. Several studies have reported their role in the reduction of incidence and/or severity of soil-borne pathogens [12–16]. In banana, they have been shown to control the nematodes *Radopholus similis* [17, 18] and *Pratylenchus goodeyi* [19] and the fungal pathogens *Cylindrocladium spathiphylli* [20] and *Fusarium oxysporum* var cubense [21].

In the recent years, an increasing number of studies have been conducted to determine whether AMF could control shoot or leaf pathogens of various hosts. For instance, higher resistance was observed in tomato against *Alternaria solani* [22] and *Botrytis cinerea* [23], in cucumber against *Colletotrichum orbiculare* [24], and in potato against *Phythophthora infestans* [25]. In this latter study, the authors investigated the interaction between *P. infestans* and an AMF under strict *in vitro* culture conditions. Potato plantlets were pre-colonized by *Rhizophagus irregularis* MUCL 41833 in a Mycelium Donor Plant (MDP) *in vitro* culture system [26]. This *in vitro* cultivation system offers numerous advantages which are, among others, the absence of any confounding effects due to unwanted microbes and the possibility for time-course gene expression analysis [27]. Recently, Koffi *et al.* [18] adapted the MDP *in vitro* culture system to bananas and investigated the impact of *R. irregularis* MUCL 41833 on *R. similis*, opening further perspectives for studies with banana leaf pathogens such as *M. fijiensis*.

The objective of the present study was to investigated the interaction between an AMF, *R. irregularis* MUCL 41833, and *M. fijiensis* MUCL 47740 on banana (*Musa accuminata* cv. Grande Naine, AAA genome, Cavendish group) grown under *in vitro* culture conditions. Leaf infection by the pathogen and AMF development within and outside the roots were simultaneously analyzed to evaluate the impact of the AMF on the early development of the Black Sigatoka disease and the impact of the leaf pathogen on the fungal symbiont.

2 Materials and methods

2.1 Biological material

2.1.1 Banana plantlets and growth conditions

Tissue-cultured banana plantlets (*Musa acuminata* Colla c.v. Grande Naine, AAA genome, Cavendish group) were graciously provided by the International Musa Germplasm collection at the Bioversity International Transit Centre (Laboratory of tropical crop improvement, KULeuven, Belgium). The plant material was proliferated and regenerated in test tubes [28] on Murashige and Skoog (MS) medium [29] supplemented with 30 g L^{-1} sucrose, 10 mg L^{-1} ascorbic acid, 10^{-3} M indole-3-acetic acid, 10^{-2} M 6-benzilaminopurine and 3 g L^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA). The pH was adjusted to 6.12–6.15 before sterilization at 121 °C for 15 min. Individual shoots (±1 cm height) were inserted in each test tube for regeneration. After 12 weeks, the banana plantlets were transferred on MS medium without growth hormones, for rooting. Rooted plantlets were obtained after a period of 4 weeks. During the different micropropagation stages, banana plantlets were placed in a growth chamber at 28/24 °C (day/night), 12 h illumination, 70% relative humidity and photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}.

2.1.2 *Medicago truncatula*

Seeds of *Medicago truncatula* Gaertn. cv. Jemalong A 17 (SARDI, Urrbrae, Australia) were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 12 min, rinsed three times in deionized sterile water and placed for germination on Petri plates (90 mm diameter – 25 seeds per Petri plates) filled with 35 mL of modified Strullu-Romand (MSR) medium [30] without sucrose and vitamins, and solidified with 3 g L^{-1} Phytagel. The Petri plates were incubated at 27 °C in the dark.

2.1.3 Arbuscular mycorrhizal fungus

A root organ culture (ROC) of *Rhizophagus irregularis* (Błaszk, Wubet, Renker & Buscot) [31] [as 'irregulare'] MUCL 41833 (Synonymy: *Glomus irregulare*) was provided by the Glomeromycota *in vitro* collection (GINCO, Louvain-la-Neuve, Belgium). The AMF was grown in association with Ri T-DNA transformed carrot (*Daucus carota* L.) roots clone DC1 on Petri plates (90 mm diameter) containing MSR medium solidified with 3 g L^{-1} Phytagel, following the method detailed in Cranenbrouck *et al.* [32]. The Petri plates were incubated several months in the dark in an inverted position at 27 °C to obtain thousands of spores.

