Arbuscular mycorrhizal fungi pre-colonisation for improving the growth and health of strawberry (*Fragaria* x *ananassa*)

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Abstract

Pre-colonisation of plants with arbuscular mycorrhizal fungi (AMF) before trans-planting has been proposed as a method for protecting crops against biotic and abiotic stresses and/or increasing plant productivity. Strawberry (*Fragaria* x *ananassa*) production systems make AMF pre-inoculation at the weaning stage relatively straightforward for *in vitro* and runner-derived plantlets. Strawberry plugs were pre-inoculated with different AMF species to study (1) whether AMF could pre-colonise different strawberry cultivars under high moisture and soil-less substrate during the weaning process, (2) whether AMF could survive the required artificial freezing cold storage of strawberry plugs for several months, and (3) whether AMF could enhance plant tolerance against *Verticillium dahliae*, *Phytophthora fragariae* and *P. cactorum.* In addition, (4) AMF was inoculated at planting to study whether AMF could increase strawberry growth and yield when cultivated in coir, and (5) a simple *in vitro* autotrophic system was also designed to investigate strawberry-AMF-pathogen interactions under axenic and controlled conditions. The study demonstrated that the soil-less substrates tested and high moisture conditions during tipping did not prevent different AMF from colonising roots of strawberry plugs. Pre-inoculated AMF species could also survive cold storage at -2°C with strawberry plugs for several months. However, AMF pre-colonisation and/or AMF inoculation at planting did not increase plant tolerance against root pathogens. It was demonstrated that AMF inoculation in coir did not significantly increase plant growth and yield. Finally, micropropagated strawberry were successfully infected by *P. fragariae in vitro* with the corresponding disease symptoms, while *V. dahlia* and AMF could germinate but did not colonise the strawberry roots in the autotrophic culture system. This is, to the best of our knowledge, the first research focusing on the AMF-strawberry interaction as a model system to study the possibility to pre-colonise strawberry plug materials to increase plant productivity and tolerance against major strawberry root diseases.

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Authors declaration

I, Benjamin Langendorf declare that all the material contained within this thesis except for the work outlined below is a result of my own work and has been written solely by myself. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as references. For all experiments, maintenance of the plants (watering/feeding) was planned by Benjamin Langendorf, and carried out by both Benjamin Langendorf and Joyce Robinson. Root sampling, root staining and root mounting on slides were carried out by Benjamin Langendorf and Joyce Robinson, but colonisation counts were carried out by Benjamin Langendorf. The field experiment outlined in Chapter 4 was designed by Benjamin Langendorf but set up with the help of Dr Feng Wei. The experiments outlined in Chapter 5 were designed by Benjamin Langendorf but carried out with the help of Joyce Robinson, Tom Passey and Dr Louisa Boyer. All calculations, statistical analyses were carried out by Dr Phil Brain and Benjamin Langendorf.

Chapter 1. General introduction

1.1. *Fragaria* x *ananassa* in commercial horticulture

The commercial strawberry (*Fragaria* x *ananassa* Duch) is a perennial crop that belongs to the *Rosaceae* family. It comes from a cross between *F. virginiana* and *F. chiloensis* (Hancock, 1999). Strawberry is an important horticultural crop worldwide in terms of its commercial, nutritional and medicinal values (Hancock, 1999). According to FAO statistics the total global land area used for strawberry cultivation was 373,435 ha in 2014 (FAO, 2017). In the UK alone, strawberry cultivation in 2014 accounted for ca. 4500 ha of land with a yield of ca. 23 t ha^{-1} (FAO, 2017). Strawberry represented 78% of all soft fruit production in the UK, worth an estimated £253 million in 2016 (DEFRA, 2017), and this is expected to rise significantly over the coming few years (Boyer *et al.*, 2016).

Strawberry transplants are usually obtained via micro-propagation using meristems or by vegetative multiplication using tips or cuttings from strawberry runners. Since the early 1990s, rooted module plants called strawberry plugs have become the most commonly used planting materials in Europe (Durner *et al.*, 2002). Plug transplants are currently replacing the bare-rooted transplants commonly lifted from the field during winter. The popularity of plug transplants is mainly explained by the fact that bare-rooted strawberries are harder to store for a long period due to absence of substrate around the roots and because they are often infected by root pathogens (Lieten, 2000; Durner *et al.*, 2002). The production of strawberry plugs usually occurs between July-August and it is divided into several stages. First, unrooted runner tips with root pegs are collected from mother plants that are actively producing stolons. Tips are planted in cells of specially designed plastic trays usually filled up with a peat/perlite mix. These tips are then weaned under misting for at least two weeks to allow rooting. Once the runner tips have rooted they are called

plugs. Plugs are then usually kept for another four weeks under glasshouse conditions to grow and establish a stronger root system. Finally, plugs are then ready for shipping, transplantation or cold storage. Before transplantation strawberry plugs are usually cold stored at -2°C for various lengths of time (on average four months) to achieve sufficient chilling to induce flower buds and to schedule cropping (Lieten *et al*., 2005).

Strawberries generally prefer sunny locations with well drained, sandy loam soils with an optimum pH range of 5.5-7.0 (Hancock, 1999). In the UK, strawberries were commonly cultivated in open fields in matted rows and raised beds up until 1991-1992 (Carter *et al*., 1993). By the end of the 1990s, protected table-top cropping systems using soil-less substrates become more common, and represent today two-thirds of the total UK strawberry acreage and more than 50% of the total European soil-less acreage (Boyer *et al*., 2016; López-Aranda *et al*., 2016).

Many cultivars including 'Vibrant', 'Elsanta', 'Red Glory' and 'Malling Centenary' are cultivated in the UK. Strawberry cultivars can be generally classified into two categories: a) short day June-bearers that can grow and initiate flower buds during short daylight seasons, giving a single, but large yield, and b) long day ever-bearers insensitive to light, producing fruits over a much longer period of time (usually 4-5 months; Hancock, 1999). Strawberries are high in vitamin C, phenolic compounds (e.g. anthocyanins) and minerals such as potassium and manganese (Debnath & Teixeira da Silva, 2007). The red colour of strawberries is due to the anthocyanins, pelargonidin-3-glucoside and cyanidin-3-glucoside (Debnath & Teixeira da Silva, 2007). The medicinal value of strawberries results from their high level of phenolic compounds, reported to have anti-cancer, antioxidant and anti-inflammatory effects (Debnath & Teixeira da Silva, 2007; Giampieri *et al.*, 2013).

1.2. Diseases in strawberry and disease control

Fungal pathogens are the principal cause of disease on strawberry (Sigee, 2005). In Europe, the main fungal pathogens of strawberry include: *Verticillium dahliae* Kleb. (resulting in strawberry wilt), *Phytophthora fragariae* (causing red core or red stele), *Phytophthora cactorum* (causing crown rot), *Podosphaera aphanis* (causing powdery mildew) and *Botrytis cinerea* (causing grey mould; Parikka, 2004). Different control measures (e.g. cultivation practices, breeding, crop rotation and use of pesticides and biocides) are practised to mitigate crop losses caused by these pathogens (Guerena & Born, 2007).

In the field, the soil-borne pathogen *Verticillium dahliae* causes a serious threat to strawberry growing in field soil (Pegg & Brady, 2002), and the European and Mediterranean Plant Protection Organization (EPPO) has listed *Verticillium* spp. as the 'principal strawberry disease' (Garrido *et al*., 2011). It forms conidia and microsclerotia that can germinate in the presence of root exudates and enter the plant through primary roots or wounds. *Verticillium* will then invade the vascular tissues of the roots and crown, depriving the leaves and stems of water and inducing wilt symptoms (Bhat & Subbarao, 1999; Lovelidge, 2004). Other symptoms such as reddish-yellow leaves curling up along the mid vein, and stunted growth are also observed. The pathogen overwinters in the soil in the form of microsclerotia on dead plant tissues. The microsclerotia can remain viable for 10 years or more even in the absence of a host plant (Pegg & Brady, 2002).

Previously, the soil fumigant methyl bromide (MB) was routinely applied to control strawberry wilt. However, MB was banned in 2008 from Europe due to its high ozone-depleting potential, but its chemical alternatives, 1,3-dichloropropene and chloropicrin, face an uncertain future due to potential changes in the legislation (López-Aranda *et al*., 2016). Therefore, alternative methods are urgently needed to control *V. dahliae*

(Klosterman *et al.*, 2009). Different strategies, such as biofumigation, solarisation, catch/cover crops, anaerobic soil disinfestation and crop rotation, contribute to disease control, but they are usually not as effective as chemical fumigants (Tahmatsidou *et al.*, 2006; Korthals *et al.*, 2014; López-Aranda *et al*., 2016). Consequently, extensive effort has gone into finding other economically effective alternatives to mitigate the threat of strawberry wilt (Martin, 2003; Goicoechea *et al.*, 2010). Two main methods are currently being explored to reduce the risk of strawberry soil-borne diseases in the UK. The first proposed method relies on the exploitation of beneficial microbial organisms against strawberry root pathogens by introducing biological control agents at planting or during propagation. Therefore, sufficient colonisation of strawberry plug roots before transplanting is expected to increase the positive effects of inoculated beneficial microbes on plant health. The second approach is to move away from traditional field soil cultivation, towards table-top systems, where strawberry plants are grown in soil-less substrates (Boyer *et al.*, 2016). This second approach is increasingly being adopted in the UK and more than 66% of the UK strawberry production is now produced in soil-less substrates, usually coir (coconut fibre), and mainly under polythene tunnels or in glasshouses on table-top systems (López-Aranda *et al*., 2016). There are also several significant benefits in adopting soil-less substrates in commercial strawberry production including: reduced cost of picking, better control of fertigation and pollination regimes as well as the possibility to extend the growing season and reduce the risk of *V. dahliae* infections (Boyer *et al.*, 2016). However, soil-less substrates are usually depleted of beneficial microbes, while root pathogens such as *P. fragariae* and *P. cactorum* continue to pose a serious threat (Schnitzler, 2004; Martínez *et al.*, 2010) as they can infect planting materials in nurseries if the water supply is contaminated (Durner *et al.*, 2002). In addition, this practice relies on high inputs of water and nutrients through fertigation, which are estimated to be more than double those of field grown crops (Boyer *et al.*, 2016).

Therefore, inoculation of beneficial microbes in soil-less substrate offers a potential means to both increase plant tolerance to root pathogens and reduce fertiliser and water inputs (Boyer *et al.,* 2016).

1.3. Role of arbuscular mycorrhiza fungi

Arbuscular mycorrhizal fungi (AMF) are ubiquitous and form obligate symbioses with over 75% of all vascular plants (Smith & Read 2008). The origin and divergence of AMF is dated to more than 480 million years and AMF symbiosis is believed to have helped the adaption of the first land plants to the terrestrial environment (Pozo *et al*., 2013; Schüßler & Walker, 2011). AMF belong to the phylum *Glomeromycota* (divided into five orders: *Glomerales*, *Gigasporales*, *Archaeosporales*, *Paraglomerales* and *Diversisporales*) and present very peculiar biological and genetic traits (Schüßler & Walker, 2011). For years AMF were considered asexual. However, the recent identification of dikaryote-like *Rhizophagus irregularis* isolates and the discovery of a mating type (MAT) locus like region in its genome, together suggested the potential existence of AMF mating (Corradi & Brachmann, 2016). Sequences within individual AMF spores have also shown to present multiple variants, as well as within and between species of the phylum *Glomeromycota* (Rodriguez *et al.,* 2015).

AMF colonisation can be initiated from three main types of propagules: spores, extraradical hyphae and colonised root fragments. When AMF propagules approach a host root (asymbiotic stage) an exchange of molecule signals between the plant and the fungi occurs and several plant and fungal regulatory genes are activated (Pozo *et al*., 2013). AMF responds to the presence of the plant roots by an intense branching of the hyphae (pre-symbiotic phase). Strigolactones contained in the host root exudates were identified to be the signalling compounds that induce AMF hyphal branching and respiration (Besserer *et*

al., 2006). When AMF hypha finally get in contact with a plant root an hyphopodium (or appressorium) is formed, marking the initiation of the symbiotic phase which terminates with the formation of arbuscules, where most of the nutrient exchange between the host plant and the AMF is thought to occur (Smith & Read, 2008; Wang *et al*., 2017). AMF are found associated with several key crop families (e.g. *Gramineae*, *Palmae*, *Leguminosae* and *Rosaceae*), including some tree species and many vegetable and ornamental plants (Prakash *et al.*, 2015). AMF colonise the root cortex and produce extraradical hyphae that are specialised in the acquisition of mineral nutrients and increase the exchange surface between root and surrounding soil (Smith & Read, 2008). AMF associations are of high interest for agriculture and horticulture. In fact, AMF can support plants in increasing nutrient uptake, particularly of poorly mobile phosphate ions. AMF can also help plants in tolerating drought and metal toxicity, as well as pathogen and herbivore attacks both above- and below-ground (Smith *et al.*, 2010; Prakash *et al.*, 2015). More recently, AMF were shown to actively assist plant in nitrogen and zinc uptake (Hodge & Fitter, 2010; Prakash *et al.*, 2015). In return, AMF obtain a carbon supply from their associated host plant. The importance of AMF in crop growth and development is becoming increasingly clear and AMF are now recognised as a vital component in agroecosystems. Traditionally, the role of AMF was believed to be one of nutrient provision only. However, it is now recognised that AMF contribute a wider range of benefits to their host plant as well as playing an important role in ecosystem services (Gianinazzi *et al*., 2010; Smith *et al.*, 2010). One of these benefits is in priming, inducing or otherwise improving plant defences against attacks from pathogens and/or insects (Pozo *et al*., 2013).

1.4. The interaction between strawberry and AMF

The oldest description of AMF structures in strawberry roots was reported in 1924 (Jones, 1924), while the first detailed description of an AMF-strawberry interaction was done by

O'Brian and McNaughton (1928), who regarded AMF as a root pathogen and believed it to be the fundamental cause of the Lanarkshire strawberry disease. In 1953, Mosse became the first researcher to describe hyphal connections between spores and strawberry roots (Mosse, 1953). This study by Mosse was conducted at East Malling Research (now called NIAB EMR), where the current project was carried out. From 1924 to 2017 there were approximately 150 publications dealing with strawberry-AMF interactions. Most of those studies have reported the beneficial effects of AMF symbiosis on strawberry plants: (a) increased fruit colouring and concentration of phenolic compounds (Plenchette *et al.*, 1983; Castellanos-Morales *et al*., 2010), (b) increased runner production (Niemi & Vestberg, 1992), (c) increased berry yield (Boyer *et al*., 2016) and (d) improved fruit quality (Lingua *et al*., 2013). AMF inoculation has also shown to increase both growth (crowns, roots and leaf area) and tolerance to water stress in micro-propagated strawberry plants (Borkowska, 2002). AMF colonisation prior to transplantation of micro-propagated strawberries also helped plants to tolerate water stress during the weaning stage (Hernández-Sebastià *et al*., 1999). Finally, AMF inoculation has shown to reduce the incidence of various strawberry root pathogens (Murphy *et al*., 2000; Vestberg, *et al.*, 2004; Sowik *et al.*, 2016) and even reduced larval survival and biomass of a black vine weevil (Gange, 2001). Interestingly, there is a limited number of reports of neutral effect and conclusive negative effect of AMF inoculation on strawberry health and/or productivity report in the literature (O'Brian & McNaughton,1928; Nemec, 1974; Bååth & Hayman *et al*., 1984; Vestberg, *et al.*, 2004). Could it be a bias in reporting or the evidence that AMF inoculation have a great potential to be used as a biocontrol agent and/or as a bio-fertiliser in strawberry production? Therefore, strawberry appears to be an ideal crop system to study the degree to which cultivar and/or growing practices affects the formation of the AMF symbiosis and its functioning. However, several essential

questions regarding the ecological and molecular aspects of this interaction remain to be answered.

The model plant *Medicago truncatula* has been a useful system to investigate AMF symbiotic interactions at a molecular level (Rose, 2008), but there is not, as yet, a standard non-legume model plant. The wild strawberry, *F. vesca* could be proposed as a versatile plant model for investigating molecular aspects of the AMF symbiosis in fruit crop belonging to the *Rosaceae* family. In fact, the wild strawberry is an herbaceous perennial with a small genome (240 Mb) that was sequenced in 2011, amenable to genetic transformation and shares substantial sequence identity with *F.* x *ananassa* as well as other economically important rosaceous crops and ornamentals (e.g. apples, pears, peach, apricot, raspberries, roses; Shulaev *et al.*, 2011).

