Effect of cultural practices on maize mycorrhization and presence of *Cephalosporium maydis*

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Abstract

The sustainability of agriculture requires the adoption of agricultural soil conservation practices, which generate positive impacts on soil quality, since they promote an increase in the microbiota and its diversity, essential for the establishment and development of any culture. This study aims to assess the influence of the presence of intact extraradical mycelium as a preferential source of inoculum of the native arbuscular mycorrhizal fungi in order to guarantee a better colonization of maize as well as its possible bioprotective effect against *Cephalosporium maydis*. In order to vary the available extraradical mycelium, two experiments, with and without cover crop, were carried out, in which two soil tillage systems and two maize varieties, susceptible and tolerant to the phytopathogenic fungus, were assessed. The capitalization of the benefits, in terms of grain production and *C. maydis* presence, derived from the use of a cover crop were only achieved with minimum tillage. Therefore both cultural practices are necessary to reduce the presence of *C. maydis*, coupling the effect of mycorrhization together with other benefits associated with the cover crop. In these circumstances, the use of tolerant or susceptible varieties seems to be indifferent. Although in the absence of a cover crop and using conventional tillage, yields and lower levels of *C. maydis* in the plants are possibly achieved, this system is more dependent on the maize variety used, does not benefit from the advantages associated with the cover crop, is more costly and environmentally unsustainable.

Keywords: Arbuscular mycorrhizal fungi, late wilt, biotic stress, cover crop, extraradical mycelium, soil tillage.

1. Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops worldwide [1] and plays a growing role in industry and energy resources. However, animal pests, weeds and pathogens have an impact on its yield and quality [2].

In recent years, late wilt disease caused by the soil borne and seed borne fungus *Cephalosporium maydis* Samra, Sabet & Hingoran (synonym *Harpophora maydis*), morphologically and molecularly closely related to Gaeumannomyces-Harpophora species complex [3,4], has emerged as an important disease of maize in the Iberian Peninsula [5], but has also been reported in India, Israel and Hungary [6]. This disease is characterized by rapid wilting of maize plants. In infested fields, incidences up to 60% in susceptible varieties can cause 50% grain yield losses [6]. Initial symptoms of maize wilt appear around flowering, and, from tasseling to maturity, they steadily progress from the lower to upper leaves. As leaf wilting advances, yellowish or reddish-brown streaks appear on the basal internodes of the stalk, which dries up and shrinks [6]. Because of the delay in appearance of initial symptoms until about flowering, this disease has been designated as “late wilt” [7]. *C. maydis* is a soilborne vascular pathogen that penetrates the root tissues and colonizes the xylem [8]. Infection by *C. maydis* results in a reduced number of vascular bundles in the plants and in occlusion of xylem vessels by the growth of fungal mycelia [9]. The most effective way of controlling late wilt is the use of tolerant maize varieties [10,11]. Although the complete absence of symptoms in tolerant maize until the end of the crop season is not frequent, material displaying moderate to high tolerance should be included within an integrated strategy for the control of late wilt of maize [12]. Since the disease causes rapid and sudden wilting, an early diagnosis in plants is needed and may help to restrict disease spread. Due to the fact that infected seeds can carry the pathogen and spread the disease [13], molecular assays are important in recognizing infected seeds and prevent spread to areas where the disease does not occur. In maize plants, the rot of the base of the affected stem and associated roots, is partly due to secondary organisms (e.g. *Fusarium* spp.) from stalks primarily affected by late wilt [14].

Arbuscular mycorrhizal fungi (AMF) are an important component of the soil biota in most agroecosystems and colonise plant roots forming a mutualistic symbiosis. Arbuscular mycorrhiza (AM) likely made possible the conquest of land by the first bryophyte-like plants around 470 million years ago [15] and now colonize more than 80% of plants [16]. These microbial symbionts are present across all soil types and biomes, in natural and anthropogenic ecosystems. These features are, according to current knowledge, unique among other mutualistic symbioses and account for the pivotal importance of AM in earth’s ecosystem [17]. The identification of these fungi was long hampered by their obligate endotrophic habit. These fungi, comprised of multinucleate and largely aseptate hyphae, are grouped in the phylum Glomeromycota [18].

Spores, fragments of AM colonized roots and extraradical mycelium (ERM) are the possible inoculum sources, collectively termed propagules [16]. They are all able to start

new mycorrhizal colonizations of plant roots, although the different propagule forms exhibit different colonization capabilities [19,20]. The ERM might be important for enhancing the roles of AM under field conditions. Root colonization from intact ERM starts earlier and develops faster than from other types of propagule [21,22]. Additionally, ERM formed by indigenous AMF will encompass the functional diversity of the local fungal population and its associated microbes, which is expected to be greater than that of any introduced commercial inoculum. ERM is such an effective propagule that even plant species usually not hosting mycorrhizal fungi can be colonized [23]. Under agricultural systems, ERM can develop on mycotrophic crops, cover crops [24] or natural vegetation that grows before seeding [25] and can be kept intact if appropriate tillage techniques are used.

Many benefits can accrue to plants from their association with AMF, depending on the environmental conditions. In natural ecosystems, the most important role of AM may be in bioprotection rather than in the acquisition of nutrients [26]. The role of AMF in protecting their host against pathogens is well documented for several combinations of cultivated plants and fungal or nematode diseases [27]. Despite the complexity of all these interactions, it is recognized that a well-established AM is crucial for an adequate degree of protection [26]. The mycorrhiza must be created and be well established before contact with the stressor, to achieve a high level of protection [28]. Given that large-scale inoculation with AMF is generally impractical in most regions, the development of management practices that maximize the benefits of this naturally occurring symbiosis is important. Tillage and crop rotation or the use of cover crops are key agronomic practices that need to be considered in developing sustainable production systems. The cultivation of crops that are their natural hosts can increase the population of AMF [24] and thus help to maintain or increase mycorrhizal inoculum present in soil [29].

Tillage system influences the physical, chemical and biological environment of the soil, but their consequences for crop performance depend also on multiple interactions involving the soil, the climate and the crop itself. Therefore, different tillage systems will have different effects on the crop performance. For AMF, the direct effects of the conventional tillage systems are related to physical disruption of the hyphal network and to the mixing of surface residues within the soil profile. These can negatively impact the effectiveness of AMF, particularly the timing of colonization [30], because the ERM is broken and consequently the colonization is essentially initiated by sources of slow-growing inoculum. When the ERM network integrity is affected there is a less efficient crop protection, due to the lower colonization of the plant by AMF [31]. Preserving the hyphal network created by the previous crop, through soil conservation techniques, will increase the probability of infection by AMF [30], because the ERM remains intact. When host plants are present and the soil is not disturbed, hyphae from colonized roots and soil mycelium network are the main source of mycorrhizal inoculum [20].

Since ERM colonizes earlier and develops faster than others sources of propagule, we hyptothesized that AM formation starting from a well-established ERM from AMF and its associated microbial population, would provide to maize plants a more efficacious protection against *C.maydis*. The use of a cover crop to develop a ERM network, which can be maintained intact with a minimum tillage system, could be the way for the early AMF colonization of maize and, consequently, increase the potential bioprotective effect against *C. maydis*. The goal of this study is to understand the potential associated with AMF and, through appropriate agricultural practices, maximize the benefits provided by mycorrhization in the control of *C. maydis* in maize.