2.1.4 *Mycosphaerella fijiensis* inoculum preparation and inoculation

The strain *Mycosphaerella fijiensis* Morelet MUCL 47740 was provided by the Mycothèque de l'Université catholique de Louvain (MUCL, Louvain-la-Neuve, Belgium). The strain was cultured in Petri plates (90 mm diam.) on 3% water-agar (Sigma-Aldrich, St. Louis, USA) medium and incubated in the dark at 22 °C to produce conidia. Two weeks later, conidia were transferred onto 39 g L^{-1} potato dextrose agar (Scharlau Chemie S.A., Barcelona, Spain) and grown for an additional two weeks. The strain was then transferred onto V8 juice agar medium [33] in Petri plates (90 mm diameter) for another 10 days under continuous light at 22 °C.

Inoculum was prepared by grinding fungal mycelium in 10 mL of sterile water with a mortar and pestle. The ground material was then passed through sterile cheesecloth (one layer pore size of approximately 150 µm) to separate the mycelium from conidia. The conidial suspension was concentrated by centrifugation at 3 700 rpm for 10 min at 4 °C. The concentration of conidia was determined using a Fuchs-Rosenthal counting chamber and further adjusted to obtain a suspension of 10^3 conidia mL^{-1} [34]. Gelatin (1%) (Merck, Darmstadt, Germany) was added to the final conidial suspension for better adhesion of the conidia on the leaves.

2.2 Experimental set-up

2.2.1 Mycelium donor plant (MDP) *in vitro* culture system

The mycelium donor plant (MDP) *in vitro* culture system, developed by Voets *et al.* [26] and adapted by Koffi *et al.* [18] for bananas in a completely closed set-up, was used to produce mycorrhizal banana plantlets. Briefly, the system consisted of a vented Petri plate (145 mm diam. × 20 mm height) (Greiner Bio-One®, Kremsmünster, Austria), in which the base of a 55 mm diam. Petri plate was placed to physically separate a root compartment (RC, *i.e.* the base of the 55 mm diam. Petri plate) from a hyphal compartment (HC, *i.e.* the 145 mm diam. Petri plate minus the base of the 55 mm diam. Petri plate). In both compartments, MSR medium without sugar and vitamins solidified with 3 g L^{-1} Phytagel was added (*i.e.* 20 mL in the RC and 150 mL in the HC). The medium in the HC was further amended with 10 mM of 2-morpholinoethanesulfonic acid monohydrate (MES; Fluka, Bornem, Belgium) to stabilize the pH at 6. A four-day old *M. truncatula* seedling was transferred to the RC with the roots placed on the surface of the MSR medium and the shoot extending outside the culture system *via* a hole (2–3 mm in diameter) made in the side of the lid and the base of the 145 mm diam. Petri plate. The plantlets were then inoculated with a plug of gel containing extraradical hyphae and approximately 100 spores of the AMF. The systems were sealed with Parafilm (Pechiney, Chicago, IL, USA) and the holes carefully plastered with silicon grease (VWR International, Leuven, Belgium). The systems were covered with black plastic bags to protect the roots and AMF from light, leaving the shoots exposed. The systems were subsequently transferred to a growth chamber (21/18°C day/night, 16 h illumination, 70% relative humidity and PPF of 300 µmol m^{-2} s^{-1}). Starting from week 3, 10 mL of MES buffered (pH 6) MSR medium without sugar and vitamins and solidified with Phytagel was added weekly into the RC. In parallel, control cultures plates were set up following the same process as above but the RC remained empty.