1.5. Plant root diseases controlled by AMF

Plant root diseases caused by soil-borne pathogens (including fungi, nematodes and bacteria) are by far the hardest to control (Koike *et al.*, 2003). The importance of AMF in protecting plants from soil-borne pathogens has been reported on different crops, including strawberry (Cano, 2014; Prakash *et al.*, 2015). For example, inoculating AMF at planting increased strawberry plant tolerance to *V. dahliae* (Ma *et al.*, 2004; Tahmatsidou *et al.*, 2006; Sowik *et al.*, 2016). AMF inoculation also reduced incidence of *P. cactorum* and/or *P. fragariae* (Norman *et al*., 1996, Murphy *et al*., 2000; Vestberg, *et al.*, 2004). In addition, similar protective effects were reported in other crops (e.g. tomato, potato, aubergine and cotton) and various aspects of this concept have been extensively reviewed (Borowiez, 2001; Whipps, 2004; St-Arnaud & Vujanovic, 2007; Akhtar & Siddiqui, 2008; Pozo *et al*., 2013). Although different mechanisms have been proposed to be involved in protection against soil-borne pathogens these are still poorly understood particularly with the regard

to: (a) inhibiting pathogen growth, (b) damage compensation, (c) increasing development of plant growth promoting rhizobacteria (PGPR) and/or suppressive microbial population, (d) increasing nutrients uptake, (e) competing for photosynthates, (f) competing for exudates external to the root, (g) competing for colonisation/infection sites, (h) inducing plant hormonal changes, (i) inducing changes in root morphology, branching and/or root exudation pattern, and (j) inducing systemic resistance (ISR) and/or changes associated with plant defences mechanisms, (Pozo *et al*., 2002; Whipps, 2004; Pozo *et al*., 2013).

All in all, inoculating the rhizosphere of strawberry plants with AMF is expected to increase protection against biotic and abiotic stresses (Vestberg, *et al.*, 2004; Boyer *et al.*, 2016). Strawberry is an ideal production system to study beneficial effects of AMF because planting materials (micro-propagated or runner-derived plugs) can be easily inoculated during their propagation and/or at planting.

1.6. Factors limiting AMF establishment in commercial strawberry production

Although the availability of commercial AMF inocula has increased in the past decade, AMF products are still rarely used in commercial horticulture. There are several factors that may limit the use of AMF in commercial strawberry production and other crops: (a) difficulties in producing high quality AMF inocula in large quantities, (b) high cost for growers, (c) variable beneficial effects, (d) uncertainties in the benefits of added AMF in the presence of resident indigenous AMF populations, and (e) unwillingness of growers to risk low production through reduced fertiliser and pesticide inputs that are often necessary for functioning symbiosis (Ryan & Graham, 2002; Boyer *et al.*, 2016).

Many horticultural practices as well as environmental conditions can also influence the outcome of the symbiosis in term of plant productivity and protection against root diseases (Johnson & Pfleger, 1992). Several characteristics of artificial growing media and substrates commonly used in strawberry nurseries can influence the formation of and/or effect AMF symbiosis (Azcon-Aguilar & Barea, 1997; Boyer *et al*., 2016). For example, certain types of peat have been reported to have negative effects on AMF root colonisation of strawberry *in vitro* derived plantlets during propagation (Niemi & Vestberg, 1992; Vestberg *et al*., 2000; Corkidi *et al*., 2004; Palencia *et al.*, 2013). It remains unclear, however, whether the negative effect of peat on AMF colonisation was due to high input of fertilisers (e.g. phosphorus), high humidity during the propagation phase and/or biological properties of the peat itself (Martinez *et al.*, 2013; Palencia *et al.*, 2013). Because the majority of the strawberry runner-tips are rooted in peat-based media (Durner *et al*., 2002), tests are therefore required to verify whether or not strawberry runner-tips can be pre-colonised by AMF under such conditions.

Moreover, the high fertilisation regime usually used in commercial strawberry production could inhibit the establishment of AMF symbiosis. For example, it has been demonstrated for several crops that an increase of soluble phosphate fertilisers reduces the overall level of AMF colonisation (Barea, 1991). Excess of nitrogen fertilisers has also been reported to decrease AMF root colonisation in strawberry and other crops (Azcon-Aguilar 1997; Salgado-Barreiro *et al*., 2012). Therefore, the fertilisation regime should be adjusted to achieve maximum strawberry yield and maintain AMF colonisation.

Aside from the effect of growing media and fertiliser application on AMF, other cultivation practices of strawberry may affect the ability of AMF to establish a symbiosis. Among these are irrigation practices (e.g. misting), cold storage of plug transplants and pesticide

application. Plant propagators or misting systems are required to maintain damp conditions for at least two weeks to ensure acclimatisation and rooting of strawberry tips (Durner *et al.*, 2002; Treder et *al.*, 2015). Some evidence suggests that AMF root colonisation may be limited under wet conditions because of a lower oxygen availability thereby reducing AMF propagule survival and root colonisation (Thormann *et al*., 1999; Miller, 2000). Before transplantation, strawberry plugs need to be cold stored at -2°C for various lengths of time. The potential consequences of a prolonged cold storage at freezing temperatures on the survival and infectivity of AMF propagules (i.e. spores, colonised roots and extraradical hyphae) in the root ball of strawberry plugs is unknown. However, several studies have suggested that AMF propagules of *Glomus* species have the ability to endure cold, including winter freezing, conditions (Safir *et al.*, 1990; Addy *et al.*, 1994; Addy *et al.*, 1997; Kabir *et al.*, 1997; Addy *et al.*, 1998; Klironomos *et al.*, 2001; Juge *et al.*, 2002). Therefore, tests need to be carried out to study the freeze tolerance of AMF colonising strawberry plug roots.

Conventional strawberry production usually requires high input of pesticides that may affect the AMF symbiosis establishment and/or its functioning. The composition of the pesticides, the doses applied, the combination applied, the application methods, the substrate types, the growing conditions, the cropping systems and AMF species within the system might all mediate the effect that pesticides may have on AMF symbiosis (Johnson & Pfleger, 1992). Therefore, there is not general rules about the effect the pesticides may have on the AMF symbiosis. Some pesticides (e.g. Captan) have been shown to be compatible or even stimulate the development of AMF mycelium in horticultural substrates (Lovato *et al*., 1995). In contrast, foliar applications of fosetyl-Al (fosetyl aluminium) on strawberry reduced AMF root colonisation in a pot experiment (Mark & Cassells, 1999).

Future research efforts should focus upon understanding the outcomes of the interaction between AMF species (or strains) and cultivation practices to confirm whether or not the AMF have a great potential to be used as biocontrol agent and/or bio-fertiliser in various strawberry production systems and other horticultural crops. The outcomes of this type of research will help growers to select the best AMF species (or isolates) combinations and cultivation practices to maximise AMF beneficial effects during propagation and/or after transplantation.

1.7. Project objectives

Sustainable horticulture has become high on the agenda for global governments and policy makers. The growing demands on water supply, land use, fertilisers and pesticides all lead to an increasing concern about global food security and environmental impact (Gianinazzi *et al*., 2010; Fitter, 2012). Strawberry is an important horticultural crop worldwide with a high economical, nutritional and medicinal value. Nevertheless, soil-borne pathogens such as *V. dahlia, P. fragariae* and *P. cactorum* cause a serious threat to strawberry production especially since the soil fumigant methyl bromide was banned in Europe due to its high ozone-depleting potential and risk to human health (Ristaino & Thomas, 1997; Martin, 2003). In addition, strawberry production is currently moving toward cultivation in substrates such as peat and coir, which are usually devoid of beneficial microbes such as AMF; therefore, introducing AMF and/or PGPR into soil-less substrates is more likely to generate benefits (Boyer *et al*., 2016). AMF associations are multi-functional, assisting the plants in nutrient and water uptake, and they can act as biocontrol agents by protecting roots from pathogens. Most importantly, the earlier AMF colonisation is established the greater the benefit (Azcón-Aguilar & Barea, 1997). In this context, the early introduction of sufficient AMF establishment of initial propagation and subsequent planting materials

in nurseries could be a useful strategy. If successful, AMF pre-inoculation could become an integral part of strawberry production in a near future.

However, several studies have highlighted the variability of the beneficial effects offered by AMF against root pathogens in different crops (Akhtar & Siddiqui, 2008). For example, the effects of AMF against root pathogens has been shown to differ among AMF species as well as among root diseases (Whipps, 2004). In addition, such disease suppressive effects may be further dependent on substrates, host cultivars and crop management practices (Baum *et al*., 2015). Therefore, there is still limited knowledge on the interaction among AMF, strawberry cultivars and root pathogens and the mechanisms underlying AMF-induced bio-protection under commercial conditions.

In an attempt to fill these knowledge gaps, the present study used the AMF-strawberry association as a model system to investigate the possibility of pre-colonising strawberry plugs to increase tolerance against major strawberry root diseases and/or increase strawberry productivity. Specifically, a series of experiments were conducted either under controlled conditions or under open field situations to investigate the following hypotheses (Figure 1.1):

- H1: It is feasible to apply AMF inoculum during strawberry tipping in different soil-less substrates under misting conditions and obtain highly AMF-colonised strawberry plugs. (Chapter 3)
- H2: AMF in colonised strawberry plug roots can survive a prolonged period of storage at -2°C. (Chapter 3)
- H3: AMF pre-colonisation of strawberry plugs increases plant tolerance against *V. dahlia* under glasshouse and open field conditions. (Chapter 4)

- H4: AMF pre-colonisation and/or inoculation of AMF and/or PGPR at planting increases plant tolerance against *P. fragariae* and *P. cactorum* in soil-less substrates. (Chapter 5)
- H5: AMF and/or PGPR inoculations increase strawberry productivity in coir bags under glasshouse conditions. (Chapter 5).
- H6: In an attempt to control for the influence of fluctuating environmental conditions that occur under both field and glasshouse conditions a simple *in vitro* autotrophic system can be established and used as a tool to investigate different aspects of the strawberry-AMF-pathogen interactions. (Chapter 6)

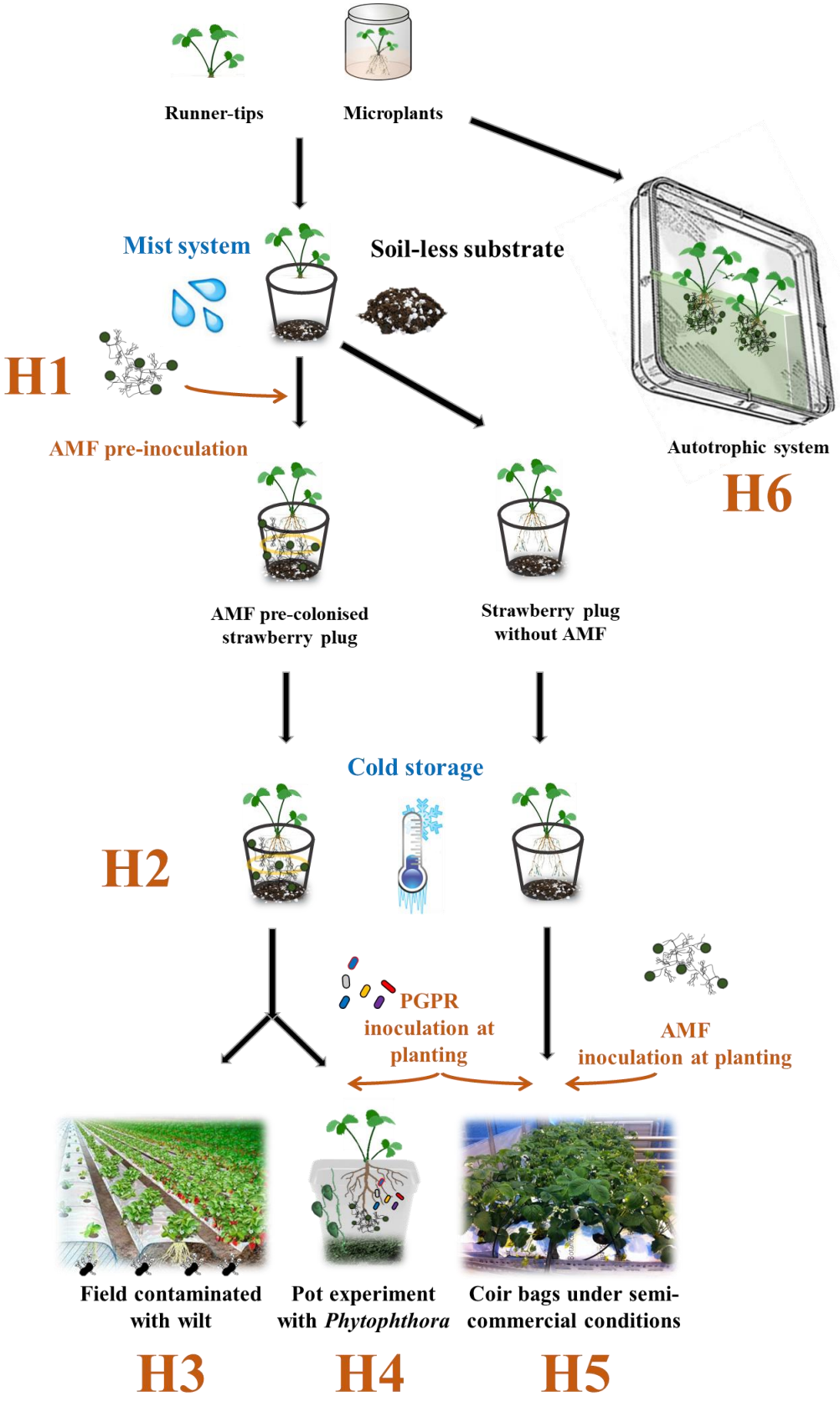

Figure 1.1: Overview of the strawberry cultivation systems examined and the six hypotheses. Hypotheses are not fully stated for clarity (Section 1.7).

Chapter 2. General methods

2.1. Arbuscular mycorrhiza fungi inoculum

2.1.1. Inoculum source and inoculation

Pure cultures of five AMF species (Table 2.1) and a commercial mix of the same five species were obtained from Plantworks Ltd, Kent, UK, as attapulgite clay/pumice/zeolite mix containing spores, mycelium, and colonised host plants root fragments. For strawberry plugs, AMF inoculum was incorporated as a powder layer comprising 10% (v/v) and applied ca. 1 cm below the surface of the potting substrate, before transplantation of the strawberry runner-tips. The use of different AMF species and even different isolates of the same species have showed to provide different beneficial effects on the same plant host due to their different ecological strategies (Rodriguez & Sanders, 2015). Nevertheless, the link between function and taxonomy of AMF species is still a poorly resolved subject (van der Heijden *et al*., 2004). Therefore, the AMF species used in this study were primarily selected for their commercial availability and because of beneficial effects on strawberry productivity reported in a previous work (Boyer *et al*., 2016). Therefore, if beneficial effects on strawberry health and/or productivity are identified, strawberry growers will be able to source and use those inocula.

Table 2.1: Arbuscular mycorrhiza fungi (AMF) species used in the studies (courtesy of Plantworks Ltd, Kent, UK).

AMF species	Authorities
Funneliformis mosseae	[T.H. Nicolson & Gerd.] C. Walker & A. Schüeßler 2010
Rhizophagus irregularis	[N.C. Schenck & G.S. Sm.] C. Walker & A. Schüeßler 2010
Claroideoglomus claroideum	[N.C. Schenck & G.S. Sm.] C. Walker & A. Schüeßler 2010
Funneliformis geosporus	[T.H. Nicolson & Gerd.] C. Walker & A. Schüeßler 2010
Glomus microaggregatum	Koske, Gemma & P.D. Olexia 1986

2.1.2. Most probable number bioassay

A most probable number (MPN) bioassay was undertaken to determine the infectivity and estimate the number of propagules in each inoculum sample (Cochran, 1950; Alexander, 1982). Samples were diluted to 1/10, 1/100 and 1/1000 using autoclaved (two cycles at 121°C for 20 min with 4 d between cycles) attapulgite clay substrate (AgSorb®, Oil-dri Ltd, Cambridgeshire, UK). For this bioassay, maize (*Zea mays* L.) was used as the trap plant as it was described to be a suitable host for *Glomus* spp. (Vestberg, 1995; Boyer pers. comm.); there were five replicate pots, each planted with three maize seeds (Figure 2.1). The pots were then placed either in a glasshouse (temperature 20-23°C, light:dark 16 h/8 h, additional 400 W halogen bulbs were also used) or a growth room (day and night 21-22°C, ca. 70% relative humidity (RH), light:dark 16 h/8 h, photosynthetic photon flux density (PPFD) of ca. 40 μmol m^{-2} s^{-1}). Plants were watered as required with tap water and roots were harvested six weeks after sowing. Harvested roots from each pot were then stained with trypan blue (see Section 2.3.1) and assessed microscopically for the presence of AMF structures. Based on the incidence of the microscopic presence of AMF structures in the sampled roots, MPN was then estimated, using MPN tables (Cochran, 1950).