2. Materials and Methods

Study sites and experimental design of field experiments

For the present study, maize was used as the host plant and two field experiments were performed. The experiments were carried out in two different properties in Golegã, Ribatejo. These areas are previously known to be infected with *C. maydis*. In one property (site 1), the cover crop was not cultivated, presenting natural vegetation, whereas in the other (site 2), the cover crop (*Lolium multiflorum*) was previously cultivated. The experimental design of field experiments were installed in two-factorial bands of four treatments with 5 and 4 replications (site 1 and site 2, respectively), in which the soil tillage system and the maize variety, tolerant and susceptible to the fungus *C. maydis,* were the study factors. The maize cultivars P1574 and P1570 (Pioneer DuPont) were used in the experiments and are considered tolerant and susceptible to *C. maydis,* respectively.

Sample collection

Samples (biological replicates) consisted of maize roots and shoots. The samples of these two components were collected in bulked samples of six plants, at three different times: two, four and six weeks after emergence of the crop. The sample collection procedure was the same at all sampling dates. Initially, the shoot was cut close to the neck and placed in an identified paper sachet. The root of the plant was harvested carefully, to avoid breaking or damaging the smaller and thinner roots, and placed in a plastic bag. Five or four replications were considered in the sample collection (site 1 and site 2, respectively), two tillage systems (minimum and conventional tillage), two maize varieties (tolerant and susceptible to fungus *C. maydis*) and three sampling dates, which completes a total of 60 samples collected for site 1 and 48 for site 2. Roots were transported to the laboratory and stored at 4ºC until further analysis that occurred within 48 h.

Root samples were used for both fungal isolation and genomic DNA (gDNA) extraction.

Fungal isolates and growth conditions

The surface of maize roots were disinfected to suppress epiphytic microorganisms. Disinfection was carried under a sterile laminar airflow chamber and consisted in a sequential series of two minutes immersion in 96% ethanol, 3% sodium hypochlorite solution, 70% ethanol, followed by final wash repeated three times in ultra-pure water [32]. After disinfection, root pieces were dried in sterile Whatman paper, cut into sections and placed on 90 mm diameter Petri dishes containing Potato Dextrose Agar medium (PDA, Merck, Germany) and incubated for 1-2 weeks at 23-25°C. Morphologically different colonies were transferred to new Petri dishes (60 mm diameter) containing fresh PDA medium. From these plates, by successive repetitions, isolations were made until obtaining pure cultures. The morphological characteristics used to distinguish the different colonies were the rate of growth, mycelium color, texture, nature of the growing margin, and color of the reverse side. Shape of conidia was observed under an Olympus BX-50 compound microscope (1000x magnification).

Additionally, for demonstration of the reliability of the method, three morphologically different strains of *Fusarium* spp. that belong to the collection of the Mycology Laboratory, Institute of Mediterranean Agricultural and Environmental Sciences (ICAAM), University of Évora, Portugal, were included in the experiment, and were grown on the same conditions described above. The fungus *C. maydis* was not possible to be morphologically identified in the experiment and a reference strain (in PDA plates) was kindly given by Dr. Ortiz-Bustos (Department of Crop Protection, Institute of Sustainable Agriculture, Córdoba, Spain) (GenBank Acc number KP164518) [33].

gDNA extraction

Roots, *Fusarium* spp. mycelium and *C. maydis* mycelium and others fungal structures that grew in pure culture in Petri dishes were ground in liquid nitrogen and stored at -80ºC until further analysis. gDNA was extracted using the CTAB (hexadecyltrimethylammonium bromide) method described by Doyle & Doyle (1987) [34], with some modifications. Briefly, about 100 mg of material was placed in 1.5 mL microtones containing pre warmed 600 µL 3% CTAB extraction buffer (10% CTAB, 5 M NaCl, 0.5 M EDTA pH 8.0, 1 M Tris-HCl pH 8.0, plus 4% PVP and 0.1% β-mercaptoethanol added just before use). The solution was incubated at 55ºC for 90 min, gently mixing by inversion every 15 min; 600 µL of chloroform-isoamyl alcohol (24:1) was added to the tubes and gently mixed for 10 min. Samples were centrifuged for 10 min at 12000 rpm (Himac CT 15RE centrifuge), the supernatant was then transferred to a fresh tube following the addition of 800 µL of cold ethanol (-20ºC). Samples were gently mixed by inversion and centrifuged at 13000 rpm (Himac CT 15RE centrifuge) for 20 min. The liquid solution was released and the DNA pellet washed with 500 µL of 70% ethanol to eliminate salt residues adhered to the DNA. Samples were centrifuged again at 13000 rpm (Himac CT 15RE centrifuge) for 15 min and the supernatant were discarded. The pellet was dried in a speed vacuum for 15 min at 55ºC. The pellet was resuspended in 40 µL of ultrapure water and stored at -20ºC. To quantify and assess gDNA purity, the absorbance was measured on a NanoDrop-2000C spectrophotometer (Thermo Scientific). gDNA integrity was checked by 0.8% agarose gel electrophoresis and visualized using GeneTools (Syngene). Tris-Borate-EDTA (TBE) 0.5x buffer (1x: 1 M Tris-HCl, 0.83 M boronic acid, 10 mM EDTA, pH 7.5) was used as electrophoresis buffer. For visualization of the bands, the gel was stained with 1.5 µL of GreenSafe (NZYTech), which binds to the DNA and, when exposed to ultraviolet radiation, emits fluorescence. GeneRuler™ 1 kb Plus DNA Ladder was used as molecular marker. Gel ran for 1 h 20 min at 80 V. Samples were diluted to a concentration of 20 ng/µL.

Amplification of the ITS region by PCR

The ribossomal internal transcribed spacer (ITS) region of nuclear rDNA was amplified through PCR from gDNA by using ITS1 and ITS4 primers [35]. PCR reactions consisted of 0.5 µL of gDNA, 10X DreamTaq buffer (Thermo Scientific), 25 mM $MgCl_2$ (Fermentas), 0.4 mM of each dNTP (Thermo Scientific), 1 µM of each primer, 2.5 U of DreamTaq DNA polymerase (Thermo Scientific), in a total reaction volume of 50 µL. Amplification was carried out in a MyCycler™ Thermal Cycler (Bio-Rad) at 95ºC for 3 min followed by 39 cycles at 95ºC for 30 s, 55ºC for 45 s, and 72ºC for 2 min and a final extension at 72ºC for 10 min. Amplified products were analysed by 1% agarose gel electrophoresis, performed at 80 V for 1 h 20 min, and visualized as described previously. PCR products were purified with the DNA purification kit of enzymatic reactions (NZYTech), according to the manufacturer's protocol, and then sent for sequencing for the company Macrogen Inc., Madrid. Sequence analysis of the ITS sequences was based on a ClustalW Multiple alignment made in BioEdit Sequence Alignment Editor v.7.2.3 software [36]. The search for homologous sequences was done using Basic Local Alignment Search Tools (BLAST) from National Center for Biotechnology Information (NCBI). All fungal sequences considered were at least 98% identical to the best hit in the NCBI database.