Six weeks after inoculation, the extraradical mycelium (ERM) of the AMF started to develop in the HC. When half of the surface of the HC was covered by the ERM, three banana plantlets were introduced in this compartment. To accommodate the banana plantlets in the system, the lid of the 145 mm diam. Petri plate was replaced by another one surmounted with a Full Gas Microsac bag (300 mm high and 90 mm diam. at the base – SACO2-Combiness, Eke, Belgium) with strips of membrane filters (0.22 μm over a total length of 40 cm and a width of 0.5 mm) allowing gas exchanges while maintaining the RC and HC under sterile conditions. To attach this Full Gas Microsac bag, the lid of a 145 mm diam. Petri plate was cut to make a hole of 85 mm in diam. over which a plastic ring (90 mm diam. and 15 mm high) was fixed with thermofusible plastic glue. The Full Gas Microsac bag was then fixed on the ring with Parafilm. The modified lid was sterilized at 25 kGy before use on the culture systems. The systems were then placed in a growth chamber (28/24 °C day/night, 12 h illumination, 70% relative humidity and PPF of 300 µmol m^{-2} s^{-1}). Besides the weekly addition of 10 mL of buffered (pH 6) MSR medium in the RC, 50 mL of the same medium was added weekly to the HC after the introduction of the banana plantlets. Ten culture plates, each containing three banana plantlets, with or without AMF, were prepared.

2.2.2 Half-closed arbuscular mycorrhizal plant (H-AMP) *in vitro* culture system

After 3 weeks in the MDP *in vitro* culture system, the banana plants were removed from the HC and introduced into H-AMP *in vitro* culture systems consisting of Petri plates (145 mm diam.) containing 200 mL of buffered (pH 6) MSR medium without sugar and vitamins and solidified with 3 g L^{-1} Phytagel. A hole was made on the lid of the Petri plate to allow the shoot to grow outside. The hole was plastered with silicon grease to avoid contaminations in the Petri plates. The H-AMP *in vitro* culture systems were placed in a growth chamber under the same conditions as described above. Two weeks later, four plantlets were randomly selected in each treatment to evaluate plantlet development and AMF root colonization. Two additional plantlets per group (mycorrhizal and non-mycorrhizal plants) were randomly discarded to equalize the number of plantlets per treatment.

2.2.3 *M. fijiensis* inoculation

After 2 weeks of growth, half of the banana plantlets were inoculated with one ml (*i.e.* 10^3 conidia) of the conidial suspension of *M. fijiensis* sprayed on the underside of the third leave [35], while the other half remained free from the pathogen. The plantlets were placed under the same growing conditions as above but the relative humidity was set at 95% during the first 3 days following inoculation of the pathogen and 70% thereafter. In total, four treatments were considered with 12 replicates each: mycorrhizal banana plantlets inoculated (+AMF +*M. fijiensis)* or not (+AMF -*M. fijiensis*) with *M. fijiensis* and non-mycorrhizal banana plantlets inoculated (-AMF +*M. fijiensis*) or not (-AMF -*M. fijiensis*) with *M. fijiensis*. For each treatment, 6 banana plantlets were randomly sampled at day 21 and day 35, following inoculation with the pathogen.

2.3 Data assessment

Plant dry weight was measured following drying at 60 °C for 72 h. The pseudostem height and diameter, and the surface area of the last unwrapped leaf were measured. The pseudostem height was measured from the base of the rhizome to the crossing point of the two last leaves. The pseudostem diameter was measured at 2 cm from the base of the rhizome. The length (l) and width (w) of the leaves were measured and the leaf area was calculated as leaf area = $\alpha\ lw$, where $\alpha = 0.7$ [36]. The number of extra-radical spores of AMF produced in the culture systems was estimated according to Declerck *et al.* [37]. The AMF root colonization was also estimated on the dried roots at day 21 and day 35. The roots were immersed in 10% KOH overnight at ambient temperature. The roots were rinsed several times with deionized water and incubated in 3% H_2O_2 for 30 min [38]. They were subsequently stained for 45 min in blue ink (Parked Quink, Saint Herblain, France) diluted (1:50 v:v) in 1% HCl [39]. Root colonization was estimated according to McGonigle *et al.* [40]. For each sample, a minimum of 150 root intersections was assessed.