Figure 2.1: *Zea may*s used as trap plants growing under glasshouse conditions to estimate AMF inoculum concentration. Five replicate pots were used at each AMF inoculum dilution: 1/10, 1/100 and 1/1000.

2.2. Plant materials

2.2.1. Production of micro-propagated strawberry plants

To be certain of the absence of pre-existing mycorrhizal colonisation, micro-propagated plants (hereafter named microplants) were used in several experiments. Microplants of *Fragaria* x *ananassa* cv. 'Calypso' and of *F. vesca* var. *alpina* were purchased from Hargreaves Plants Ltd, Norfolk, UK, whilst microplants of *F.* x *ananassa* cv. 'Vibrant', 'Red glory' and accession 'EM-1996', and of *F. vesca* clone VSI were provided by the NIAB EMR tissue culture laboratory, Kent, UK. Microplants were all established for at least two months on Murashige & Skoog (M&S) medium (Murashige & Skoog, 1962) supplemented with 0.75% agar, 3% sucrose, 1.2 mL L^{-1} GA_3 (phytohormone) and 8 mL L^{-1} IBA (phytohormone) to induce rooting. *In vitro* plants were incubated in a growth room

(21°C, light:dark 16 h/ 8 h PPFD of 40 μmol m^{-2} s^{-1}) until roots had developed sufficiently for plantlets to be transplanted.

2.2.2. Production of runner-tips

Pre-established strawberry mother plants (cv. 'Elsanta', 'Malling Centenary', 'Vibrant', 'Red Glory') were grown in coir bags (Botanicoir Ltd, London, UK) in a poly-tunnel or a glasshouse compartment at NIAB EMR. Runner-tips were produced within three months either under greenhouse conditions (in winter; temperature 20-23°C, light:dark 16 h/8 h, additional lightning in the form of 400 W halogen bulbs was used with ample irrigation, appropriate fertilisation regimes and pest control) or poly-tunnel conditions (in spring and summer; natural light and temperatures, ample irrigation, appropriate fertilisation regimes and pest control). Inflorescences emerging from the mother plants were removed regularly to stimulate runnering. Once runner plantlets contained at least three compound leaves they were cut away from the mother plants and used for experiments (weaning and AMF inoculation). Runner-tips were also purchased from two commercial nurseries: R W Walpole Ltd, Norfolk, UK for 'Vibrant' and Edward Vinson Plants Ltd, Kent, UK for 'Red Glory'.

2.2.3. Growth medium and strawberry plantlet weaning

The roots of the microplants were washed with purified water to remove any adhering agar and nutrients and transplanted into individual tray cells (40 cells, ca. 46 cm^3 per cell, B&Q 40 Cell Insert 08535B, Kent, UK; or 56 cells, 70 cm^3 per cell, Agrii Ltd, Kent, UK). Cells were filled with either non-autoclaved coir (Botanicoir Ltd, London, UK) or autoclaved (two cycles at 121°C for 20 min with 4 d between cycles) vermiculite medium (Sinclair horticulture Ltd, Lincoln, UK) fertilised with 0.25 g L^{-1} of autoclaved (one cycle at 121°C, 20 min) bone-meal, a complex nitrogen (N) and phosphorus (P) source to encourage AMF

development (3.5% N, 7.4% P; Verve, Hampshire, UK). Plantlets were then weaned in plastic propagator units with transparent vented lids (52 × 42.5 × 24 cm, Stewart Plastics Ltd, Oxon, UK) kept in a growth room (Meridian Refrigeration Ltd, Croydon, UK; day and night 21-22°C, ca. 70% RH, light:dark 16 h/8 h, PPFD of ca. 40 μmol m^{-2} s^{-1}; Figure 2.2). Both adjustable vents present on the lid of the propagator were kept closed initially (1 week), and then left open (1 week) before the lid was completely removed. Each plantlet was then watered as needed with 10 mL of purified water and no additional fertiliser was added. Inter plant contamination was prevented by spacing the plantlets over the plastic module tray (i.e. one empty tray cell between each plant), by using a syringe to water each plant and by avoiding a direct contact of the module tray cell with the bottom of the propagator.

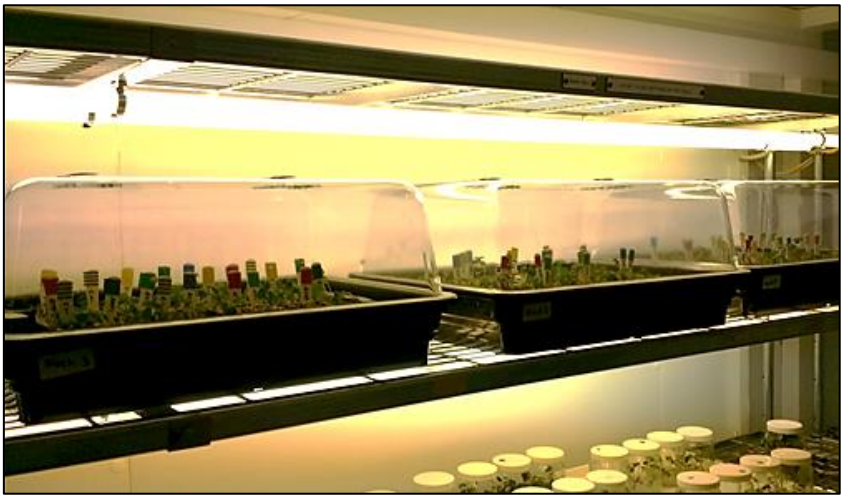

Figure 2.2: Microplants weaned inside plant propagators that were kept under growth room conditions.

Freshly cut runner tips were immediately pinned-down in standard plastic module trays (56 cells, 70 cm^3 per cell, Agrii Ltd, Kent, UK; or 48 cells, 70 cm^3 per cell, Desch Plantpak Ltd, Essex, UK) filled with the potting mix consisting of 7 parts Irish dark peat (Clover Peat Products Ltd, Dungannon, Ireland) and 3 parts 2.0-5.0 mm perlite (Sinclair

Horticulture Ltd, Lincoln, UK; Figure 2.3A). The potting mix was limed with 16 g L^{-1} of non-autoclaved dolomite lime (Omya UK Ltd, Derbyshire, UK) to provide a pH of 7 and fertilised with 0.25 g L^{-1} of autoclaved (one cycle at 121°C, 20 min) bone-meal (Verve, Hampshire, UK). Immediately after transplantation in plastic trays, runner-tips were placed in a misting cabinet (daily mean temperature: > 20°C, no artificial light, daily mean RH > 90%) and intermittently sprayed with tap water using a Macpenny Solarmist VTL misting system (Wright Rain Ltd, Hampshire, UK) for 15 d (misting continuously for ca. five seconds at a frequency depending on light conditions, ranging from six minute intervals on bright days to 20 minutes intervals on dull days according to the manufacturer guidelines; Figure 2.3B). Plant propagation was carried out in a glasshouse compartment (ca. 19°C, daily mean RH of 70%, 16 h/8 h light:dark cycle with additional lighting supplied in the form of 400 W halogen bulbs; Figure 2.3C). The plug plants were watered once a day with tap water. No additional fertiliser was added. Inter plant contamination was prevented by spacing the plants over the plastic module tray (i.e. one empty tray cell between each plant) and by avoiding a direct contact of the module tray with the bottom of the propagator and/or greenhouse tables.

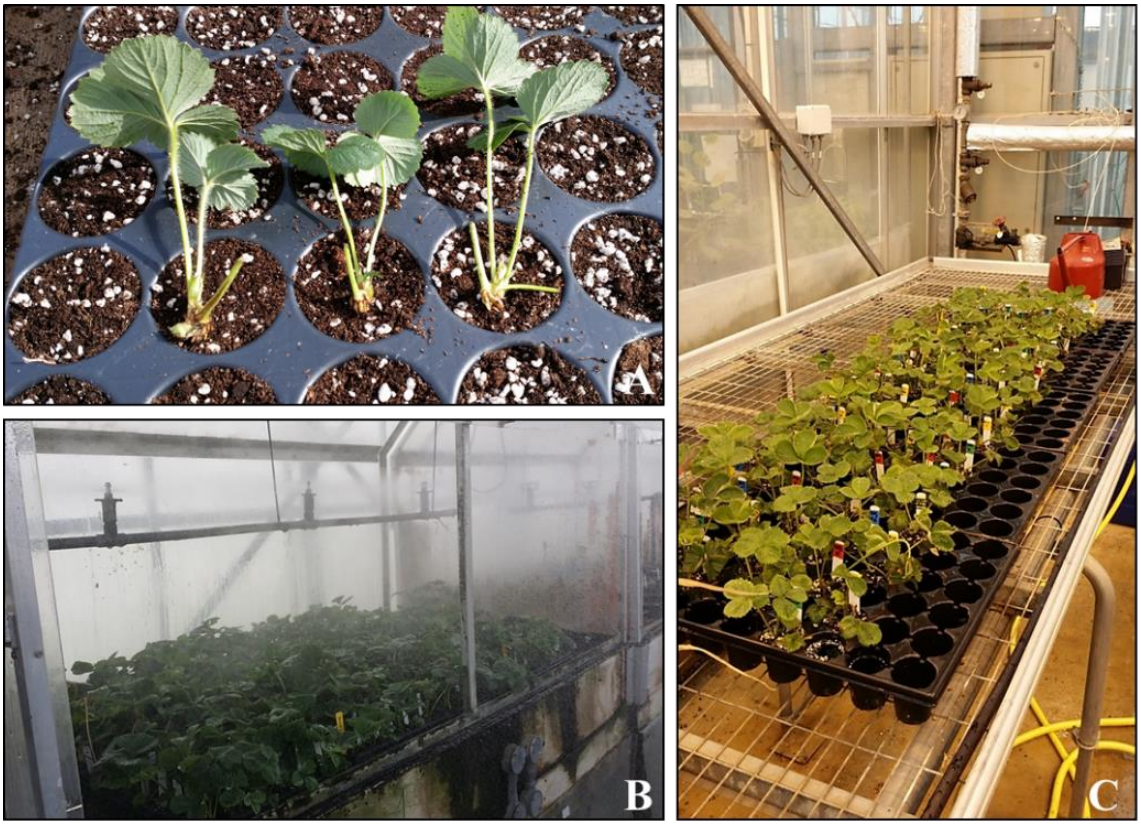

Figure 2.3: (A) Runner-tips ready to be pinned down on Irish dark peat/perlite mix (7:3, v/v). (B) Strawberry plug transplants inside misting system cabinet. (C) Plug plants growing under glasshouse condition at 7 weeks post transplantation.

2.3. Arbuscular mycorrhiza fungi quantification

2.3.1. Root sampling and staining

To observe AMF structures within strawberry roots, root samples were randomly picked, placed into histocassettes (Simport, Beloeil, Canada) to facilitate handling and cleaned with tap water to remove substrate particles. The roots were then cleared in 2% (w/v) potassium hydroxide solution (KOH) for 1 h at 90°C. Cassettes containing roots were then rinsed three times with tap water before being submerged for 30 min in 2% (v/v) hydrochloric acid (HCl) at room temperature. The HCl was then discarded and the root samples covered with 0.05% (w/v) trypan blue in lactoglycerol (lactic acid, glycerol, water – 1:1:1 as Kormanik & McGraw, 1982, but omitting phenol) for 1 h at 90°C in water bath. After de-staining in 50% (v/v) glycerol-water, root segments (30 per sample) were permanently mounted (using polyvinyl alcohol lactoglycerol (PVLG)) on two slides (15 root fragments per slide = ca. 60 cm of root in total per slide) as described in www.invam.wvu.edu/methods/recipes. Root cells were gently separated by applying slight pressure to the root (Figure 2.4).

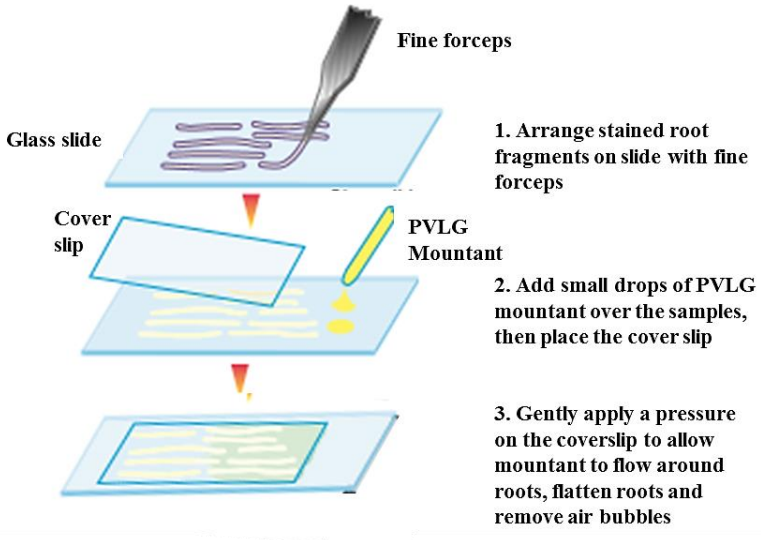

Figure 2.4: Method of stained root mounting on slides (figure adapted from www2.dijon.inra.fr/mychintec/Protocole/Image3.pdf)

2.3.2. Root length colonisation assessment

To quantify total root length colonised by AMF (% RLC) in the sample, the grid-line intersect method of McGonigle *et al*. (1990) was used. AMF colonisation was assessed on 100 intersects of root tissue and expressed as a percentage of root length colonisation. Slides were examined under a Leitz Diaplan microscope (Leitz, Wetzlar, Germany) with 250 × magnification. Sections of root were recorded as either positive or negative for any mycorrhizal structures as they crossed an intersect line of an eyepiece gratitude (McGonigle *et al.*, 1990). One hundred intersects were assessed for each sample. Figure 2.5 shows examples of intersects observed.

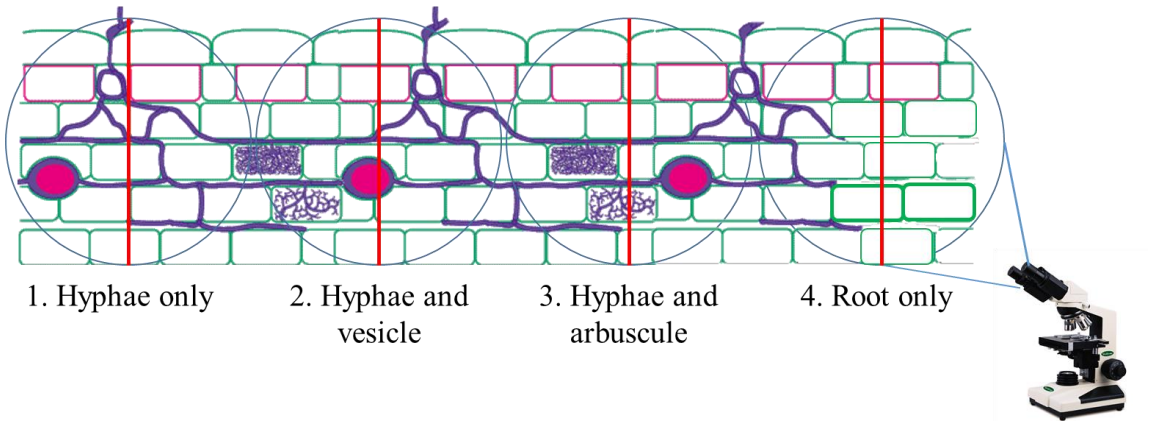

Figure 2.5: A microscopic method to assess strawberry root length colonisation (% RLC) by arbuscular mycorrhiza fungi. Randomly selected microscope field of view and cross-hair position are showing different possible intersects.

Chapter 3. Strawberry plug weaning practices and freezing cold storage do not prevent arbuscular mycorrhiza fungi root colonisation deriving from early inoculation

3.1. Introduction

Pre-inoculation of horticultural crops with AMF before transplanting has been proposed as an environmentally-friendly method to promote plant growth and health by protecting crops against biotic and abiotic stresses (Varma & Schüepp, 1994; Corkidi *et al.*, 2004; Vestberg *et al.*, 2004; Rouphael *et al.*, 2015). Strawberry (*Fragaria* x *ananassa*) production systems make AMF pre-inoculation at the weaning stage relatively straightforward for both *in vitro* and runner derived plantlets. AMF can colonise strawberry roots in different types of substrates and growing conditions (Holevas, 1966; Daft & Okusanya, 1973; Robertson *et al.*, 1988; Hršelová *et al.*, 1989; Vestberg, 1992a; Williams *et al.*, 1992; de Silva *et al.*, 1996). However, similar studies have not been carried out with runner-derived strawberry plants during the weaning stage.