Parameters Evaluated

Shoot Dry Weight (SDW)

When the shoot was harvested, it was placed in paper bags corresponding to each composite sample, which were then

placed in the drying oven at 60ºC for approximately 72 h. After that period, the material contained in each sachet was weighed with a semi-analytical balance (KERN & Sohn GmbH, KB 600-2) and the results of dry matter production (DM) were recorded.

Mycorrhizal Colonization

For analysis of the mycorrhizal colonization, it is necessary to stained the roots to bring out the fungal structures. Thus, Trypan Blue was used, which binds only to these structures due to the affinity to chitin. The staining procedure consisted of the following steps: a) place about 0.7 g of roots of each composite sample in a histology cassette; b) dip all cassettes into 10% (w/v) potassium hydroxide (KOH); c) autoclaving for 12.5 min at 121ºC to degrade and eliminate cellular constituents; d) wash thoroughly with tap water to remove excess KOH and drain; e) stain in a solution containing 0.1% Trypan Blue in lactoglycerol in the proportion of (1: 1: 1) (glycerol, 80% lactic acid and water) for about 11 min at 70ºC in a water bath. In this step, the Trypan Blue will bind to the chitin from the cell wall of the fungus; f) remove the cassettes containing the stained roots from the solution described above and store in a 50% (v/v) glycerol solution. The roots can be observed under the microscope after 48 h but may remain submerged in the 50% (v/v) glycerol solution for long periods of time. It should be noted that this solution dissolves the Trypan Blue which is not bound to fungal structures, whereby a better contrast can be obtained between the roots and these structures.

To determine the mycorrhizal colonization, the intersection method described by McGonigle et al. (1990) was used [37]. In this method, the stained roots were mounted on microscope slides and covered with a 24x60 mm coverslips. Roots were aligned parallel to the long axis of the slides and observed under an optical microscope at magnification x200. For each sample, two slides were made and observed, both of which were treated as a single unit. The quantification of the mycorrhizal colonization was made by complete passes across each slide perpendicular to its long axis. The distance between passes is not critical but should be constant for a subsample. The number of intersections of roots with vertical crosshair was counted in the following categories: “negative” (no fungal material in root), “arbuscules”, “vesicles”, and “hyphae only”. The arbuscular colonization (AC) and vesicular colonization (VC) were calculated by dividing the count for the “arbuscules” and “vesicles” categories respectively by the total number of intersections examined. Hyphal colonization (HC) was calculated as the proportion of non-negative intersections. Of all the possible forms of mycorrhizal colonization, only the presence of arbuscules and hyphae were considered, since these are representative of the degree of colonization of the root of a plant. All of the data collected using the magnified intersections method was examined in a random order with the identity of the roots unknown to the observer.

Quantification of *C. maydis* gDNA by qPCR

Sequences obtained from the ITS regions, using ITS1 and ITS4 primers, were aligned with *C. maydis* sequences present in the database (NCBI), as well as with the assigned sequence [33], using the ClustalW algorithm [38]. Based on the alignment, specific primers were designed for *C. maydis* using Primer Express 3.0 software for Real-Time PCR (Applied Biosystems), the primer forward being 5'-GGGGCCCCCAAGTACATC-3’ and the primer reverse 5'-GGGTTTAGCGGCTGGAAG-3', and the SYBRGreen technology was initially tested. However, specificity for *C. maydis* was not achieved and TaqMan technology was followed, the primers and probe were designed for *C. maydis* with the same software (Table 1).

Table 1 | Sequences of qPCR oligonucleotide primers and probe designed on ITS gene region for *C. maydis* [39].

Primers (5’→ 3’)	Probe (5’→ 3’)
Fw: XXXXXXXXXXXXXXXXX Rv: XXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXX

qPCR reactions were carried out on a 7500 Real Time PCR System (Applied Biosystems) using 100 ng of gDNA as template, 2x NZY qPCR Probe Master Mix (Nzytech), 400 nM of each primer and 100 nM of probe (Nzytech), in a total volume of 20 µL. The threshold cycle (Ct) values were acquired for each sample with the Applied Biosystems 7500 software, with the following cycling conditions: 10 min at 95ºC for initial denaturation and an amplification program of 40 cycles at 95ºC for 15 s and 60ºC for 1 min. The fluorescence threshold was manually set above the background level. To quantify the presence of the *C. maydis* the Ct values were used. Four biological replicates and three technical replicates were considered for each sample. Fungi positive target controls and no template controls were included in all plates.

As a measure of sensitivity and the quantitative range of the developed qPCR procedure, the limit of detection was determined. A total of 16 standards were prepared by a two-fold serial dilution (2^{-1}, 2^{-2}, ..., 2^{-16}) of the *C. maydis* gDNA extracted.

Grain Production

Grain production was obtained from 5 and 4 replicates, respectively, at sites 1 and 2 of an area of approximately 612 m^2 per replicate.

Statistical analysis

Data analysis was performed using the MSTAT-C program (version 1.42; Michigan State University), followed by a randomized ANOVA. Fisher's Least Significance Difference (LSD) was used to separate the means.

3. Results

Morphological and molecular identification of the isolates obtained

From the total of the isolates obtained through morphological identification, only those that showed visual differences between them were selected, leading to a total of 12 isolates for site 1 and 10 isolates for site 2. The gDNA of the 22 isolates was extracted and amplified the ITS region by PCR using ITS1 and ITS4 primers. The analysis of the ITS region from the isolates obtained in the experiments allowed the identification of the two *Fusarium* species: *F. oxysporum* (GenBank Acc number MH094661, 99% identity) and *F. verticillioides* (GenBank Acc number MH094662, 99% identity). The analysis of the strains of *Fusarium* spp. from the collection of the Mycology Laboratory allowed to additionally identify the species *F. graminearum* (GenBank Acc number MH094665, 100% identity), *F. incarnatum* (GenBank Acc number MH094664 99% identity) and *F. solani* (GenBank Acc number MH094664, 99% identity), which were included in the TaqMan probe specificity analysis designed for *C. maydis*.

C. maydis TaqMan probe specificity

The specificity of the *C. maydis* assay in relation to *Fusarium* species was confirmed, firstly *in silico* and then experimentally. The probe was also tested for cross-reactivity with gDNA of *Alternaria tenuissima*, *Cladosporium cladosporioides* and *Epicoccum nigrum*, belonging to the Mycology Laboratory collection, and no cross-reaction was found.

Sensitivity and linearity of qPCR procedures

As a measure of sensitivity and linearity of the qPCR procedures, dilution series of *C. maydis* extracted gDNA were used. gDNA tested in PCR ranged from final concentration of 1.53×10^{-3} ng to 100 ng, in a total of 16 data points. Standard curves were automatically generated by the instrument software (Applied Biosystems). *C. maydis* presented a linear correlation (R^2=0.960) between Ct and template gDNA amount, confirming the reliability of the assays and suggesting absence of PCR inhibitors. Ct values between 34 and 35 were taken as indicative of traces amounts, while a Ct=35 was considered the cut off limit, defining no detection [40]. The method showed a high sensitivity and allowed the detection of 3.91×10^{-1} ng of *C. maydis*.