The severity index (SI) of banana plants was rated visually at day 21 and day 35 in presence (+AMF +*M. fijiensis*) or absence (-AMF +*M. fijiensis*) of AMF. The SI of the disease was determined following the method described by Gauhl *et al.* [41], using the formula:

$$\mathrm{SI} = \sum n\mathrm{b} \times 100/(N-1)T$$

where: n = number of leaves for each scale degree, b = degree of scale (0 = no symptom, 1 < 1% of leaf presenting symptoms, 2 = 1 to 5% of leaf presenting symptoms, 3 = 6 to 15% of leaf presenting symptoms, 4 = 16 to 33% of leaf presenting symptoms, 5 = 34 to 50% of leaf presenting symptoms, 6 = 51 to 100% of leaf presenting symptoms). N = number of degree used in the scale and T = number of total leaves assessed.

The rate of disease development was also assessed by the area under disease progress curves (AUDPC) proposed by Campbell and Madden [42] at day 21 and 35:

$$\mathrm{AUDPC} = \sum_{i=1}^{n_i-1}((Y_i + Y_{i+1})/2)(X_{i+1} - X_i)$$

where: Y_i = disease severity at the i^{th} observation, X_i = time at the i^{th} observation, n_i = number of times and i = order index for the times.

2.4 Statistical analysis

The data were analyzed with the software Statistica for Windows (StatSoft, 2001). Data for percentage hyphae, arbuscules, and spores/vesicles were arcsin $\sqrt{(x/100)}$ transformed and data for SI and AUDPC were square root transformed prior to analysis. Data were subjected to a one-way analysis of variance (ANOVA). Means were separated by Tukey's Honest Significant Difference (HSD) to identify significant differences ($P \leqslant 0.05$) between the treatments.

Table I. Root colonization of banana plantlets associated with *Rhizophagus irregularis* MUCL 41833, 21 and 35 days after their inoculation (+AMF +*M. fijiensis*) or not (+AMF –*M. fijiensis*) with *Mycosphaerella fijiensis* MUCL 47740.

Treatment	Structure	Days after *M. fijiensis* inoculation	
		21	35
+AMF –*M. fijiensis*	Hyphae	17.09 ± 2.60 a / A*	12.00 ± 2.43 a / A
	Arbuscules	45.64 ± 4.63 a / A	43.47 ± 5.66 a / A
	Spores/vesicles	4.81 ± 2.16 a / A	7.57 ± 3.14 a / A
+AMF +*M. fijiensis*	Hyphae	13.52 ± 1.12 a / A	11.30 ± 0.83 a / A
	Arbuscules	59.53 ± 1.80 b / A	58.68 ± 1.19 b / A
	Spores/vesicles	8.02 ± 2.04 a / A	8.19 ± 3.26 a / A

* Values (mean ± standard error of 6 replicates). For each structure across the treatments, data in the same column followed by identical small letters do not differ significantly ($P \leqslant 0.05$). For each structure within a treatment, data in the same line followed by identical capital letters do not differ significantly ($P \leqslant 0.05$). Both analyses were conducted as a one-way ANOVA (Tukey's HSD).

3 Results

Two days after transfer of the banana plantlets from the MDP *in vitro* culture systems into the H-AMP *in vitro* culture systems, a few hyphae started to emerge from the banana roots. Hyphal length increased rapidly thereafter and the first newly produced spores were observed 4 days after the transfer of the banana plantlets in the H-AMP *in vitro* culture systems. After two weeks in the H-AMP *in vitro* culture systems, the length of hyphae and number of extraradical spores produced were 582 ± 27 cm and 220 ± 41 individuals, respectively, while the percentages of hyphae, arbuscules, and spores/vesicles were 12 ± 2%, 56 ± 2%, and 10 ± 2% respectively.

At day 21 (*i.e.* 3 weeks after inoculation of *M. fijiensis* or 5 weeks after plating in the H-AMP *in vitro* culture systems), the number of extraradical spores produced in the +AMF +*M. fijiensis* treatment was significantly higher (*i.e.* 16.200 ± 460) than in the +AMF -*M. fijiensis* treatment (*i.e.* 6.687 ± 403). The hyphal length could not be estimated due to its profuse development. Regarding the colonization of the roots, no significant differences were observed in % hyphae and % spores/vesicles in presence or absence of *M. fijiensis* (*table I*). To the contrary, the % arbuscules was significantly higher in the plantlets in presence of the pathogen.