The substrates commonly used to propagate strawberry plantlets deriving from *in vitro* and runner-tips are coir, peat, perlite and vermiculite (Vestberg *et al.*, 2000; D'Anna *et al.*, 2002; Corkidi *et al.*, 2004; Rouphael *et al.*, 2015; Treder *et al.*, 2015), but AMF propagules are not usually present in these growing media (Azcón-Aguilar & Barea, 1997). AMF inoculation of substrates containing peat mixed with sand, perlite, zeolite and/or vermiculite has been demonstrated to result in successful AMF colonisation of strawberry plantlets (Vosatka *et al.*, 1992; Williams *et al.*, 1992). In contrast, other studies have reported that certain types of peat had negative effects on AMF root colonisation of strawberry *in vitro* derived plantlets during propagation (Niemi & Vestberg, 1992; Vestberg *et al.*, 2000; Corkidi *et al.*, 2004; Palencia *et al.*, 2013). Therefore, it remains

unclear whether the negative effect of peat on AMF colonisation was due to high input of fertilisers (e.g. phosphorus) or to other chemical and biological properties of the peat itself (Martinez *et al.*, 2013; Palencia *et al.*, 2013). Because the majority of the strawberry runner-tips are rooted on peat-based media (Durner *et al.*, 2002), tests are therefore required to verify whether or not strawberry runner-tips can be pre-colonised by AMF under such conditions. Moreover, plant propagators or misting systems are required to maintain damp conditions for at least two weeks to ensure plant acclimatisation and rooting (Durner *et al.*, 2002; Treder *et al.*, 2015). Whereas many studies have been conducted on the effect of water deficiency on AMF development and influence on the host plant fitness (e.g. Boyer *et al*., 2015), less work has been done on the conditions of excess water. There are evidences to suggest that AMF root colonisation may be limited under wet conditions as a result of lower oxygen availability reducing AMF propagule survival (Thormann *et al.*, 1999; Miller, 2000), althought some wetland and aquatic plants often associate with AMF (Clayton & Bagyaraj, 1984; Miller, 2000).

Differences in the levels of AMF root colonisation among different strawberry cultivars have previously been documented under field (Robertson *et al.*, 1988) and glasshouse conditions (Chávez & Ferrera-Cerrato, 1990; Vestberg, 1992b), while other studies have found no significant variation under glasshouse conditions (Robertson *et al.*, 1988; Cekic & Yilmaz, 2011). Furthermore, the positive effect of AMF inoculation on strawberry plant growth is still open to debate. Several studies demonstrated the beneficial effects of AMF inoculation on strawberry growth during propagation (Kiernan *et al.*, 1984; Chavez & Ferrera-Cerrato, 1987; Hršelová *et al.*, 1989; Niemi & Vestberg, 1992; Vestberg, 1992b; Vestberg *et al.*, 2000; Borkowska, 2002; Stewart *et al.*, 2005; Castellanos-Morales *et al.*, 2010; Fan *et al.*, 2011; Boyer *et al.*, 2015), whilst others reported either limited (Cekic &

Yilmaz, 2011; Garland *et al.*, 2011; Palencia *et al.*, 2015) or negative effects (Chávez & Ferrera-Cerrato, 1990; Hršelová *et al.*, 1990; Vestberg *et al.*, 2004).

Before transplantation strawberry plugs need to be cold stored at -2°C for various lengths of time in order to achieve sufficient chilling to enhance flower initiation and to schedule cropping (Lieten *et al.*, 2005). The potential consequences of a prolonged cold storage at freezing temperatures on the survival and infectivity of AMF propagules (i.e. spores, colonised roots and extraradical hyphae) in the root ball of strawberry plugs are unknown, although several studies suggested that AMF propagules of *Glomus* species have the ability to endure cold, including winter freezing conditions (Safir *et al.*, 1990; Addy *et al.*, 1994; Addy *et al.*, 1997; Kabir *et al.*, 1997; Addy *et al.*, 1998; Klironomos *et al.*, 2001; Juge *et al.*, 2002).

Microscopic assessments of strawberry root have previously showed the presence of dark septate endophytes (DSE; Lizarraga *et al.*, 2015; Boyer pers. comm.). These DSE fungi have been described as 'miscellaneous fungi' that colonise the root tissue of a large array of plant species without causing any noticeable damage to their host (Jumpponen, 2001; Newsham, 2011). DSE have been frequently reported to co-exist with AMF under field conditions (Urcelay, 2002; Lizarraga *et al.*, 2015; Boyer pers. comm.). Hence, it is reasonable to expect that AMF and DSE share the same spatial niche and interact with each other. Whilst the AMF symbiosis is well investigated, the DSE association remains relatively under-studied, despite the fact that it seems to be as common as mycorrhizas in the field (Newsham, 2011). There is some evidence that DSE can improve plant growth under controlled conditions, but other reports indicate that these endophytes can also have negative or neutral effects on plant fitness (Jumpponen, 2001). Further studies on plant responses to DSE inoculation and/or interaction with AMF are clearly needed.

Four experiments were conducted to investigate whether inoculation with AMF during weaning of ex-tissue culture (hereafter named microplants) and runner-tip derived strawberry plantlets of different cultivars could result in plug transplants well-colonised by AMF. The following hypotheses were tested: (1) AMF can colonise roots of strawberry plantlets under damp conditions in different soil-less substrates; (2) inoculated AMF species differ in their ability to colonise strawberry cultivars; (3) AMF species differ in their capacity to colonise roots of different plant sizes; (4) early AMF colonisation increases growth of strawberry plugs during the weaning and propagation. Finally, one experiment was carried out to study the freezing tolerance (i.e. tolerance to the formation of ice in the strawberry root ball for several months) of AMF in colonised strawberry plug roots. Three commercially available AMF species were screened to test the hypothesis that AMF can survive several months at -2°C.

3.2. Materials and methods

A total of five experiments were carried out in 2014-2015; the duration of each experiment varied from six to 45 weeks. The objective of experiments 1-4 was to investigate the capacity of AMF to colonise strawberry plugs in different soil-less substrates under damp conditions, while the aim of experiment 5 was to study the freezing tolerance of AMF in colonised strawberry plug roots. A schematic representation of the experiment setup is shown in Figure 3.1 (colonisation during the weaning stage) and Figure 3.2 (freezing tolerance of AMF), whilst Table 3.1 gives the summary of experimental details for each experiment.

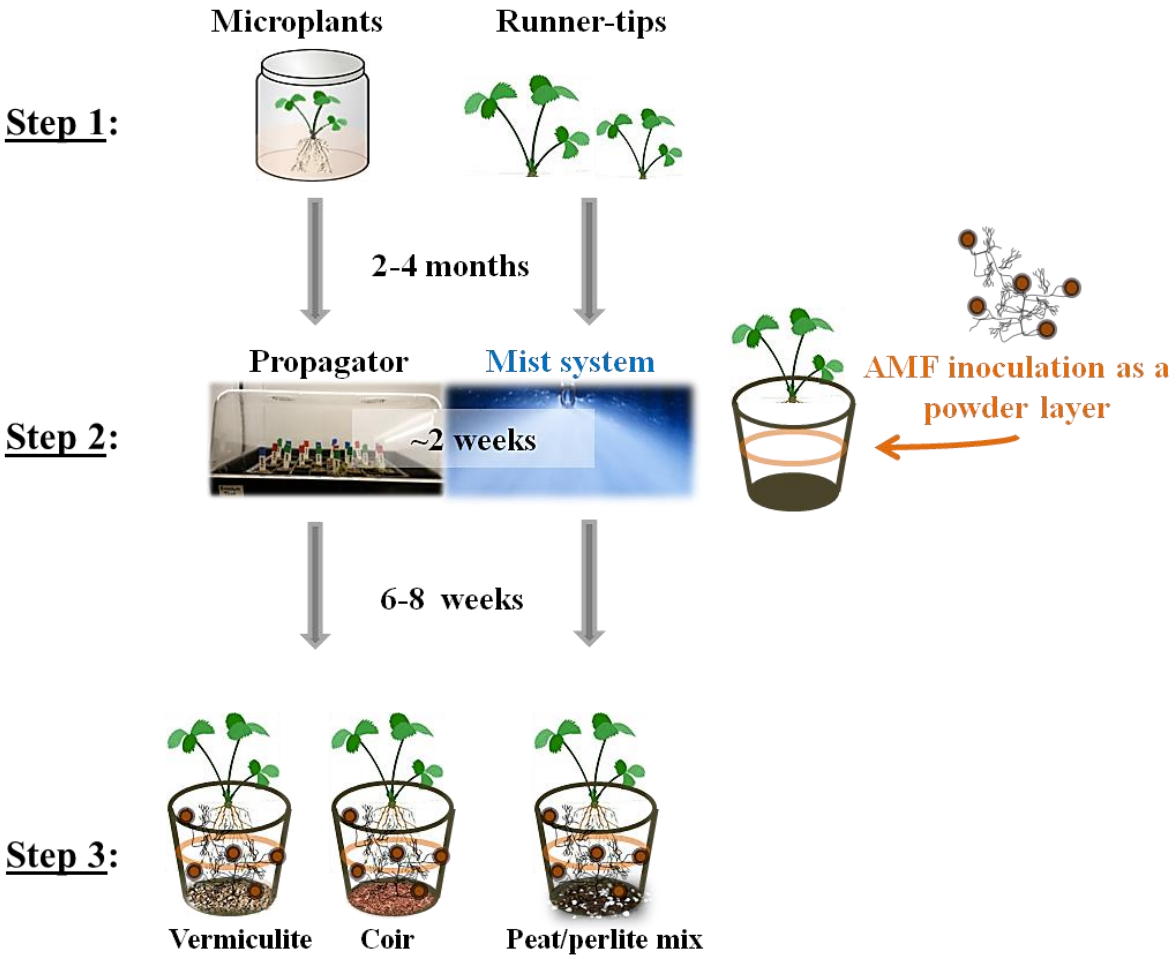

Figure 3.1: Schematic representation of the experimental setup to study the effect of arbuscular mycorrhiza fungi (AMF) pre-inoculation of strawberry transplants during weaning and propagation. Microplants and runner-tips were produced (Step 1), pinned down in vermiculite, coir, or Irish dark peat/perlite mix (7:3, v/v) and then weaned for ca. 2 weeks inside a propagator or misting cabinet (Step 2). Newly formed roots were assessed for the extent of AMF colonisation at the end of the plug transplant propagation (Step 3).

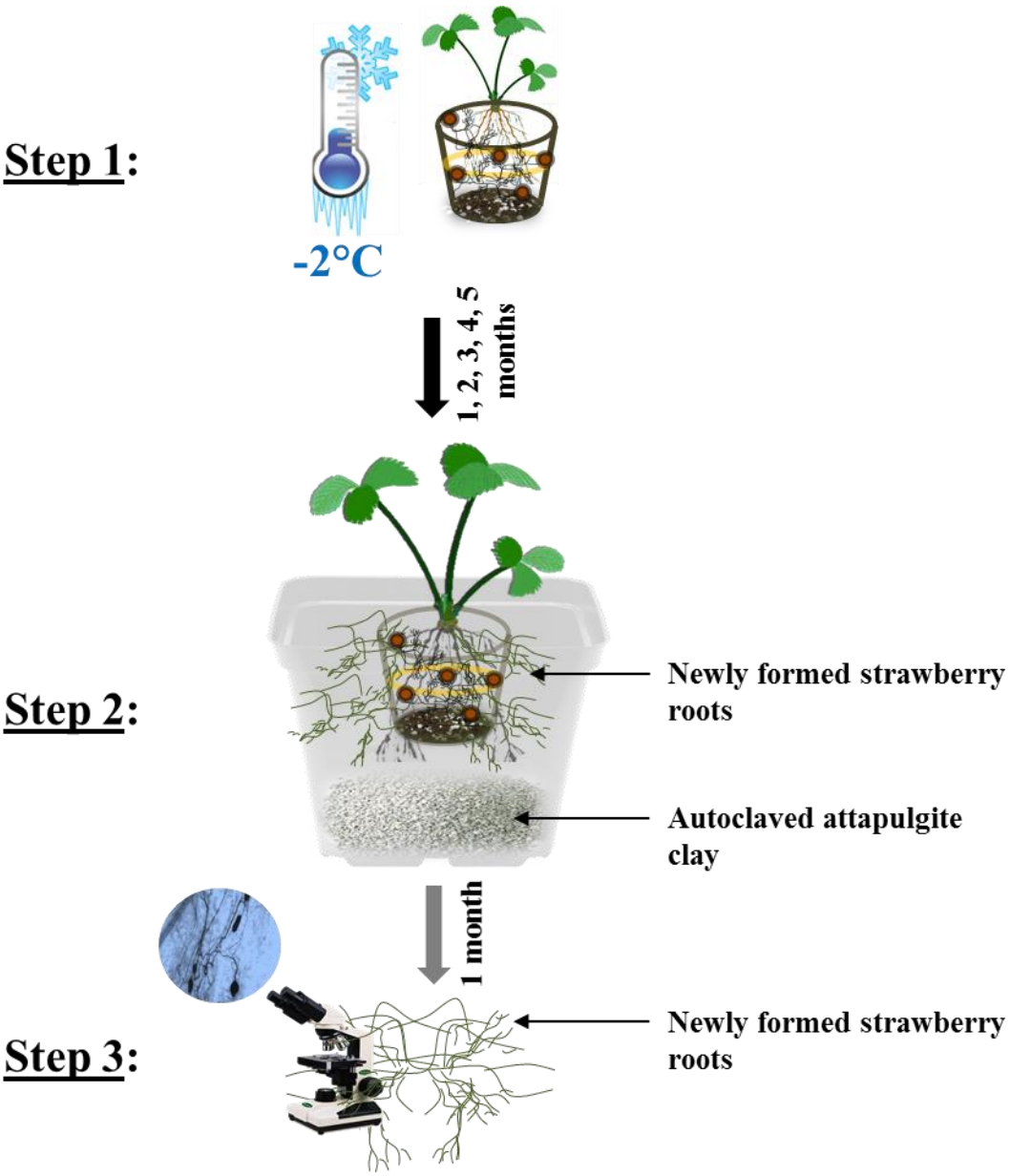

Figure 3.2: Schematic representation of the experimental setup to study the effect of cold storage on the survival of arbuscular mycorrhizal fungi (AMF) in colonised strawberry roots. Cold storage at -2°C of AMF pre-inoculated strawberry plug transplants (Step 1). Plants were re-potted at monthly intervals (1 to 5 months) in autoclaved attapulgite clay and placed in a growth room at 22°C (Step 2). Newly formed roots were assessed for the extent of AMF colonisation one month after transplantation (Step 3).

Table 3.1: Details of five experiments to study arbuscular mycorrhizal fungi (AMF) pre-inoculation of strawberry plug during the weaning stage (experiment 1-4) and the effect of cold storage at -2°C on the survival of AMF in colonised strawberry roots (experiment 5).

	Experiment				
	1	**2**	**3**	**4**	**5**
Total no. treatments	5	4	10	48	30
AMF species and controls[a]	F.m, R.i, C.c, Cb^-, Cb^+	R.i, Cb^+	F.m, R.i, C.c, Cb^-, Cb^+	F.m, R.i, C.c, G.m, F.g, Cb^+	F.m, R.i, C.c
Plant cultivars[b]	EM-1996	V	RG, V	E, MC, RG, V	RG, V
Plant (runner) size categories	-	-	-	Small/Large	-
Weaning substrates[c]	Vermiculite	Vermiculite or Coir	Peat/perlite mix	Peat/perlite mix	Peat/perlite mix
Re-growth substrate	-	-	-	-	Attapulgite clay
Cold storage duration at -2°C (months)	-	-	-	-	1, 2, 3, 4, 5
No. replicates per treatment	12	21	10	6	11
No. blocks (trays)	3	3	5	6	-
Plant material types[c]	Microplant	Microplant	Runner-tip	Runner-tip	Runner-tip
Weaning substrate autoclaved	Yes	No	No	No	No
AMF inoculum washing per plant (v/plant)	1 mL	10 mL	1.5 mL	10 mL	-
Plastic tray size (no. cells)[d]	40	56	48	48	56
Substrate fertilisation[d]	Bone-meal	-	Bone-meal	Bone-meal	Bone-meal
Dolomite lime amendment[d]	-	-	Limed	Limed	Limed
Weaning methods[d]	Propagator	Propagator	Misting system	Misting system	Misting system
Experiment locations	Growth room	Growth room	Glasshouse	Glasshouse	Growth room
Start dates	31/01/14	31/03/15	03/03/14	03/06/14	23/09/14
Duration of weaning + propagation (weeks)	2 + 4	2 + 6	2 + 4	2 + 5	2 + 16

[a] Abbreviations F.m, R.i, C.c, G.m and F.g respectively stand for the single AMF species inoculated: *Funneliformis mosseae*, *Rhizophagus irregularis*, *Claroideoglomus claroideum*, *Glomus microagregatum*, *Funneliformis geosporum*. Cb^-: a control inoculated with autoclaved attapulgite clay; Cb^+: a control inoculated with an autoclaved equal mix of AMF species used and inoculated with bacterial washing.