Analysis of the parameters evaluated

The effect of the factors under study (tillage system, maize variety and sampling date) for each of the parameters analysed, namely HC and AC, DM and Ct values, indicators of *C. maydis* gDNA amount in qPCR reaction, was evaluated in each experiment (site 1 - without cover crop and site 2 - with cover crop). For the grain production, the influence of tillage system and maize variety was evaluated.

Site 1

In site 1, without cover crop, HC and AC were significantly affected by the three factors under study. Colonization rates were significantly higher for the minimum tillage and for the susceptible variety. Regarding the sampling date, both colonization rates increased, but not significantly, from date 1 to date 2, in which the highest values were obtained, and decrease significantly from date 2 to date 3. The DM was only influenced by sampling date, increasing throughout the plant cycle. Ct values were not influenced by tillage system or sampling date. On the other hand, the tolerant variety has a significantly higher Ct value than the susceptible variety (Table 2).

The interaction between tillage system and sampling date was significant in relation to AC. At date 1 and date 3 there were no significant differences between two tillage systems, however at date 2 the AC was significantly higher in minimum tillage (Figure 1).

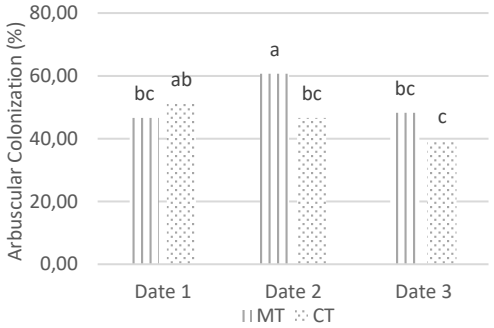

Figure 1 | Effect of interaction between tillage system and sampling date in the arbuscular colonization on site 1. Different lowercase letters indicate significant differences for p<0.05. Caption: MT – minimum tillage; CT – conventional tillage.

The interaction between tillage system and variety was also reflected in significant differences in Ct values (Figure 2). With minimum tillage there were no significant differences in the abundance of *C. maydis* between two varieties. However, in the conventional tillage, the tolerant variety differs significantly from the susceptible variety, presenting a lower amount of the phytopathogenic fungus.

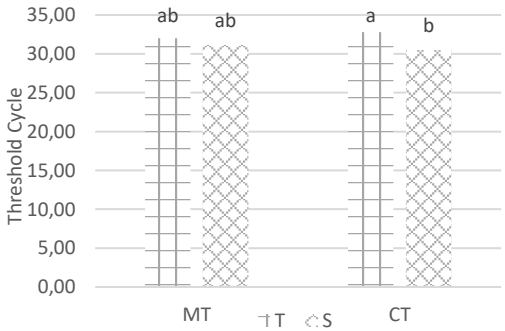

Figure 2 | Effect of interaction between tillage system and variety in threshold cycle values on site 1. Different lowercase letters indicate significant differences for p<0.10. Caption: MT – minimum tillage; CT – conventional tillage; T – tolerant variety; S – susceptible variety.

Regarding grain production, it was influenced by tillage system, contrary to what happened in relation to the variety. Conventional tillage led to significantly higher grain production than minimum tillage (Table 2).

The interaction between tillage system and variety was significant, indicating that the variety production depended on the tillage system (Figure 3). Under minimum tillage, the tolerant variety produced more grain, whereas with conventional tillage, it was the susceptible variety that presents greater production. Although, overall, there were no differences in grain production in variety, the analysis of the interaction tillage system x variety shows distinct behaviours.

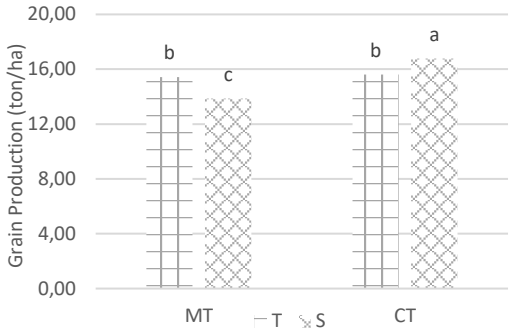

Figure 3 | Effect of interaction between tillage system and variety in the grain production on site 1. Different lowercase letters indicate significant differences for p<0.05. Caption: MT – minimum tillage; CT – conventional tillage; T – tolerant variety; S – susceptible variety.

Table 2 | Hyphal and arbuscular colonization (HC and AC), dry matter (DM), threshold cycle (Ct) and grain production values obtained for each factor under study on site 1. For each parameter, different lowercase letters indicate significant differences for p<0.05.

	HC (%)		AC (%)		DM (g/plant)		Ct		Grain Production (ton/ha)	
Treatment										
Soil Tillage										
Minimum Tillage (MT)	54.40	a	51.80	a	14.64	a	31.58	a	14.64	b
Conventional Tillage (CT)	48.60	b	45.70	b	16.20	a	31.64	a	16.20	a
Variety										
Tolerant (T)	48.60	b	45.80	b	15.51	a	32.39	a	15.51	a
Susceptible (S)	54.30	a	51.70	a	15.33	a	30.83	b	15.33	a
Sampling Date										
Date 1	50.60	ab	48.90	ab	4.14	c	32.39	a		
Date 2	57.30	a	53.70	a	18.33	b	31.52	a		
Date 3	46.40	b	43.70	b	48.71	a	30.92	a		

Site 2

In site 2, with cover crop, HC and AC were significantly affected by variety and sampling date, showing higher values in the tolerant variety and at date 1. Regarding sampling date, it was verified that both colonization rates decrease significantly from date 1 to date 2 and increase, but not significantly, from date 2 to date 3. No effect of tillage system was observed in these parameters. The DM was influenced by tillage system and by sampling date, with higher values in the minimum tillage and at date 3, and was not affected by variety. The DM increased significantly over three sampling dates. Ct values were only affected by tillage system, and with minimum tillage the Ct value was significantly higher than the observed with conventional tillage (Table 3).

Although the difference is not significant, it should be noted that AC value on date 1 was higher in minimum tillage (data not shown).

The DM, besides the effect of soil tillage and sampling date, was also significantly affected by the interaction between these two factors. Although on date 1, there were no significant differences between two tillage systems, on the two subsequent dates, minimum tillage presented values significantly higher than conventional tillage (Figure 4). It should be noted that despite no significant differences observed on date 1, the value of DM in minimum tillage was higher than in conventional tillage and this trend was intensified on dates 2 and 3, resulting in significant differences.

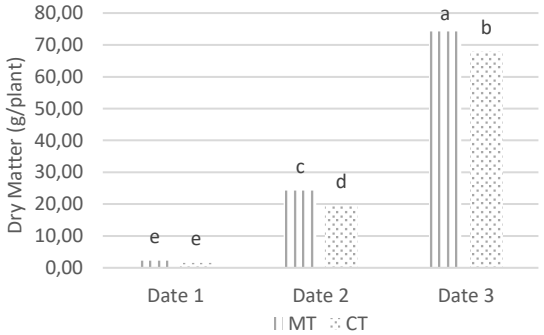

Figure 4 | Effect of interaction between tillage system and sampling date in the dry matter production on site 2. Different lowercase letters indicate significant differences for p<0.05. Caption: MT – minimum tillage; CT – conventional tillage.