Similar observations were made at day 35 (*i.e.* 5 weeks after inoculation of *M. fijiensis* or 7 weeks after plating in the H-AMP *in vitro* culture systems). The number of extraradical spores produced was significantly higher (*i.e.* 26.485 ± 1.851) in presence of *M. fijiensis* (*i.e.* +AMF+*M. fijiensis* treatment) as compared to the treatment in absence (*i.e.* +AMF-*M. fijiensis* treatment) of the pathogen (*i.e.* 18.683 ± 1.558). The length of hyphae could not be estimated for the same reason as above. No significant differences were noted in % hyphae and % spores/vesicles, while the % arbuscules was significantly higher in the plantlets inoculated with the pathogen (*table I*). The % hyphae, arbuscules and spores/vesicles did not differ between day 21 and day 35 in presence as well as in absence of *M. fijiensis* (*table I*).

The banana plantlets transferred from the MDP *in vitro* culture systems into the H-AMP *in vitro* culture systems produced new leaves and primary, secondary and tertiary roots until the end of the experiment. At day 21 and 35, no significant differences were observed in plantlet dry weight, pseudostem height and diameter, and leaf area between the four treatments (*table II*). Between day 21 and day 35, plantlet dry weight significantly increased except in the +AMF +*M. fijiensis* treatment, where no significant difference was observed. Whatever the treatment, pseudostem height did not differ significantly between day 21 and day 35, while the diameter of pseudostem increased significantly only in the treatment -AMF -*M. fijiensis*. Whatever the treatment, the leaf emission rate was about one new leave every week (data not shown). Finally, the area of the last unwrapped leaf decreased significantly between day 21 and day 35 for the treatments with AMF in presence (+AMF +*M. fijiensis*) as well as absence (+AMF -*M. fijiensis*) of the pathogen, while it remained unchanged in the two other treatments.

Whatever the treatment, no symptoms were observed before day 21. The first lesions on the leaves were observed at day 21 in the -AMF +*M. fijiensis* treatment on all banana plantlets replicates and only one replicate in the +AMF +*M. fijiensis* treatment (*figure 1*). Brown dots approximately 1 mm in diameter were noticed on the underside of the leaves. These small dots evolved rapidly in streaks and larger spots that started to coalesce (*figure 1*). With the increase of lesions, the leaves changed color from green to yellow. The same symptoms were observed in presence of AMF, although these symptoms appeared later and the number of dots and spots were less numerous (*figure 1*). At day 21 as well as day 35, SI and AUDPC values were significantly lower for the mycorrhizal banana plantlets (*table III*). The value of SI and AUDPC significantly increased between day 21 and 35 in presence as well as in absence of the AMF (*table III*).

4 Discussion

In this study we report for the first time on the interaction between an AMF (*R. irregularis* MUCL 41833) and the hemibiotroph *M. fijiensis*, the causal agent of Black Sigatoka, on banana plantlets (c.v. Grande Naine) grown under *in vitro* culture conditions. The banana plantlets were colonized by the AMF prior to any interaction with the pathogen. Typical necroses were noticed on leaves, 21 and 35 days after infection with *M. fijiensis*. However, the SI as well as the AUDPC was drastically reduced in presence of the AMF, 21 (> 80%) as

Table II. Growth parameters of banana plantlets associated (+AMF) or not (-AMF) with *Rhizophagus irregularis* MUCL 41833, 21 and 35 days after their inoculation (+*M. fijiensis*) or not (–*M. fijiensis*) with *Mycosphaerella fijiensis* MUCL 47740.