[b] Abbreviations E, MC, RG, and V respectively stand for the strawberry cultivars: 'Elsanta', 'Malling Centenary', 'Red Glory' and 'Vibrant'.

[c] See Section 2.2.1 for vermiculite or coir and Section 2.2.2 for peat/perlite mix.

[d] See Section 2.2.3 for experimental details.

3.2.1. Inoculation of arbuscular mycorrhiza fungi

All AMF species used in the experiments (Table 3.1) were provided by Plantworks Ltd, Kent, UK, and inoculated as described in Section 2.1.1. The number of infective propagules of each AMF inoculum (Table 3.2) was determined using a most probable number (MPN) bioassay procedure described in Section 2.1.2. In addition, two non-AMF controls were included: (1) control inoculated with autoclaved (two cycles at 121°C for 20 min with 4 d between cycles) attapulgite clay (AgSorb®, Oil-dri Ltd, Cambridgeshire, UK) to assess possible physico-chemical effects of the main AMF inoculum carrier (Cb^-), and (2) control inoculated with autoclaved (one cycle, 121°C, 20 min) inoculum or an equal mix of the single AMF species used (Cb^+; Table 3.2). To equalise the starting microbial community, 1 mL, 1.5 mL or 10 mL of AMF inoculum washing solution was also added to each plantlet (Table 3.1). This was produced by suspending 1 g of live inoculum in 10 mL of purified water, then vortexed for ca. 10 min and filtered through a 45 μm sieve (Laboratory test sieve, Endecotts Ltd, London, UK).

Table 3.2: Number of arbuscular mycorrhiza fungi (AMF) infective propagules per mL of inoculum substrate carrier used to inoculate strawberry plants in experiments 1-5.

Experiment	**AMF species**	**Propagules mL^{-1}**
1, 3, 4	*Funneliformis mosseae*	> 1600
5	*Funneliformis mosseae*	170
1, 3, 4	*Rhizophagus irregularis*	> 1600
2	*Rhizophagus irregularis*	70
5	*Rhizophagus irregularis*	170
1, 3, 4	*Claroideoglomus claroideum*	> 1600
5	*Claroideoglomus claroideum*	23
4	*Glomus microagregatum*	79
4	*Funneliformis geosporum*	350

3.2.2. Source of plant materials, weaning and propagation

Microplants (Table 3.1; Figure 3.3; 3.4) or runner-tips (Table 3.1; Figure 3.5; 3.6; 3.7A) were used for the experiments. A randomised block design was used except for experiment 5 (see Section 3.2.4 below). In experiment 4, there were also two plant crown size groups named grade 'Large' and 'Small' (Table 3.3). Experiments were all carried out in plastic trays that were either filled up with vermiculite, coir or Irish dark peat/perlite mix (Table 3.1). The pH (e.g. low pH: < 5.1) and nutrient status (e.g. high P level: > 25 ppm) of the growing media can negatively influence AMF symbiosis and its effect on plant growth (Azcón-Aguilar & Barea, 1997; Horneck *et al*., 2011). Therefore, the background nutrient status of the weaning substrates was analysed before plant transplantation and showed a neutral or alkaline pH and a low P level for all substrates tested (Table 3.4). The microplants or runner-tips were transplanted and weaned as described in Section 2.2.3. In experiment 1 and 2, each plantlet was watered as required with 10 mL of purified water, while in the other three experiments plants were watered once a day with tap water. No additional fertiliser was added. However, the absence of fertilisation resulted in rapid occurrence of nutrient deficiency symptoms in experiment 1. Thus, each plant received 10 mL of half-strength Rorison's nutrient solution minus phosphate, three times per week (Hewitt & Bureaux, 1966), which rapidly alleviated the nutrient deficiency symptoms. During the growing period of experiment 2, the plants were infested by spider-mites, which was controlled by weekly release of spider-mite predators (*Phytoseiulus persimilis*; Phytoline p; Syngenta Bioline Ltd, Essex, UK) until the end of the experiment.

In experiment 5, after weaning plants were first grown for 48 d under glasshouse conditions (see Section 2.2.3) and then transferred to a poly-tunnel with natural shorter days and cooler temperatures for 51 d to induce plant dormancy (Figure 3.7A). Plants continued to be watered daily with tap water. At the end of the propagation period, plants were

acclimated to lower temperatures for 24 d in a dark compartment cooled to 2°C. Watering ceased from this point onwards. Finally, strawberry plugs were cold stored at -2°C for different periods of time (Figure 3.7B; see Section 3.2.4 below).

Table 3.3: Average strawberry runner crown diameter for the explant size of 'large' and 'small' in experiment 4.

Strawberry cultivar	**Crown diameter (mm)***	
	'Large'	**'Small'**
'Elsanta'	8.9 ± 0.4	5.2 ± 0.3
'Malling Centenary'	6.9 ± 0.4	4.4 ± 0.2
'Red Glory'	5.9 ± 0.2	4.1 ± 0.2
'Vibrant'	7.8 ± 0.3	4.4 ± 0.1

* mean ± SE, n = 21

Table 3.4: Background nutrient status analysis[a] of the growing media used in the experiments.

Experiment	**Medium**	**pH**	**NO_3**	**NH_4**	**P**	**K**	**Mg**	**Ca**
			ppm = mg kg^{-1}					
1, 2	Vermiculite	8.1	< 1.2	29.8	< 1.2	36.2	21.9	1.2
2	Coir	6.6	< 3.7	19.8	< 6.2	30.9	< 1.2	1.9
3	Irish dark peat/perlite mix	7.0	13.9	63.1	2.1	8.8	40.2	25.1
4	Irish dark peat/perlite mix	7.2	39.9	35.2	2.5	6.2	58.4	37.7
5	Attapulgite clay	6.4	< 0.7	18.8	3.2	81.0	117.6	142.9

[a] Available nutrient status of each growth medium was provided by NRM Laboratories (Berkshire, UK) in mg L^{-1} and it was converted to mg kg^{-1}. NO_3 was determined by ion chromatography and NH_4 by colorimetric analysis. P, K, Mg and Ca were analysed by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy). Note that the nutrient status analysis of each growing medium could not be replicated due to high cost. Only the substrate attapulgite clay used for plant re-growth was analysed in experiment 5. Hence, the Irish dark peat/ perlite mix used for plant weaning in experiment 5 was not analysed for background nutrient status.

Figure 3.3: (A) Microplants (experiment 1) weaned on autoclaved vermiculite inside plant propagators kept under growth room conditions at 22°C. (B) Strawberry plugs at 6 weeks post transplantation.

Figure 3.4: (A) Strawberry plugs at the end of experiment 2 (8 weeks post transplantation). (B) A strawberry plug grown on non-autoclaved vermiculite. (C) A strawberry plug grown on non-autoclaved coir with the AMF inoculum powder layer visible (see red arrow).

Figure 3.5: (A) Experiment 3 strawberry plug transplants weaned in Irish dark peat/perlite mix (7:3, v/v) inside a misting cabinet. (B) Plug plants growing under glasshouse conditions for ca. 4 weeks with additional lightning. (C) A strawberry plug cv. 'Red Glory' from the non-mycorrhizal treatment and (D) strawberry plug cv. 'Red Glory' inoculated with *Claroideoglomus claroideum* at 6 weeks post transplantation.

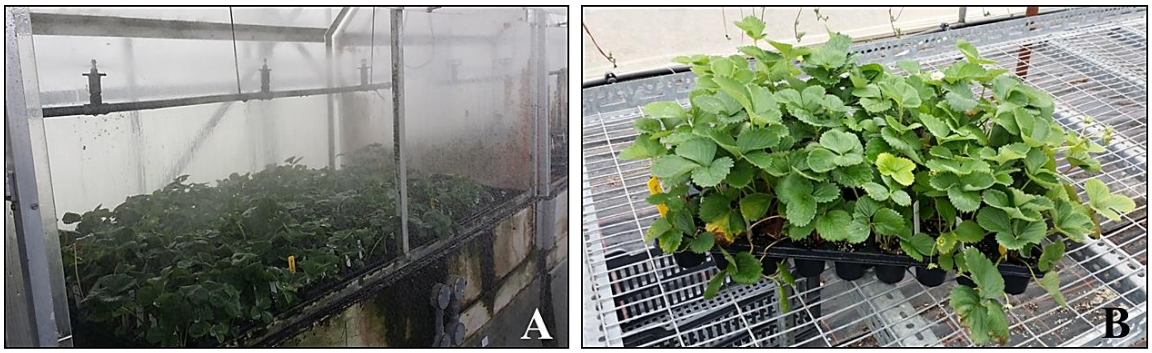

Figure 3.6: (A) Experiment 4 strawberry plug transplants weaned in Irish dark peat/perlite mix (7:3, v/v) inside a misting cabinet. (B) Plug plants were grown under glasshouse conditions for 7 weeks.

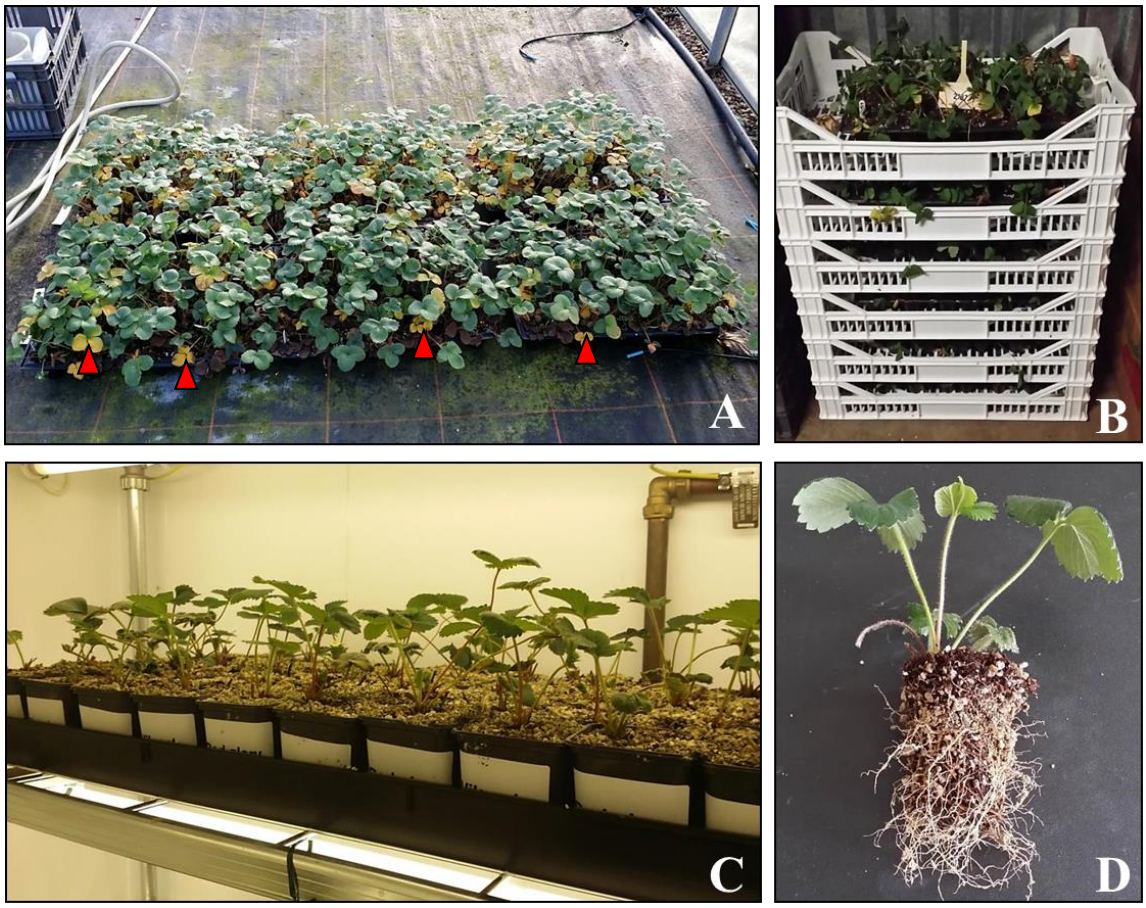

Figure 3.7: (A) Experiment 5 strawberry plug transplants propagated in Irish dark peat/perlite mix (7:3, v/v) under natural temperature and light conditions in a poly-tunnel. Note the first signs of dormancy shown by yellow leaves (red arrows). (B) Module trays cold stored in the dark at -2°C. (C) Strawberry plugs re-potted in autoclaved attapulgite clay producing new leaves after one week in a growth cabinet at 22°C. (D) A strawberry plug producing new roots after one month of re-potting.

3.2.3. Re-growth of strawberry plugs after freezing cold storage

In experiment 5, strawberry plants stored at -2°C were moved out of the cold store, re-potted into 250 mL plastic pots (7 × 7 × 8 cm, Desch Plantpak Ltd, Essex, UK) and placed to a growth room after 1, 2, 3, 4 or 5 months in the cold store. The dead leaves were cut-off and the attapulgite clay substrate (AgSorb®, Oil-dri Ltd, Cambridgeshire, UK) was autoclaved (two cycles at 121°C for 20 min with 4 d between cycles). A square piece of filter paper was placed inside each pot to avoid substrate loss and so inter contamination

during watering. Pots were randomised on a single plastic tray (Figure 3.7C). Plants were grown for one month in the growth room (Meridian Refrigeration Ltd, Croydon, UK; day and night 21-22°C, light: dark 16 h/8 h; photosynthetically active radiation (PPFD) of 40 μmol m^{-2} s^{-1} with RH ca. 70%; Figure 3.7C) and watered from the bottom twice a week with 4 L of purified water. No additional fertiliser was added.

3.2.4. Root sample analysis and plant growth

For all experiments, fresh roots were sampled at the end of the experimental period (with an additional root sampling before cold storage in experiment 5) then stained (Section 2.3.1) to assess endophyte and AMF colonisation (Section 2.3.2). Aseptate inter- or intracellular linear hyphae associated with vesicle and/or arbuscule structures were characterised as AMF colonisation. Microsclerotium like structures, moniliform group of fungal cells and non-linear hyphae not associated with vesicles or arbuscules were recorded as dark septate endophyte (DSE) colonisation. In experiment 1, each root sample were assessed (n = 12) for each treatment, whereas a subset of individual samples was used in experiment 2 (n = 9), experiment 3 (n = 3) and experiment 4 (n = 3) for each AMF treatment and plant cultivar tested. In experiment 5, each root sample originated from two pooled individual plants and a subset (n = 3) was assessed for each AMF treatment and plant cultivar tested.

Plant crown size was measured by a calliper measurement at the widest point (experiment 1-4). Plant height was measurement by a calliper measurement of the highest leaf stalk, measured from the crown to the tip of the stalk (experiment 1 and 3). To measure the total plant fresh weight (experiment 1 and 2), plants were harvested, and roots were gently washed with tap water to remove substrates particles and plants were weighted. For the

dry weight (experiment 2), plants were oven dried at 80°C for at least 3 d before weighing. To calculate the root weight ratio (RWR: root dry weight as a fraction of total plant dry weight), roots were weighted after being separated from the shoot. Strawberry plugs that were non-destructively harvested in experiment 1 and 3 were used in other experiments (See Chapter 4).