Regarding grain production, it was only influenced by tillage system, for p<0.10. Minimum tillage led to significantly higher grain production than conventional tillage (Table 3).

Table 3 | Hyphal and arbuscular colonization (HC and AC), dry matter (DM), threshold cycle (Ct) and grain production values obtained for each factor under study on site 2. For each parameter, different lowercase letters indicate significant differences for p<0.05. [1)] Significant differences for p<0.10.

	HC (%)		AC (%)		DM (g/plant)		Ct		Grain Production (ton/ha)	
Treatment										
Soil Tillage										
Minimum Tillage (MT)	39.90	a	38.00	a	33.55	a	33.36	a	11.03	a[1)]
Conventional Tillage (CT)	41.80	a	39.30	a	29.73	b	30.48	b	9.38	b[1)]
Variety										
Tolerant (T)	45.20	a	42.80	a	31.98	a	31.97	a	9.73	a
Susceptible (S)	36.50	b	34.60	b	31.29	a	31.87	a	10.69	a
Sampling Date										
Date 1	50.40	a	47.70	a	1.89	c	31.77	a		
Date 2	34.90	b	33.80	b	21.92	b	31.47	a		
Date 3	37.30	b	34.60	b	71.09	a	32.52	a		

Site 1 and Site 2

In order to compare the grain production of two sites, the production values were considered as a % of the maximum. In the absence of cover crop, it was observed that there are no significant differences in production in both tillage systems. However, with cover crop, there was a significant advantage of minimum tillage (Figure 5).

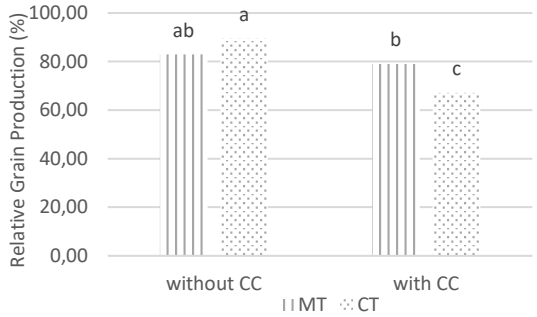

Figure 5 | Effect of interaction between soil cover and tillage system in the relative grain production. Different lowercase letters indicate significant differences for p<0.05. Caption: MT – minimum tillage; CT – conventional tillage; CC – cover crop.

The relationship between tillage system and variety also influenced grain production (Figure 6). In minimum or conventional tillage, there are no differences in the relative grain production between tolerant and susceptible variety. However, tolerant variety with minimum tillage leads to significantly higher relative yield than with conventional tillage.

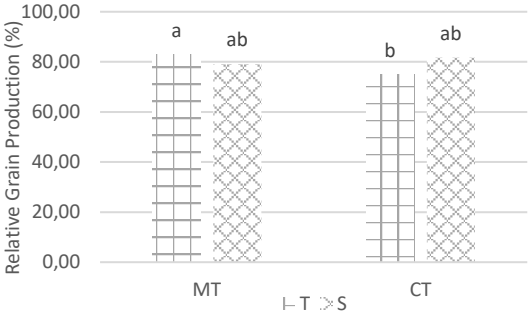

Figure 6 | Effect of interaction between tillage system and variety in the relative grain production. Different lowercase letters indicate significant differences for p<0.10. Caption: MT – minimum tillage; CT – conventional tillage; T – tolerant variety; S – susceptible variety.

The results of AC were analysed at both sites and for both tillage systems at date 1 (Figure 7). With cover crop and minimum tillage the colonization is higher. Although there were no significant differences, the differences between two tillage systems are reduced in non-cover crop experiment. However, with cover crop, the differences between tillage systems are more pronnounced, and minimum tillage leads to an higher colonization rate of maize.

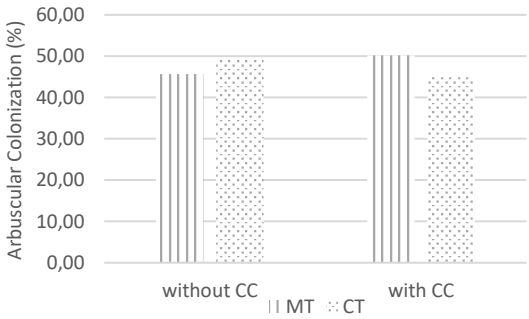

Figure 7 | Effect of interaction between soil cover and tillage system in the arbuscular colonization at date 1. Caption: MT – minimum tillage; CT – conventional tillage; CC – cover crop.

The results obtained for Ct values at both sites and tillage systems at date 1 were also analysed (Figure 8) and it was observed that, without cover crop, there were no differences in the amount of the fungus for both tillage systems. In contrast, with cover crop, minimum tillage had a significantly lower amount of *C. maydis* than conventional tillage.

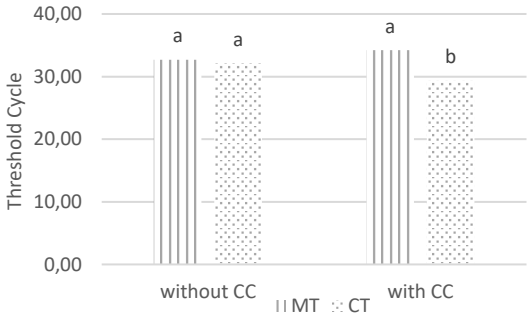

Figure 8 | Effect of interaction between soil cover and tillage system in threshold cycle values at date 1. Different lowercase letters indicate significant differences for p<0.05. Caption: MT – minimum tillage; CT – conventional tillage; CC – cover crop.

4. Discussion

It was not possible the identification of the *C. maydis* on the fungal isolates obtained in the experiments. Nevertheless, the sampling area where the experiment took place is known to be infected with this fungus. Recovery of *C. maydis*, even from heavily infested material, was already referred as difficult due to its slow growth and to the relative abundance of other more rapidly growing fungi, most commonly *Fusarium* spp. [41]. The similarity between *Fusarium* spp. and *C. maydis* ITS sequences turned difficult the design of species-specific probes and did not allow the use of SYBRGreen methodology.

The degree of complexity of the etiology of late wilt in maize regarding the determination of the role of each fungal species on the development of the disease and/or the severity of symptoms, should be studied by confirming the pathogenicity of the soil-borne fungi [42]. The methodology here presented gives an important contribution towards a better understanding on the interaction between both fungus.

Since *C. maydis* is difficult to detect and isolate [41] and present the ability to establish and survive on stored corn seeds (even in apparently healthy parent plants), there is a demand to develop new ways to monitor the health status of the seeds and to control the spread of diseases [6]. Thus, qPCR, through the use of TaqMan probes, is suggested as a promising tool for the identification and specific quantification of soil pathogenic fungi.