Treatment	Growth parameters	Days after *M. fijiensis* inoculation	
		21	35
-AMF –*M. fijiensis*	Plant dry weight (g)	4.43 ± 0.90 a / A*	5.29 ± 0.22 a / B
	Pseudostem height (cm)	7.27 ± 0.08 a / A	7.90 ± 0.25 a / A
	Pseudostem diameter (cm)	1.13 ± 0.03 a / A	1.28 ± 0.05 a / B
	Last unwrapped leaf area (cm^2)	38.36 ± 1.66 a / A	36.90 ± 3.14 a / A
-AMF +*M. fijiensis*	Plant dry weight (g)	4.51 ± 0.14 a / A	5.38 ± 0.13 a / B
	Pseudostem height (cm)	7.37 ± 0.16 a / A	7.67 ± 0.23 a / A
	Pseudostem diameter (cm)	1.17 ± 0.05 a / A	1.20 ± 0.04 a / A
	Last unwrapped leaf area (cm^2)	38.01 ± 3.71 a / A	34.25 ± 2.54 a / A
+AMF –*M. fijiensis*	Plant dry weight (g)	4.42 ± 0.08 a / A	5.13 ± 0.07 a / B
	Pseudostem height (cm)	7.67 ± 0.23 a / A	7.45 ± 0.15 a / A
	Pseudostem diameter (cm)	1.2 ± 0.04 a / A	1.18 ± 0.04 a / A
	Last unwrapped leaf area (cm^2)	39.64 ± 1.99 a / B	31.51 ± 2.02 a / A
+AMF +*M. fijiensis*	Plant dry weight(g)	4.84 ± 0.13 a / A	4.92 ± 0.14 a / A
	Pseudostem height (cm)	7.43 ± 0.14 a / A	7.43 ± 0.14 a / A
	Pseudostem diameter (cm)	1.17 ± 0.04 a / A	1.17 ± 0.04 a / A
	Last unwrapped leaf area (cm^2)	42.51 ± 2.62 a / B	30.40 ± 1.67 a / A

* Values (mean ± standard error of 6 replicates). For each growth parameter across the treatments, data in the same column followed by identical small letters do not differ significantly ($P \leqslant 0.05$). For each growth parameter within a treatment, data in the same line followed by identical capital letters do not differ significantly ($P \leqslant 0.05$). Both analyses were conducted as a one-way ANOVA (Tukey's HSD).

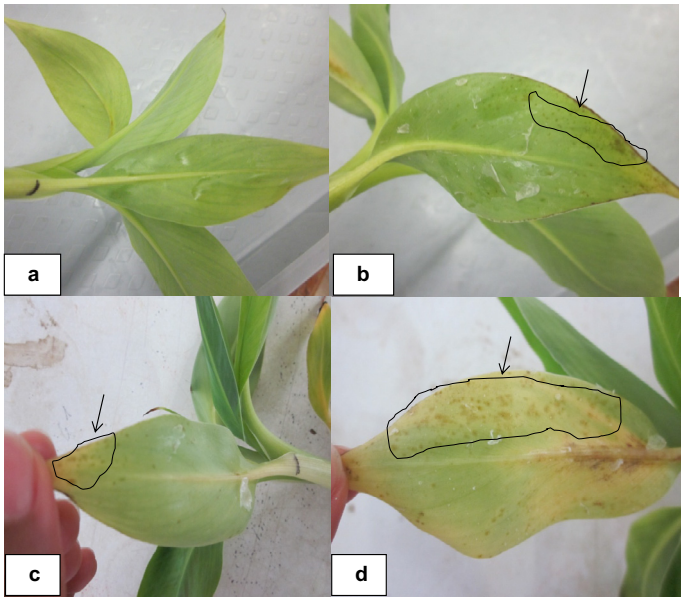

Figure 1. Leaf symptoms of 21 day- old plantlets infected by *Mycosphaerella fijiensis* MUCL 47740 and associated (a) or not (b) to the AMF *Rhizophagus irregularis* MUCL 41833 and of 35 day-old plantlets infected by the pathogen and associated (c) or not (b) to the mycorrhizal fungus. Black arrow shows symptoms.

well as 35 (~60%) days after infection with the pathogen. This clearly demonstrated the bio-control effect of the AMF upon *M. fijiensis* during its early stages of development and under controlled conditions.

The MDP *in vitro* culture system developed by Voets *et al.* [26] and recently adapted by Koffi *et al.* [18] allowed for the dense and homogenous pre-mycorrhization of the plantlets within a period of three-weeks. Following their subsequent transfer to individual Petri plates, the banana plantlets and their root fungal associate further developed with increased plant biomass over time and the production of numerous spores and an extensive network of hyphae. This corroborates an earlier study demonstrating that an ERM network composed of numerous actively growing hyphae arising from a donor plant is the most adequate source of inoculum for the extensive colonization of banana plantlets within a period of time compatible

Table III. Severity index (SI) and area under disease progress curve (AUDPC) values of banana plantlets associated (+AMF) or not (-AMF) with *Rhizophagus irregularis* MUCL 41833, 21 and 35 days after their inoculation (+*M. fijiensis*) with *Mycosphaerella fijiensis* MUCL 47740.