3.2.5. Data analysis

All data were analysed using GenStat 13th edition (VSN International Ltd, Hemel Hempstead, UK). AMF root length colonisation (% RLC) was analysed by ANOVA after arcsine square root transformation to satisfy normality. In experiment 1, treatment effects were tested using a one-way ANOVA while a two-way ANOVA was used to test for treatment effects in experiment 2 and 3. In experiment 4, treatment effects were analysed using an unbalanced three-way ANOVA. In experiment 5, the effect of cold storage duration on % RLC was assessed using a three-way ANOVA with storage time (1 to 5 months), AMF treatment (single AMF species inoculated: *F. mosseae* or *R. irregularis* or *C. claroideum*) and strawberry cultivar ('Vibrant' or 'Red Glory') as factors. The main objective of this analysis was to test the effect of cold storage duration on AMF survival in colonised roots. However, it was suspected that plant roots with high DSE colonisation were more likely to have lower AMF root colonisation due to competition for space or other indirect effects. Therefore, DSE root colonisation level was included as a covariate in the ANOVA to remove its influence on AMF root colonisation. The significant differences among individual treatments were determined using a least significant difference (LSD) post-hoc test once the overall effect was significant for a specific treatment factor. Additionally, proportions of plant survival between AMF inoculated and control treatments in experiment 4 were analysed using a generalised linear model (GLM)

with residual errors assumed to follow binomial distributions; the logit link function was used.

Only significant differences are reported in the text. For the plant growth parameter data, there was no significant difference among the AMF species, the data from different AMF species were hence pooled to form a single group (AMF inoculated). The non-mycorrhizal (NM) control (Cb^+ and Cb^-) data were treated similarly. Therefore, only the overall effect AMF cf. NM for plant growth parameters has hereafter been presented.

3.3. Results

3.3.1. Influence of weaning conditions on AMF colonisation

Microscopic assessments of strawberry plug roots showed the presence of arbuscules and/or vesicles for all the plants tested irrespective of the type of plant material, growing substrate, plant size and cultivar, confirming the presence of AMF (Figure 3.8; 3.9; 3.10). The occurrences of microsclerotium-like structures and/or moniliform group of fungal cells were sporadically observed in the root cortex of plants in experiment 4, while they were more abundant in experiment 5, suggesting the presence of DSE (Figure 3.11).

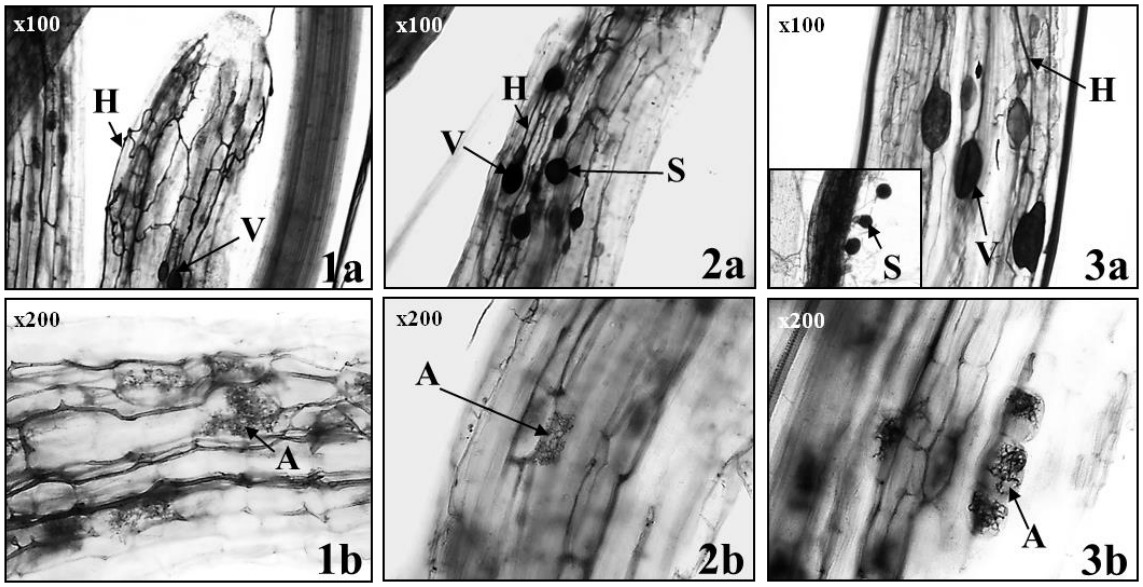

Figure 3.8: Root colonisation by arbuscular mycorrhiza fungi (AMF) of *Fragaria* x *ananassa* plug ('EM-1996') after 6 weeks cultivation in autoclaved vermiculite in a growth room at 22°C (experiment 1). The numbers represent the AMF species: (1) *Funneliformis mosseae*; (2) *Rhizophagus irregularis*; (3) *Claroideoglomus Claroideum* while the adjoining letters represent various mycorrhizal structures (a) or arbuscules (b). Letters next to black arrows are A: arbuscule, V: vesicle, H: hypha, S: spore. The scale bar feature was not available in the camera used for image acquisition. Thus, the magnification is reported instead.

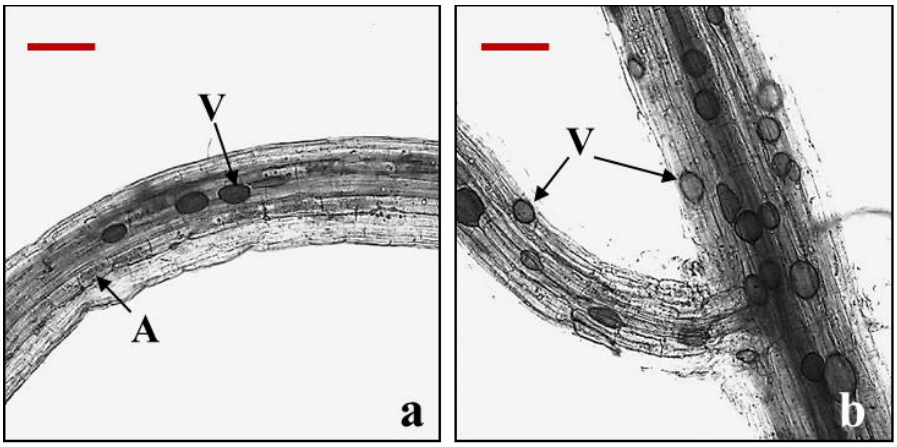

Figure 3.9: Root colonisation by arbuscular mycorrhiza fungi (AMF) of *Fragaria* x *ananassa* plug cv. 'Vibrant' after 8 weeks cultivation in (a) coir and (b) vermiculite in a growth chamber at 22°C (experiment 2). Longitudinal squash of roots stained with trypan blue colonisation by single AMF species *Rhizophagus irregularis*. Letters next to black arrows are A: arbuscule and V: vesicle (red scale bars represent 100 µm).

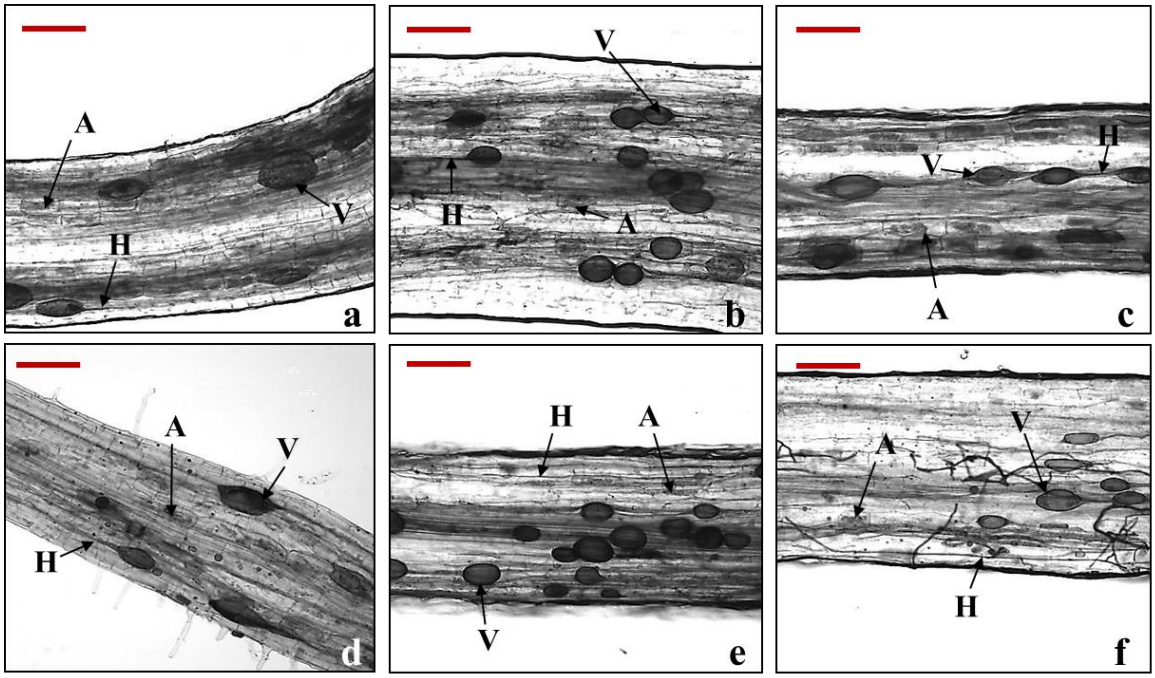

Figure 3.10: Root colonisation by arbuscular mycorrhiza fungi (AMF) of *Fragaria* x *ananassa* plug cv. 'Vibrant' (a-c) and 'Red Glory' (d-f) after 6 weeks of cultivation in the Irish peat/perlite mix (7:3; v/v) under glasshouse conditions (experiment 3). Longitudinal squash of roots stained with trypan blue colonisation by single AMF species: (a, d) *Funneliformis mosseae*; (b, e) *Rhizophagus irregularis*; (c, f) *Claroideoglomus claroideum*. Letters next to black arrows are A: arbuscule, V: vesicle, H: hypha (red scale bars represent 100 μm).

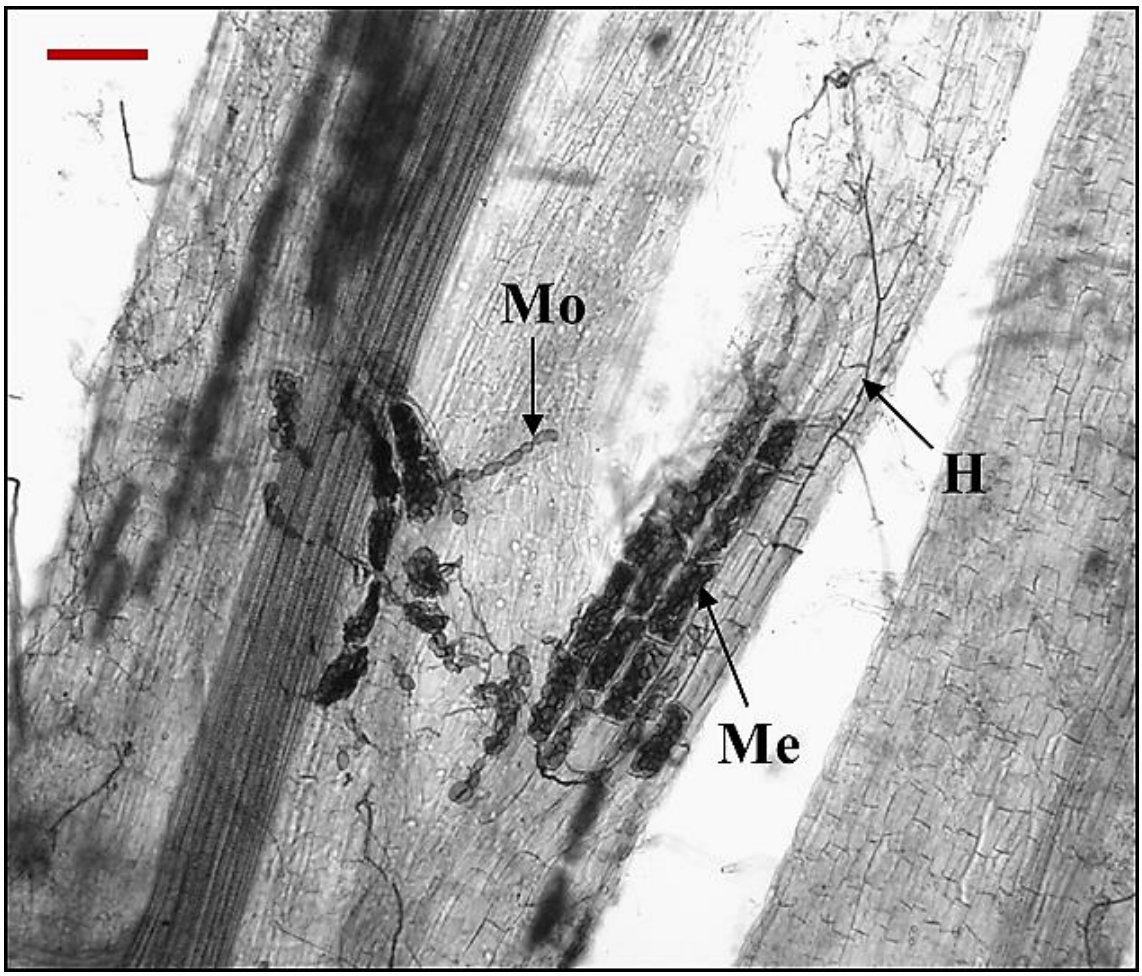

Figure 3.11: Root colonisation by dark septate endophytes (DSE) of *Fragaria* x *ananassa* of cv. 'Elsanta' after 7 weeks of cultivation in the Irish dark peat/perlite mix (7:3; v/v) under glasshouse conditions (experiment 4). Letters next to black arrows are H: hypha, Mo: moniliform cell and Me: microsclerotium (red scale bars represent 100 μm).

In experiment 1, the use of vermiculite as a substrate proved equally optimal for *F. mosseae*, *R. irregularis* and *C. claroideum* root colonisation. Irrespective of the AMF species tested, average % RLC at 6 weeks was ca. 94% (Figure 3.12A) and there were no significant differences in the frequency of arbuscules or vesicles among the three AMF species tested (Figure 3.12B, C).

In experiment 2, vermiculite and coir were both conducive for root colonisation by *R. irregularis*. However, there was a significant difference in % RLC between the two

substrates (Figure 3.12D; $F_{1,14} = 54.7$; $P < 0.001$). Average % RLC for coir was 40% and 76% for vermiculite at 8 weeks post inoculation.

In experiment 3, the Irish dark peat/perlite mix used for the weaning of runner-tips allowed *F. mosseae*, *R. irregularis* and *C. claroideum* to equally colonise plant roots. No significant differences were found between 'Vibrant' and 'Red Glory' cultivars. Average % RLC at 6 weeks ranged from 67 to 81%. In addition, there were no significant interactions between AMF and cultivar (Figure 3.12E). Similarly, the frequency of arbuscules (Figure 3.12F) and vesicles (Figure 3.12G) did not depend on the AMF species inoculated and strawberry cultivar. Finally, there were no significant interactions between AMF and cultivar for arbuscule or vesicle frequencies.