Considering the results obtained in field experiments, it was possible to verify different levels of *C. maydis* infection due to the cultural techniques used, as well as different levels of colonization by AMF and variations in dry matter and grain production. Without cover crop, minimum tillage, because it did not destroy the ERM developed by the weeds that were in the field before sowing of maize, has significantly higher rates of arbuscular and hyphae colonization (Table 2). Although the mycelium developed by the weeds was eventually less abundant, it was found that maintained intact had a positive effect on maize colonization by AMF [43]. It was also verified that colonization rates presented higher values for susceptible variety, just as Ct value was significantly lower for this variety (Table 2), that is, the abundance of phytopathogenic fungus in this variety was higher as expected. Since the selection of varieties used was based only on phenotyping tests in relation to the visible symptoms of the disease, and the mechanisms of tolerance involved are unknown, it is not known to what extent they interfere with the process of mycorrhizal colonization. The fact that both colonization rates (by hyphae and arbuscules) are higher in susceptible variety and the abundance of *C. maydis* in this variety is also higher leads us to suppose that the entry mechanisms of AMF and phytopathogenic fungus may not be competitors. In this case, there is no effect of mycorrhization in the abundance of *C. maydis.* However, the lower amount of *C. maydis* in tolerant variety had no effect on dry matter production (Table 2). It is important to note that the symptomatology of this disease is only noticeable at an advanced stage of the vegetative cycle [42] and since samplings were performed at two, four and six weeks after emergence, the effect that the presence of the fungus could cause in the production of dry matter at this stage was not yet visible. It was also observed that the dry matter production increased over time (Table 2), as expected.

Mycorrhizal colonization rates, in general, decreased over time (Table 2). This fact happens since the colonization rates simultaneously integrate the effect on the growth of the two partners of the symbiosis: the plant and the AMF. Thus, a reduction in colonization rates may not mean unfavourable conditions to AMF growth, but only be the result of a further growth of the plant root system. One way to overcome this issue is to use colonized root density (CRD), which integrates root and AMF growth, allowing a three-dimensional evaluation of the progression of symbiosis and evolution of the total number of arbuscules per unit of colonized root length and per unit of volume of soil. This parameter may discriminate the effect of growth conditions on each of the symbionts [44]. However, CRD evaluation involves measuring the root system length of the host plant, which in a field experiment of this nature would be impractical. In the present study, it is probable that the presence of AMF has been diluted by a greater development of the root, since maize is a spring/summer irrigated crop that responds easily and quickly to the inputs that it is supplied and, therefore, show a great root growth.

In relation to arbuscular colonization, the interaction between tillage system and sampling date was significant (Figure 1), and on date 1 and date 3 there were no significant differences between two tillage systems; however at date 2 the arbuscular colonization was significantly higher with minimum tillage. The high colonization rates observed since the first sampling date reveal that this was a already late time point and compromised the perception of eventual differences in the early colonization of the culture, imposed by the factors under study. In fact, at the first sampling, the colonization rates observed were already relatively high, and the initial dynamics of colonization may not have been fully covered.

Without cover crop and conventional tillage, tolerant variety was significantly different from susceptible variety, presenting a smaller amount of *C. maydis* (Figure 2). In this case, it can be said that genetics is an effective tool in reducing the damage caused by late wilt in maize. However, with minimum tillage the differences between maize varieties in relation to the amount of *C. maydis* were no longer observed, which emphasizes the need to use different cultural practices, namely the use of a minimum tillage system, which allow a lower pathogenic fungus abundance, regardless of the variety used.

Regarding grain production, the values obtained in the experiment without cover crop were significantly higher when conventional tillage was used (Table 2). However, there was interaction between tillage system and maize variety, which indicates that the behaviour of variety depended on the type of soil tillage. It was verified that, with minimum tillage, tolerant variety produced more grain, whereas, with conventional tillage, it was the susceptible variety that had the highest production (Figure 3). Although there were no differences in grain production at the variety, the analysis of the interaction tillage system x variety shows distinct behaviours. In fact, while tolerant variety did not give rise to significantly different yields, regardless of tillage system, this was not the case with susceptible variety, which was significantly more productive with conventional tillage, although this was also the situation in which there was a higher amount of *C. maydis* (Figure 2). Apparently, in these circumstances, the higher amount of phytopathogenic fungus associated with the susceptible variety did not translate into a decrease in grain production.

The use of a mycotrophic cover crop allows for the development in the soil of an extensive network ERM which, if maintained intact by non-tillage or reduced soil tillage, can provide early colonization of the subsequent crop and thus better protect it against any biotic or abiotic stresses [26]. At site 2, with cover crop, there are no significant differences in colonization rates with minimum or conventional tillage (Table 3), as would be expected in the presence of a mycotrophic cover crop. This is likely related to the selection of the first sampling date, which could have been to late. This was the first work performed with a population of native AMF in maize in this region. Although there is already some knowledge in this scientific area for wheat [45], this is a winter crop with lower growth rates. Despite the anticipation of the first sampling date when compared to wheat experiments, it has proved to be innacurate, therefore an even earlier sampling will be required in future work to detect the eventual very initial colonization differences.

Contrary to the experiment without cover crop, in this experiment colonization rates were higher for tolerant variety (Table 3). This different behaviour of the variety was probably due to the presence of the cover crop, which will have many more beneficial effects on the soil [29], besides develop an extensive network ERM capable of increasing mycorrhizal colonization. It may also be because at site 2 the planting occurred more than one month later than at site 1 and as such the temperature conditions at the initial stage of the culture were different, conditioned the development of the AMF and the crop. In addition, there are no differences between varieties regarding the presence of *C. maydis* (Table 3), which leads us to think that the advantages associated with the cover crop overlap the effect of the variety. In this case, and contrary to what happened in the previous experiment, the Ct value is significantly higher with minimum tillage (Table 3). This fact highlights the need to increase the use of conservation agriculture practices to reduce the incidence of the disease. With cover crop, the ERM is developed by the AMF that colonize this crop. By conserving this well developed ERM using minimum tillage, maize roots come into contact with the inoculum source that will provide an early, faster and more intense colonization [30,45,46], so that the plants will benefit both the absorption of nutrients and protection against biotic or abiotic stresses, from the early stages of the vegetative cycle. This colonization offers the possibility of early activation of plant defence mechanisms, both locally and systemically [47].

With cover crop, dry matter production was significantly higher with minimum tillage than with conventional tillage (Table 3), which can be due to the benefits associated with cover crop [29], which translated into advantages for maize at the level of its development, but probably also due to the significantly less amount of *C. maydis* present under these circumstances. This behaviour of maize showed the benefit of the presence of an intact ERM previously developed by the cover crop.

With cover crop, it was also found that colonization rates generally declined over time, as previously reported, and that dry matter production increased over time (Table 3), as expected. Concerning dry matter production, the interaction between tillage system and sampling date did not show significant differences between two tillage systems on date 1. However, on the two subsequent dates differences were found, with minimum tillage having significantly higher values than the conventional tillage (Figure 4). The minimum tillage, because the experiment has a cover crop, presents an advantage to the conventional tillage. Since the ERM developed in the soil by the cover crop remains intact, contributes to a better development of the crop.