Treatment	Parameter	Days after *M. fijiensis* inoculation	
		21	35
-AMF +*M. fijiensis*	SI	7.14 ± 0.00 b / A*	19.04 ± 1.51 b / B
	AUDPC	74.97 ± 0.00 b / A	183.26 ± 10.54 b / B
+AMF +*M. fijiensis*	SI	1.19 ± 1.19 a / A	7.14 ± 0.00 a / B
	AUDPC	12.50 ± 12.50 a / A	74.97 ± 0.00 a / B

* Values (mean ± standard error of 6 replicates). For each parameter across the treatments, data in the same column followed by identical small letters do not differ significantly ($P \leqslant 0.05$). For each parameter within a treatment, data in the same line followed by identical capital letters do not differ significantly ($P \leqslant 0.05$). Both analyses were conducted as a one-way ANOVA (Tukey's HSD).

with the micropropagation process [18]. Interestingly, a recent study [43] demonstrated that the mycorrhization of banana plantlets at the *in vitro* phase markedly improved their growth at acclimatization (an increase of 40% as compared to non-mycorrhized plantlets), potentially representing a promising tool for the pre-adaptation of micropropagated banana plantlets to *post-vitro* conditions and potentially to speed-up their development for further transfer into the field.

The inoculation of the pathogen was achieved *via* the spraying of a conidial suspension on the underside of the leaves [35]. The first symptoms were observed after 21 days, as generally reported in the literature under controlled conditions, and evolved subsequently until the end of the experiment at day 35. The disease symptoms observed on the banana leaves were drastically reduced in presence of AMF. The AUDPC value was reduced by 59% in the leaves of the AMF banana plantlets 35 days following infection by the pathogen. Identically, the SI was decreased by more than 60% in the pre-mycorrhized plantlets at the early stage of infection (*i.e.* at 35 days following infection by the pathogen).

The percentage of arbuscules within the roots and the number of spore produced in the medium were significantly higher in the banana plantlets infected by *M. fijiensis* at both observations times (*i.e.* 21 and 35 days). Arbuscules are the primary site where minerals and carbohydrates are exchanged between the two partners of the symbiosis [11], while spores are a major sink for carbon. Thus, this may suggest a higher transfer of resources between the plant and the fungal symbiont in the presence of *M. fijiensis*.

Interestingly, these structures have been associated with increased resistance of the plants against various pathogens. Pozo and Azcón-Aguilar [44] observed that root cells of tomato containing arbuscules have a high rate of endogenous jasmonic acid and these plants showed some resistance to *Phytophthora parasitica*. These observations were in line with the results of Gallou *et al.* [27] who used a similar *in vitro* culture system with potato plants challenged with *P. infestans*. In the mycorrhizal potato plants. they observed a delay in the progression of the late blight symptoms over the 4 days of their experiment as compared with the non-mycorrhizal ones. In our study the delay in the appearance and progression of symptoms could possibly be explained by a delayed switch from biotrophic to necrotrophic phase of the pathogen in the mycorrhizal plants.

Several hypotheses can be advanced to explain the reduced level of symptoms in the mycorrhizal plantlets. Obviously, a direct suppressive effect is to be excluded; both organisms (the AMF and pathogen) developing in different niches, below and above-ground respectively. An increased biomass to compensate for symptoms is also to be excluded since no differences were noticed between the plants in presence/absence of the AMF. The most probable and frequently reported mechanism to explain the decreased disease severity of *M. fijiensis* is a systemic defense response triggered by the colonization of the roots by AMF, as shown for other interactions with either root or shoot pathogens. Liu *et al.* [14] were the first to demonstrate the induction of plant defense genes in the shoots of mycorrhizal *M. truncatula* plants. They correlated the induction of defense genes in the shoot with an increase in resistance against *Xanthomonas campestris*. Pozo and Azcón-Aguilar [44] confirmed the induction of defense genes, in particular jamonate (JA)-dependent genes, while Gallou *et al.* [25] suggested a systemic resistance in mycorrhizal potato plants infected by *P. infestans* related to the induction of salicylic acid (SA)-dependent plant defense genes. The higher resistance observed in the banana plantlets in the presence of *M. fijiensis* could be associated with an induction of specific defense genes involved in either one or both of the pathways mentioned.