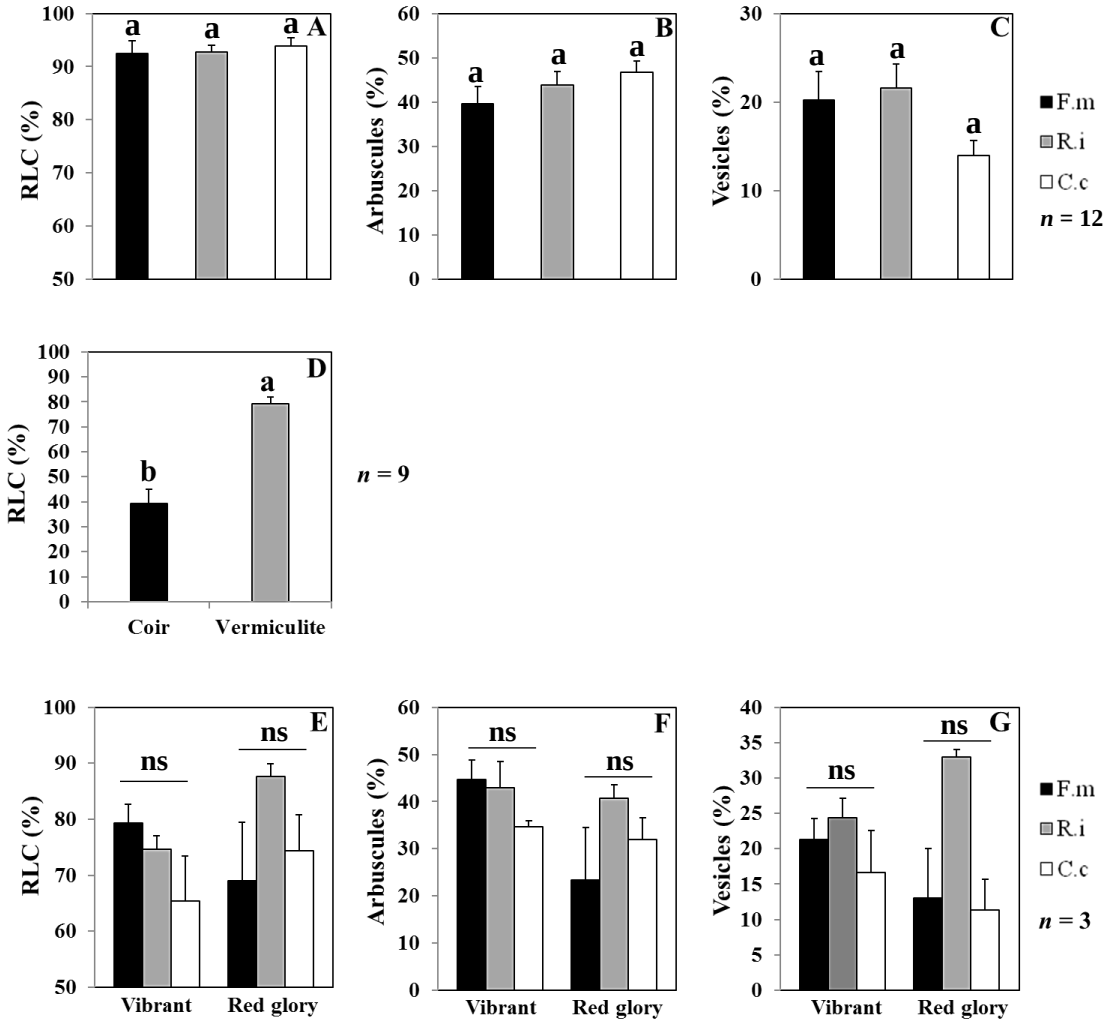

Figure 3.12: Percentage of root colonisation by arbuscular mycorrhizal fungi (AMF, % RLC), arbuscules and vesicles in experiment 1 (A-C), experiment 2 (D) and experiment 3 (E-G). The abbreviations F.m, R.i and C.c respectively stand for *Funneliformis mosseae*, *Rhizophagus irregularis* and *Claroideoglomus claroideum*. In experiment 2, *R. irregularis* was the only AMF species used. Root samples originated from two plants in experiment 2, but from individual plants in all the other experiments. No AMF colonisation observed in non-mycorrhizal controls (Cb^- and/or Cb^+). Bars represent standard error (+ 1 SE), and *n* is the number of replicates assessed (per treatment). Treatments that did not differ significantly share at least one common letter (Pairwise comparisons, $P \leq 0.05$). ns indicates the absence of statistical significance between AMF species inoculated for each strawberry cultivar tested (E-G; Pairwise comparisons, $P \leq 0.05$). Note some scales do not start at 0.

In experiment 4, the Irish dark peat/perlite mix also supported the establishment of AMF root colonisation of the strawberry plugs. However, differences among AMF species inoculated were evident (Table 3.5; $F_{4,78}$ = 19.6; P < 0.001), except between *F. mosseae* and *G. microagregatum.* Average % RLC was in the order: *R. irregularis* (64%) > *F. mosseae* (42%) > *G. microagregatum* (40%) > *C. claroideum* (26%) > *F. geosporum* (12%). A significant effect (Table 3.5; $F_{3,78}$ = 3.5; P = 0.018) of strawberry cultivar on % RLC was also detected as % RLC for cultivars 'Elsanta', 'Red Glory' and 'Vibrant' was ca. 40%, but only 27% for 'Malling Centenary'. There was no significant difference of % RLC between the two runner size groups (Table 3.5); there were no significant interactions among factors (Table 3.5).

Table 3.5: Results of the three-way ANOVA of the total percentage root colonisation by arbuscular mycorrhiza fungi (AMF, % RLC) in strawberry plugs grown for 7 weeks in Irish dark peat/perlite mix (7:3, v/v) under glasshouse conditions (experiment 4). Significant differences are shown by bold font ($P \leq 0.05$).

Source of variation	**% RLC**			
	df	***Mean square***	***F***	***P***
Cultivar	3	0.23	3.54	**0.018**
AMF	4	1.28	19.62	**< 0.001**
Plant (runner) size	1	0.06	0.92	0.340
Cultivar × AMF	12	0.08	1.21	0.294
Cultivar × Plant (runner) size	3	0.10	1.58	0.201
AMF × Plant (runner) size	4	0.01	0.09	0.986
Cultivar × AMF × Plant (runner) size	12	0.03	0.51	0.902
Residual	78	0.07		

df = degrees of freedom. Fixed effects include cultivar (refers to 'Elsanta', 'Malling Centenary', 'Red Glory' and 'Vibrant'), AMF (refers to plants inoculated singly with the AMF species: *Funneliformis mosseae, Rhizophagus irregularis, Claroideoglomus claroideum, Funneliformis geosporum, Glomus microagregatum*).

In experiment 5, AMF colonisation of roots of strawberry plugs cultivated in peat/perlite mix grown under glasshouse/poly-tunnel conditions was observed. DSE were also present in the roots. Before cold storage % RLC and % DSE colonisation ranged from 1-16%, and 11-16% respectively (Table 3.6).

Table 3.6: Percentage of root length colonisation by arbuscular mycorrhiza fungi (AMF, % RLC) and by dark septate endophytes (DSE, % DSE) of *Fragaria* x *ananassa* plug cv. 'Vibrant' and 'Red Glory' inoculated with single AMF species (*Funneliformis mosseae*, *Rhizophagus irregularis*, or *Claroideoglomus claroideum*) after 113 d of cultivation in the Irish dark peat/perlite mix (7:3, v/v) under glasshouse/poly-tunnel conditions (experiment 5).

Plant cultivar	AMF species	% RLC *	% DSE*
Vibrant	*F. mosseae*	1 ± 1	12 ± 2
	R. irregularis	16 ± 2	14 ± 2
	C. claroideum	9 ± 3	12 ± 1
Red Glory	*F. mosseae*	15 ± 5	16 ± 4
	R. irregularis	4 ± 2	15 ± 2
	C. claroideum	10 ± 5	11 ± 4

* mean ± SE; n = 3, each root sample was pooled from five individual plants.

3.3.2. Effect of AMF on strawberry plug transplant growth

In experiment 1, all plants survived and grew normally. Plant crown diameter was affected by AMF inoculation ($F_{1,56}$ = 11.7; P = 0.001). AMF inoculated plants had bigger crowns (mean 3.2 ± 0.1 mm; n = 36; Figure 3.13A) than the NM plants (mean 2.7 ± 0.1 mm; n = 24; Figure 3.13A), while plant height was not modified by AMF inoculation. Plant fresh biomass was influenced by AMF inoculation ($F_{1,56}$ = 5.8; P = 0.021). AMF-inoculated plants produced less fresh biomass (mean 2.4 ± 0.1 g; n = 36; Figure 3.13B) than the non-mycorrhizal treatment (mean 2.7 ± 0.1 g; n = 24; Figure 3.13B).

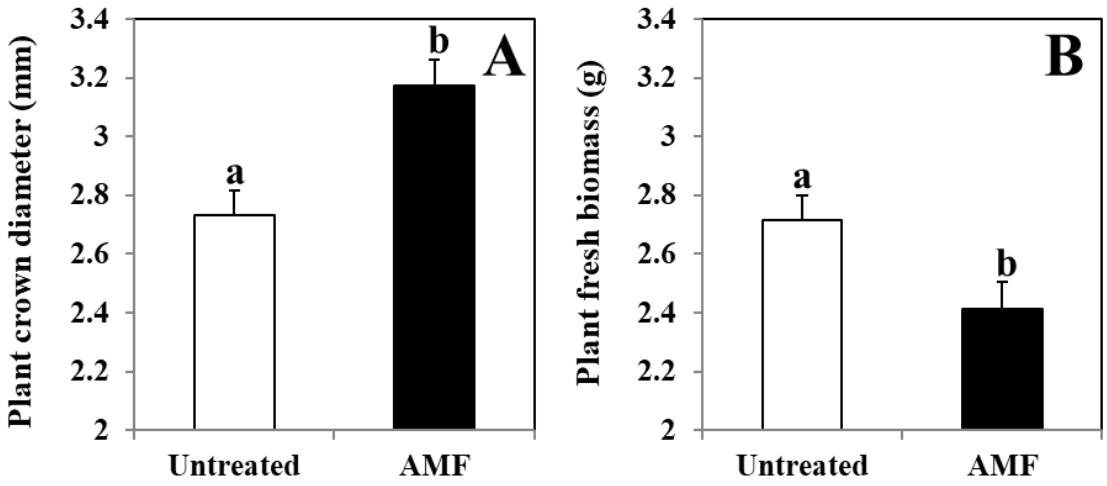

Figure 3.13: (A) Mean plant crown diameter (mm) and (B) mean fresh biomass (g) in experiment 1. Non-mycorrhizal (NM = Untreated) treatments are the white bars and the black bars represent the AMF inoculated treatments. Bars represent mean + 1 SE. For NM treatment n = 24 and for AMF inoculated treatment n = 36. Treatments that did not differ significantly share at least one common letter (Pairwise comparisons, $P \leq 0.05$). Note the scales do not start at 0.

In experiment 2, despite the infestation by spider mites, no plants died or showed visual differences in terms of growth. Plant crown diameter was not affected by AMF inoculation or by substrate. However, the interaction between the two factors was close to statistical significance ($F_{1,78}$ = 3.89; P = 0.052). Plant fresh biomass was not affected by AMF inoculation or by substrate and neither was the interaction term. Plant dry biomass did not vary with AMF inoculation and substrate. However, a significant disordinal interaction between AMF inoculation and substrate was detected for plant dry biomass ($F_{1,78}$ = 3.9; P = 0.033; Figure 3.14). Plants without AMF performed better in coir (i.e. higher dry biomass), whereas when growing in vermiculite plant with AMF did better (Figure 3.14). Finally, RWR was significantly affected by the substrate ($F_{1,78}$ = 18.3; P < 0.001). The root weight ratio (RWV) was smaller in vermiculite (mean 0.168 ± 0.004; n = 42) than in coir (mean 0.189 ± 0.003; n = 42).

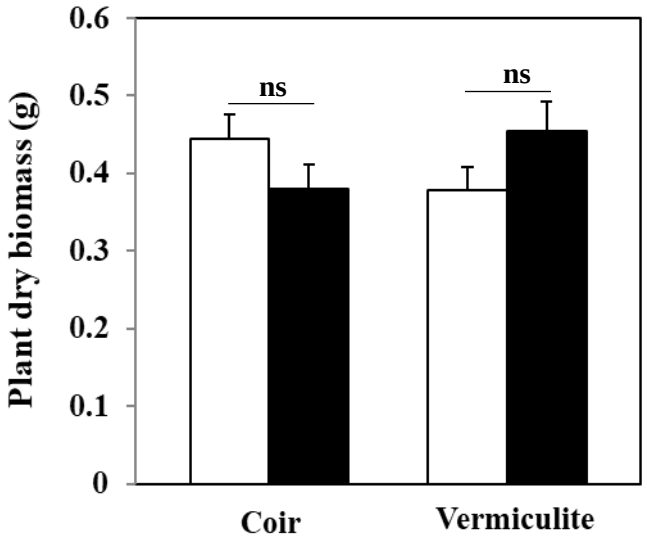

Figure 3.14: (A) Mean plant dry biomass (g) in experiment 2. Non-mycorrhizal (NM) treatments are the white bars and the black bars represent the AMF inoculated treatments. Bars represent mean + 1 SE. For NM treatment and AMF inoculated treatment n = 21. A significant disordinal interaction between AMF inoculation and substrate was detected ($F_{1,78}$ = 3.9; P = 0.033). ns indicates the absence of statistical significance between NM treatment and AMF inoculated treatment for each substrate tested (Pairwise comparisons, $P \leq 0.05$).

In experiment 3, all plants appeared to be healthy. Plant crown diameter was not affected by AMF inoculation but by cultivar (Figure 3.15A; $F_{1,89}$ = 9.5; P = 0.003). Average crown size of 'Vibrant' (mean 6.8 ± 0.1 mm; n = 47) was bigger than 'Red Glory' (mean 6.4 ± 0.1 mm; n = 50). The interaction between AMF treatment and cultivar was not significant. Similarly, plant height was not affected by AMF inoculation but by plant cultivar (Figure 3.15B; $F_{1,89}$ = 8.5; P = 0.004) with 'Vibrant' higher (mean 89.2 ± 2.2 mm; n = 47) than 'Red Glory' (mean 81.1 ± 1.8 mm; n = 50). There was no interaction between AMF and cultivar.

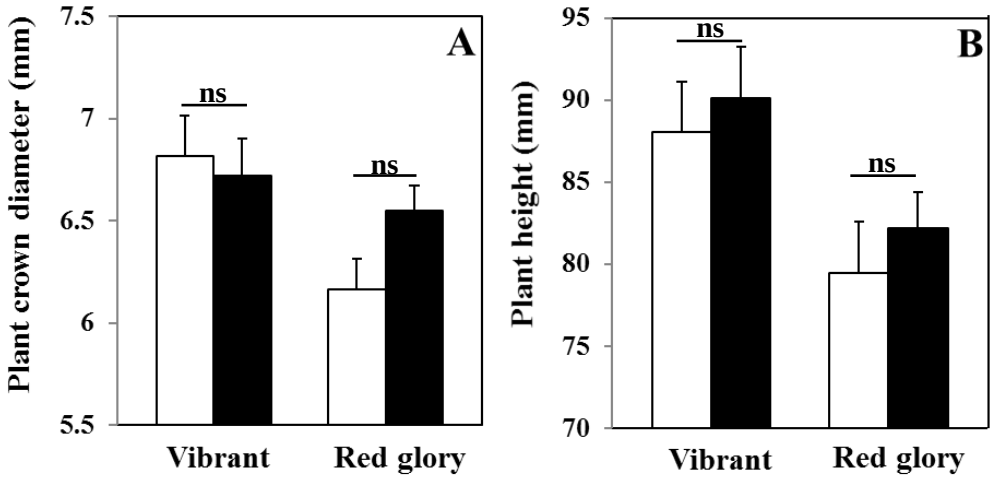

Figure 3.15: (A) Mean plant crown diameter (mm) and (B) mean plant height (mm) in experiment 3. Non-mycorrhizal (NM) treatments are the white bars and the black bars represent the AMF inoculated treatments. Bars represent mean + 1 SE. For NM treatment n = 20 and for AMF inoculated treatment n = 27-30. Cultivar had significant effects on (A) crown diameter ($F_{1,89}$ = 9.5; P = 0.003) and (B) height ($F_{1,89}$ = 8.5; P = 0.004). ns indicates the absence of statistical significance between NM treatment and AMF inoculated treatment for each strawberry cultivar tested (Pairwise comparisons, $P \leq 0.05$).

In experiment 4, both plant cultivars (P = 0.023) and plant runner-tip size (P < 0.001) significantly affected plant survival (Table 3.7). The dead plants came mostly from the plant size 'Small' and cultivar 'Malling Centenary'. Mycorrhiza inoculation did not affect plant crown size or plant survival (Table 3.7). In addition, there were no interactions between these three factors (Table 3.7).

Table 3.7: Results of three-way ANOVA of plant crown diameter (mm) and generalised linear model (GLM) of survival (%) of plants pre-inoculated with arbuscular mycorrhiza fungi (AMF) and grown for 7 weeks in the Irish dark peat/ perlite mix (7:3, v/v) under glasshouse conditions (experiment 4). Significant differences are shown by bold font ($P \leq 0.05$).

Source of variation	Plant crown diameter (mm)				Plant survival (%)		
	df	***Mean square***	***F***	***P***	***df***	***deviance***	***P***
Cultivar	3	49.5	24.2	**< 0.001**	3	9.5	**0.023**
AMF	1	2.7	1.3	0.251	1	2.1	0.146
Plant (runner) size	1	215.4	105.4	**< 0.001**	1	26.2	**< 0.001**
Cultivar × AMF	3	1.9	0.9	0.427	3	6.3	0.097
Cultivar × Plant (runner) size	3	1.4	0.7	0.555	3	6.2	0.103
AMF × Plant (runner) size	1	0.6	0.3	0.578	1	9.7	0.458
Cultivar × AMF × Plant (runner) size	3	0.5	0.2	0.872	3	0.9	0.446
Residual	239	2.0			271	125.4	

df = degrees of freedom. Fixed effects include cultivar (refers to 'Elsanta', 'Malling Centenary', 'Red Glory' and 'Vibrant'), AMF (refers to plants inoculated with AMF) and runner-tips size ('Large' and 'Small').