The sustainability of agricultural activity is closely related to the way soils are grown. Therefore, adopting conservation practices, such as the use of cover crops and minimum tillage systems, can positively affect crop productivity. The grain production values obtained in the experiment with cover crop are thus significantly higher for minimum tillage (Table 3). In this way, it was verified that, with cover crop and minimum tillage, maize presented a higher dry matter yield, a higher grain yield and a lower amount of *C. maydis* in its roots, which clearly shows the benefits of using the combination of cover crop and minimum tillage.

Comparing two experiments in terms of grain production, it was found that without cover crop, there were no significant differences in production between the two tillage systems. However, with cover crop, grain production value was closer to the maximum with minimum tillage (Figure 5). This system only presents an expressive translation in the increase of the local production with cover crop, which seems to be related to the

effect of the cover crop and all the associated benefits, as well as the presence of an intact mycelium. The capitalization of the benefits derived from the cover crop, including the reduction of *C. maydis* in maize plants, can only be achieved with minimum tillage, so it only makes sense to use this soil tillage system when a cover crop has been previously installed. Comparing two sites under study (with and without cover crop), and although there were no significant differences for varieties in each tillage system (Figure 6), tolerant variety yielded significant higher grain yield with minimum tillage. This result seems to be associated with the expected comparative advantage of the use of a tolerant variety and minimum tillage system that led to higher Ct values (lower amount of *C. maydis*), mainly with cover crop. Thus, in the environments in which the phytopathogenic fungus is present, stands out the particular importance of conservation cultural practices (cover crop and minimum tillage), which may be associated with the use of a tolerant variety. However, under the specific conditions of the experiments, there were no significant differences in grain production between varieties, and therefore appears that the use of the tolerant variety has no advantage.

Although there were no significant difference at date 1, it was found that with cover crop and minimum tillage, the arbuscular colonization is higher (Figure 7). Thus, in the presence of cover crop and when the ERM was maintained intact, serving as the preferred source of inoculum, the arbuscular colonization of maize roots at an early stage was larger and developed faster, witch is in agreement with previous work [22,30,45,46]. ERM network integrity can be affected when there is soil disturbance, thus reducing plant colonization by the AMF and, consequently, providing less efficient crop protection [31]. With conventional tillage there is a reduction in the colonization by AMF once the ERM is broken, so colonization is essentially initiated by sources of slow-growing inoculum. Therefore, the best way to guarantee and achieve good initial colonization rates of the crop is to avoid the destruction of the ERM network of the native and naturally biodiverse inoculum, using soil conservation techniques [17].

It was also verified that, without cover crop, there were no differences in the presence of phytopathogenic fungus when comparing two tillage systems. On the other hand, with cover crop, the plants cultivated with minimum tillage presented a Ct value significantly higher than with conventional tillage, pointing to a smaller amount of *C. maydis* fungus in the culture with minimum tillage, since a cover crop has been previously cultivated (Figure 8). Thus, in order to reduce the presence of *C. maydis* fungus in maize, two cultural practices, use of cover crop and minimum soil tillage system, should be associated, bringing together the beneficial effect of mycorrhization (more indirectly detected in this study) and other benefits associated with the cover crop. In these circumstances, the use of tolerant or susceptible varieties seems to be indifferent.

5. Conclusions

The presence of a well-developed intact ERM, using a cover crop and minimum tillage, is a strategy with benefits for the culture both in its growth and in the protection against biotic stresses, namely in protection against *C. maydis*. As noted, one of the limitations to the use of AMF in agricultural ecosystems is the idea that the time elapsed is high so that a sufficient level of colonization of the host plant is achieved to guarantee bioprotection of the crop. However, if symbiosis is well established from the beginning of the vegetative cycle, which is achieved by using the type of propagule that promotes an early and faster colonization, intact ERM, it is possible to overcome this challenge. It is crucial to have a better knowledge of the AM symbiosis. Many farmers are unaware of AM or that their benefits are within reach and can be obtained by the adoption of simple crop management techniques. In conclusion, in environments where *C. maydis* is known at the outset, the choice of cultural conservation practices is particularly important for maize cultivation, namely the installation of a cover crop and minimum tillage system. Although in the absence of a cover crop and using conventional tillage, it is possible to reach interesting yields and lower levels of *C. maydis*, this system is more dependent on the maize variety used, does not benefit from the advantages associated with the cover crop, has higher costs and is less sustainable.

References

1. Czembor, E., Stępień, Ł., & Waśkiewicz, A. (2015). Effect of environmental factors on *Fusarium* species and associated mycotoxins in maize grain grown in Poland. *PloS One*, 10(7): e0133644.
2. Oerke, E. C. (2006). Crop losses to pests. *Journal of Agricultural Science*, 144, 31-43.
3. Gams, W. (2000). Phialophora and some similar morphologically little-differentiated anamorphs of divergent ascomycetes. *Studies in Mycology*, 45, 187-199.
4. Saleh, A. A., & Leslie, J. F. (2004). *Cephalosporium maydis* is a distinct species in the Gaeumannomyces-Harpophora species complex. *Mycologia*, 96, 1294-1305.
5. Molinero-Ruiz, M. L. , Melero-Vara, J. M., & Mateos, A. (2010). *Cephalosporium maydis*, the cause of late wilt in maize, a pathogen new to Portugal and Spain. *Plant Disease*, 94, 379-379.
6. Drori, R., Sharon, A., Goldberg, D., Rabinovitz, O., Levy, M., & Degani, O. (2013). Molecular diagnosis for *Harpophora maydis*, the cause of maize late wilt in Israel. *Phytopathologia Mediterranea*, 52, 16-29.
7. Samra, A., Sabet, K., & Hingorani, M. (1963). Late wilt disease of maize caused by *Cephalosporium maydis*. *Phytopathology*, 53, 402–406.
8. Sabet, K. A., Zaher, A. M., Samra, A. S., & Mansour, I. M. (1970). Pathogenic behaviour of *Cephalosporium maydis* and *C. acremonium*. *Annals of Applied Biology*, 66, 257-263.
9. Abd El-Rahim, M. F., Fahmy, G. M., & Fahmy, Z. M. (1998). Alterations in transpiration and stem vascular tissues of two maize cultivars under conditions of water stress and late wilt disease. *Plant Pathology*, 47, 216-223.
10. Zeller, K. A., Ismael, A.-S. M., El-Assiuty, E. M., Fahmy, Z. M., Bekheet, F. M., & Leslie, J. F. (2002). Relative Competitiveness and Virulence of Four Clonal Lineages of *Cephalosporium maydis* from Egypt Toward Greenhouse-Grown Maize. *Plant Disease*, 86, 373-378.