In the present case, Induced Systemic Resistance (ISR) which can be activated by beneficial microorganisms [23, 44, 45], can result in an increased production of jasmonates (JA) or at least in the activation of JA- and ethylene-dependent defense responses causing increased resistance to a pathogen attack [46]. These plant hormones are key players in plant development and plant defense mechanisms [47, 48] but are also suspected to be involved in the auto-regulation of the AM symbiosis [47], in particular in arbuscular formation [49] and in controlling carbon access to the AMF [49].

To support this hypothesis, Hause *et al.* [49] suggested that JA may increase the sink strength of mycorrhizal roots and thus stimulate the production of carbon in the shoots and their subsequent transport to the roots. As such, the increased density of arbuscules in the roots may result in a higher carbon transfer to the AMF and thus in an increased production of spores. In our experiment, the significant decrease in surface of the last unwrapped leaf in the mycorrhizal banana plantlets (between day 21 and day 35) may be an indication that JA

produced by the plant controlled C-partitioning to the benefit of the mycorrhizal symbiont and thus represent a cost to the above ground development of the plant. However, it is unknown whether the shoot infection by *M. fijiensis* had a stimulating effect on the production of JA by the plant with the subsequent cascade of events resulting in a stronger root colonization and biomass production by the AMF. This hypothesis should be tested.

This could indicate that when *M. fijiensis* switches from its biotrophic to necrotrophic phase, resistance mechanisms induced by AMF would lead to a better protection of the plants and consequently to the delayed appearance of symptoms and to a stimulation of the AMF colonization. It is therefore not excluded that *M. fijiensis* may affect the AMF through a systemic effect of the above-ground on the below-ground organs of the banana plant [50]. In the case of the present interaction, we indeed observed that while mycorrhizal banana plants had lower symptoms caused by *M. fijiensis* (the benefit) they also showed sign of stunted growth (the cost). The promotion of the AMF colonization could thus add to the costs by further reducing the availability of C to the plants and could eventually turn the symbiotic interaction between AMF and plants to a more parasitic one.

Nowadays, the application of AMF in the field to control banana pathogens is seldom considered, although several convincing results have been obtained under controlled pot [20, 21] and *in vitro* [17–19] conditions against root pathogens [17–20]. It is obvious that the pre-colonization of the plantlets at the early stage of growth (hardening/post-hardening phases) and even at *in vitro* phase, as recently demonstrated by Koffi *et al.* [43], would prepare the plant to better resist adverse soil biotic and abiotic conditions. Indeed it is well known from the literature that pre-colonized plants are more adapted to resist pathogens than concomitant colonization by the AMF and infestation by the pathogen. It is thus necessary to investigate the impact of pre-colonization of plantlets at the early stage of growth on their subsequent resistance/tolerance to Black Sigatoka under pot culture and ideally, field conditions, to evaluate whether these organisms should be considered in an integrated control strategy of this major disease.

We have demonstrated that mycorrhizal banana plantlets had reduced symptoms caused by *M. fijiensis* under *in vitro* culture conditions. We also observed an increased production of arbuscules and extraradical spore in presence of the pathogen. These results emphasize the complex multitrophic interactions between above and below-ground microorganisms which are mediated by the plant. Jasmonates, which are known to play diverse roles, from regulating plant defenses upon attack to controlling root colonization by the AMF, may be crucial. The autotrophic *in vitro* cultivation system used in our experiment represents a promising option for investigating more deeply (*i.e.* at the level of defense gene expression, JA concentration and carbohydrates re-allocation) the mechanisms involved in the bio-protection conferred by AMF to the major leaf pathogen of banana.

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