11. García-Carneros, A. B., Girón, I., & Molinero-Ruiz, L. (2012). Aggressiveness of *Cephalosporium maydis* causing late wilt of maize in Spain. *Communications in Agricultural and Applied Biological Sciences*, 77, 173-179.
12. Michail, S. H., Abou-Elseoud, M. S., & Nour Eldin, M. S. (1999). Seed health testing of corn for *Cephalosporium maydis*. *Acta Phytopathologica et Entomologica Hungarica*, 34, 35-42.
13. Sabet, K. A., Samra, A. S., & Mansour, I. S. (1966). Interaction between *Fusarium oxysporum*, *F. vasinfectum* and *Cephalosporium maydis* on cotton and maize. *Annals of Applied Biology*, 58, 93-101.
14. Mohamed, H. A., Ashour, W. E., Sirry, A. R., & Fathi, S. M. (1967). Fungi carried by corn seed and their importance in causing corn diseases in the United Arab Republic. *Plant Disease Reporter*, 51, 53-56.
15. Selosse, M., Strullu-Derrien, C., Martin, F., Kamoun, S., & Kenrick, P. (2015). Plants, fungi and oomycetes: a 400 million year affair that shapes the biosphere? *New Phytologist*, 206, 501-506.
16. Smith, S., & Read, D. (2008). Mycorrhizal Symbiosis. Third Edition. Academic Press and Elsevier.
17. Goss, M., Carvalho, M., & Brito, I. (2017). Functional Diversity of Mycorrhiza and Sustainable Agriculture - Management to Overcome Biotic and Abiotic Stresses. Academic Press and Elsevier, London, pp. 231.
18. Schüßler, A., Schwarzott, D., & Walker, C. (2001). A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycological Research*, 105, 1413-1421.
19. Requena, N., Jeffries, P., & Barea, J. M. (1996). Assessment of natural mycorrhizal potential in a desertified semiarid ecosystem. *Applied and Environmental Microbiology*, 62, 842-847.
20. Klironomos, J., & Hart, M. (2002). Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. *Mycorrhiza*, 12, 181-184.
21. Martins, M. A., & Read, D. J. (1997). The effects of disturbance on the external mycelium of arbuscular mycorrhizal fungi on plant growth. *Pesquisa Agropecuaria Brasileira*, 32, 1183-1189.
22. Fairchild, G. L., & Miller, M. H. (1988). Vesicular–arbuscular mycorrhizas and the soil-disturbance induced reduction of nutrient absorption in maize II. Development of the effect. *New Phytologist*, 110, 75-84.
23. Püschel, D., Rydlová, J., & Vosátka, M. (2007). The development of arbuscular mycorrhiza in two simulated stages of spoil-bank succession. *Applied Soil Ecology*, 35, 363-369.
24. Kabir, Z., & Koide, R. T. (2000). The effect of dandelion or a cover crop on mycorrhiza inoculum potential, soil aggregation and yield of maize. *Agriculture, Ecosystems & Environment*, 78, 167-174.
25. Brito, I., Carvalho, M., & Goss, M. J. (2011). Summer survival of arbuscular mycorrhiza extraradical mycelium and the potential for its management through tillage options in Mediterranean cropping systems. *Soil Use and Management*, 27, 350-356.
26. Garg, N., & Chandel, S. (2010). Arbuscular mycorrhizal networks: process and functions. A review. *Agronomy for Sustainable Development*, 30, 581-599.
27. Harrier, L. A., & Watson, C. A. (2004). The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. *Pest Management Science*, 60, 149-157.
28. Nogales, A., Aguirreolea, J., Santa María, E., Camprubí, A., & Calvet, C. (2009). Response of mycorrhizal grapevine to *Armillaria mellea* inoculation: disease development and polyamines. *Plant and Soil*, 317, 177-187.
29. Kabir, Z., & Koide, R. T. (2002). Effect of autumn and winter mycorrhizal cover crops on soil properties, nutrient uptake and yield of sweet corn in Pennsylvania, USA. *Plant and Soil*, 238, 205-215.
30. Goss, M. J., & de Varennes, A. (2002). Soil disturbance reduces the efficacy of mycorrhizal associations for early soybean growth and N_2 fixation. *Soil Biology and Biochemistry*, 34, 1167-1173.
31. Lendzemo, V. W., & Kuyper, T. W. (2001). Effects of arbuscular mycorrhizal fungi on damage by *Striga hermonthica* on two contrasting cultivars of sorghum, *Sorghum bicolor*. *Agriculture, Ecosystems & Environment*, 87, 29-35.
32. Verma, V., Gond, S., Kumar, A., Kharwar, R., & Strobel, G. (2007). The endophytic mycoflora of bark, leaf, and stem tissues of *Azadirachta indica* A. Juss (neem) from Varanasi (India). *Microbial Ecology*, 54, 119-125.
33. Ortiz-Bustos, C. M., Testi, L., García-Carneros, A. B., & Molinero-Ruiz, L. (2016). Geographic distribution and aggressiveness of *Harpophora maydis* in the Iberian Peninsula, and thermal detection of maize late wilt. *European Journal of Plant Pathology*, 144, 383-397.
34. Doyle, J., & Doyle, J. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, 19, 11-15.
35. White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky, JJ, White, TJ (eds), PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc, New York.
36. Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95-98.
37. McGonigle, T., Miller, M., Evans, D., Fairchild, G., & Swan, J. (1990). A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist*, 115, 495-501.
38. Higgins, D. G., Thompson, J. D., & Gibson, T. J. (1996). Using CLUSTAL for multiple sequence alignments. In: Russell F. D., editor. Methods in Enzymology, Academic Press, 266, 383-402.
39. Campos, M. D., Patanita, M., Campos, C., Materatski, P., Varanda, C. M. R., Brito, I., & Félix, M. R. Detection and quantification of *Fusarium* spp. and *Cephalosporium maydis* in maize using real-time PCR targeting the ITS region. – submitted.
40. Campos, M. D., Valadas, V., Campos, C., Morello, L., Braglia, L., Breviario, D., & Cardoso, H. G. (2018). A TaqMan real-time PCR method based on alternative oxidase genes for detection of plant species in animal feed samples. *PloS One*, 13(1): e0190668.
41. Saleh, A. A., Zeller, K. A., Ismael, A. S. M., Fahmy, Z. M., El-Assiuty, E. M., & Leslie, J. F. (2003). Amplified fragment length polymorphism diversity in *Cephalosporium maydis* from Egypt. *Phytopathology*, 93, 853-859.
42. Ortiz-Bustos, C. M., García-Carneros, A. B., & Molinero-Ruiz, L. (2015). La marchitez tardía del maíz (*Zea mays* L.) causada por *Cephalosporium maydis* en la Península Ibérica, y otros hongos asociados. *Summa Phytopathologica*, 41, 107-114.
43. Yamato, M. (2004). Morphological types of arbuscular mycorrhizal fungi in roots of weeds on vacant land. *Mycorrhiza*, 14, 127-131.
44. Carvalho, M., Brito, I., Alho, L., & Goss, M. J. (2015). Assessing the progress of colonization by arbuscular mycorrhiza of four plant species under different temperature regimes. *Journal of Plant Nutrition and Soil Science*, 178, 515-522.
45. Brito, I., Carvalho, M., & Goss, M. J. (2013). Soil and weed management for enhancing arbuscular mycorrhiza colonization of wheat. *Soil Use and Management*, 29, 540-546.
46. Brito, I., Goss, M. J., & Carvalho, M. (2012). Effect of tillage and crop on arbuscular mycorrhiza colonization of winter wheat and triticale under Mediterranean conditions. *Soil Use and Management*, 28, 202-208.
47. Cameron, D. D., Neal, A. L., van Wees, S. C., & Ton, J. (2013). Mycorrhiza-induced resistance: more than the sum of its parts? *Trends in Plant Science*, 18, 539-545.