3.3.3. Effect of cold storage on AMF and DSE survival

After a month of plant re-growth under growth room conditions, microscopic assessments of newly formed strawberry roots showed the presence of arbuscules and/or vesicles for both cultivars (‘Vibrant’ and ‘Red Glory’) irrespective of the length of cold storage (up to 5 months), confirming the presence of AMF. The presence of microsclerotium-like structures and septate hyphae in the root cortex indicated the presence of DSE (Figure 3.16A-F). In addition, smaller microsclerotia and moniliform group of fungal cells coexisting with dark septate hyphae were occasionally observed (Figure 3.16B, E, F), suggesting presence of different species or developmental stages of DSE. Overlapping of AMF and DSE structures was rarely observed.

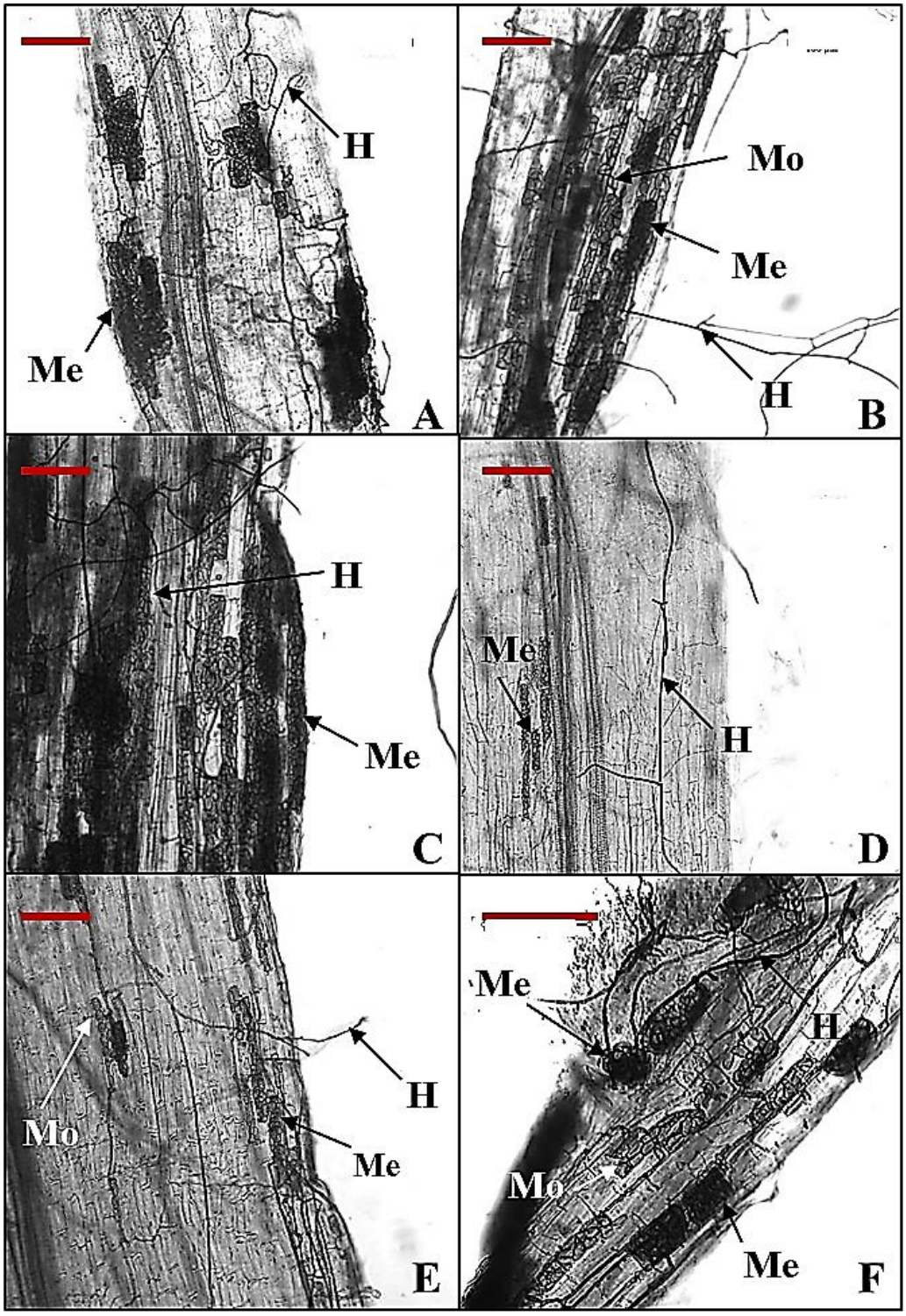

Figure 3.16: Root colonisation by dark septate endophytes (DSE) of *Fragaria* x *ananassa* plug cv. 'Vibrant' (A-C) and 'Red Glory' (D-F) cold stored at -2°C for 2 months, after 30 d of cultivation in autoclaved attapulgite clay in a growth cabinet at 22°C. Similar structures were observed in roots of plants cold stored at -2°C for 1, 3, 4 and 5 months (data not shown). Letters next to black arrows are H: hypha, Mo: moniliform cells and Me: microsclerotium (red scale bars represent 100 μm).

3.3.4. AMF root colonisation level in relation to DSE and cold storage

Strawberry plugs that were stored for five months at -2°C had the highest AMF colonisation level (mean 58 ± 4%, *n* = 18; Figure 3.17), whereas the plugs stored for one month had the lowest level of AMF colonisation (mean 24 ± 4%, *n* = 18; Figure 3.17). DSE colonisation was lowest in the strawberry plugs that were stored for five months at -2°C (mean 15 ± 3%, *n* = 18; Figure 3.17) and highest in plants stored for one month (mean 51 ± 4%, *n* = 18; Figure 3.17). When DSE was included as a covariate in the three-way ANOVA, there were significant effects of cold-storage duration ($F_{4,59}$ = 2.8, P = 0.032), AMF species ($F_{2,59}$ = 8.3, P < 0.001) and the three-way interaction between cold storage duration, cultivar and AMF ($F_{8,59}$ = 2.5, P = 0.023) on % RLC (Table 3.8). Overall, plants pre-inoculated with *C. claroideum* (mean 54 ± 3%, *n* = 30) had a significantly higher level of colonisation than the plants pre-inoculated with *F. mosseae* (mean 34 ± 4%, *n* = 30) or *R. irregularis* (mean 35 ± 3%, *n* = 30) regardless the cultivars and duration of cold storage.

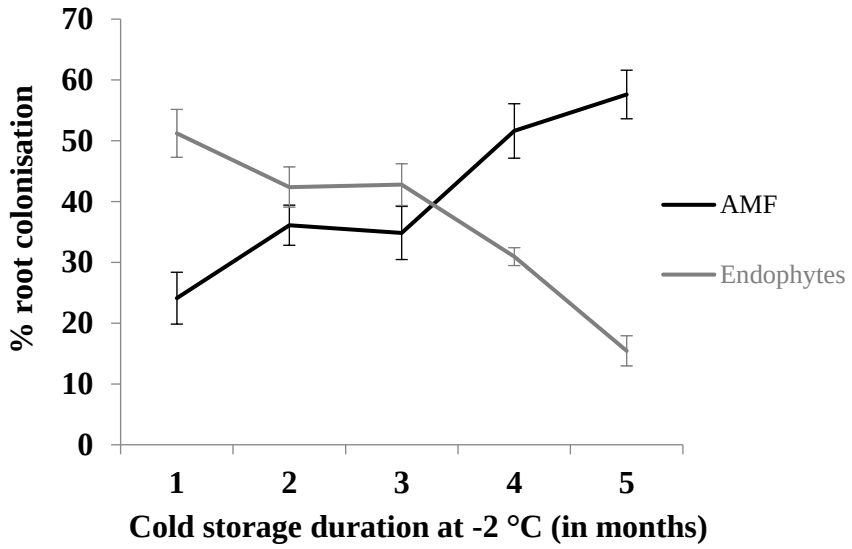

Figure 3.17: Percentage arbuscular mycorrhiza fungi (AMF, % RLC) and dark septate endophytes (DSE, % DSE) root length colonisation means (± SE, *n* = 18; each root sample was pooled from three individual plants) after different cold storage duration (one to five months) followed up by 30 d of re-growth in a growth room at 22°C.

Table 3.8: Results of the three-way ANOVA of percentage arbuscular mycorrhiza fungi (AMF, % RLC) colonisation levels in strawberry roots pre-inoculated with AMF, cold stored at -2°C for one to five months and re-grown for 30 d in a growth room at 22°C. Significant differences are shown by bold font ($P \leq 0.05$).

Source of variation	AMF colonisation (% RLC)			
	df	***Mean square***	***F***	***P***
Duration (of cold storage)	4	0.051	2.8	**0.032**
Cultivar	1	0.003	0.2	0.699
AMF	2	0.149	8.4	**<0.001**
Duration × Cultivar	4	0.023	1.3	0.293
Duration × AMF	8	0.034	1.9	0.079
Cultivar × AMF	2	0.019	1.1	0.356
Duration × Cultivar × AMF	8	0.043	2.5	**0.023**
Covariate (DSE colonisation)	1	0.695	39.2	**<0.001**
Residual	59	0.018		

df = degrees of freedom. Five cold-storage durations (1, 2, 3, 4 and 5 months) at -2°C, cultivar ('Vibrant' and 'Red Glory'), and AMF species inoculated (*Funneliformis mosseae* or *Rhizophagus irregularis* or *Claroideoglomus claroideum*); DSE colonisation percentage was included as a covariate.

3.4. Discussion

3.4.1. Influence of weaning conditions on AMF colonisation

The soil-less substrates used for weaning, propagation and/or cultivation of horticultural crops usually lack AMF (Azcón-Aguilar & Barea, 1997; Vestberg *et al.*, 2004). By introducing AMF at a very early stage of the strawberry propagation process, it might be possible to decrease fertiliser and pesticide application rates without adverse effects on plant growth and health. However, the effect of the misting conditions and/or soil-less substrates necessary for weaning strawberry plantlets may negatively impact on the establishment of AMF symbiosis. This study represents the first attempt to examine the combined effects of high moisture conditions and different soil-less substrates on strawberry root colonisation by AMF during the weaning phase. The results indicated that

AMF can colonise roots of strawberry plugs under damp conditions in different soil-less substrates.

Plant propagators or misting systems are required to maintain damp conditions for at least two weeks to ensure strawberry plant acclimatisation and rooting (Durner *et al.*, 2002; Treder *et al.*, 2015). Previous evidence suggested that AMF root colonisation is limited under damp conditions and declines with increasing amount of water in the substrate due to lower oxygen availability (Khan & Belik, 1995; Muthukumar *et al.*, 1997; Thormann *et al.*, 1999; Miller, 2000). Even though the soil-substrates tested (i.e. coir, peat/perlite mix and vermiculite) were rather wet during the weaning period, AMF could successfully colonise the strawberry plug roots in this study. These results agreed with the literature reporting AMF presence in wet land habitats (Søndergaard & Laegaard, 1977; Miller, 2000) or successful root colonisation of crops under irrigation (Baslam *et al.*, 2011). Therefore, AMF establishment is possible during the weaning stage of microplants and runner derived strawberry plants.

This study highlighted the capacity of several AMF species to colonise strawberry root when cultivated in different types of soil-less substrates. Microplants inoculated with AMF and grown in vermiculite (experiment 1 and 2) showed the highest level of AMF colonisation (80-90%). Thus, use of vermiculite as a soil-less substrate is recommended for establishment of the AMF symbiosis in the roots of strawberry microplants during their weaning stage. This agrees with previous reports describing vermiculite as a suitable substrate for commercial AMF inocula production (de Santana *et al.*, 2014; Rouphael *et al.*, 2015). Several other studies have successfully colonised strawberry plants with AMF using vermiculite as amendment in their potting mixes (Mark & Cassells, 1996; Murphy *et al.*, 2000; Sinclair *et al.*, 2013), but this study is the first to report strawberry root

colonisation by AMF in vermiculite only. In experiment 2, strawberry plantlets were also successfully colonised by AMF in coir, which agreed with the findings of other studies (Linderman & Davis, 2003a, Boyer *et al*. 2016). Nevertheless, the level of colonisation obtained in vermiculite was about twofold higher (80%) than in coir (40%). Therefore, lower levels of AMF colonisation in coir compared to vermiculite may indicate that (1) coir is a less suitable environment for AMF colonisation, (2) coir inhibits the movement or production of extraradical hyphae or other propagules, or that (3) plant root physiology may have been modified in coir. Boyer *et al.* (2016) also reported a lower level of AMF colonisation of maize and strawberry grown in coir (overall 13%) compared to attapulgite clay (overall 29%). The reasons for the negative effect of coir on AMF colonisation remain unclear, but the biological, physical and chemical properties of the substrate have been suggested as possible reasons for the phenomenon (Boyer *et al*. 2016).

Peat based mixes are commonly used by nurseries as a substrate to propagate strawberry plants (Vestberg *et al.*, 2000; D'Anna *et al.*, 2002; Treder *et al.*, 2015). The results of this study demonstrated that different AMF species could colonise strawberry roots in an Irish peat/perlite mix (7:3 v/v). The results are in contrast with many studies reporting the negative effects of peat-based substrates on AMF root colonisation in strawberry and other crops (Vestberg *et al.*, 2000; Linderman & Davis, 2003a; Linderman & Davis, 2003b; Vestberg *et al.*, 2004; Vestberg & Kukkonen, 2007). The successful AMF colonisation observed in the current study may be the result of the dilution of the peat with perlite. The commonly observed negative effect of peat substrates on AMF root colonisation was shown to be partially alleviated by mixing peat substrates with mineral components such as sand or clay (Vestberg & Kukkonen, 2008).

In experiments 3, 4 and 5 strawberry plugs were cultivated with the same limed peat/perlite mix (7:3 v/v) and weaned under misting conditions (Table 3.1). However, the % RLC in experiment 5 was lower (1-16%) compared to experiment 3 and 4 (40-81%). Several factors may explain this difference in AMF root colonisation levels among those experiments. Firstly, the AMF inoculants used had a much lower number of infective propagules in experiment 5 (23-170 propagule mL^{-1}) than in experiments 3 and 4 (1,600 propagule mL^{-1}). By increasing the number of infective propagules added to the strawberry plugs, similar colonisation percentage might have been obtained, assuming that the 'colonisation capacity' (Tommerup, 1992) is similar between *F. mosseae*, *R. irregularis* and *C. claroideum*. Secondly, the lower AMF colonisation level may be related to the growing season. In experiment 5, the runner-tips were inoculated with AMF at the end of September, while in the other trials the plants were inoculated during a period from March to June. The growing conditions of the plugs were also relatively different in experiment 5. The plugs were grown under glasshouse conditions and then under poly-tunnel conditions (with natural light and cooler temperatures). Further, plant roots were sampled in January (during vegetative dormancy), while plant roots were sampled between April-July (months of maximum vegetative growth) in the other experiments. In addition, the plants had an extended growing period in experiment 5 (18 weeks cf. 6-8 weeks in experiment 3 and 4). Seasonal variations of AMF colonisation were previously documented under glasshouse (Niemi & Vestberg, 1992) and field conditions (Branzanti *et al.*, 2002). Nevertheless, additional studies are needed to characterise the effects of the physical, chemical and biological properties of peat-based substrates and investigate how these could affect AMF colonisation.

Microscopic assessments suggested that strawberry plug roots were sometimes colonised by DSE (experiment 4 and 5). The DSE inocula seemed to originate from the Irish dark

peat used during plant weaning stage. This is supported by the fact that DSE were not observed in the other experiments with other soil-less substrates. Occurrence of DSE has mostly been described from soil systems (Wagg *et al.,* 2008; Lizarraga *et al.,* 2015; Vandegrift *et al.,* 2015) but has also been reported from peat (Fuchs & Haselwandter, 2004; Thormann, 2006; Weishampel & Bedford, 2006). The interest in DSE has recently increased but thus far only 30 DSE species have been identified and their phylogenetic identity or functional roles are uncertain (Andrade-Linares & Franken, 2013; Knapp *et al.,* 2015). In the present investigation, the DSE observed in the strawberry roots were unidentified. Future phylogenetic analysis of DSE in strawberry root and studies on their potential effect on plant growth may provide insight into their identity and functions.

3.4.2. Effect of strawberry cultivar and plant size on root colonisation level

Different strawberry cultivars were tested in combination with several AMF species (strains) to study the potential presence of specific interactions between the hosts and the fungal symbionts (experiment 3 and 4). Results showed that AMF species generally did not differ in their ability to colonise strawberry cultivars. In experiment 3, there were no cultivar effects on AMF colonisation. This contrasted with the results from experiment 4 where cultivar 'Malling Centenary' had a lower level of colonisation, while 'Elsanta', 'Red Glory' and 'Vibrant' had similar levels of AMF colonisation. Variability of AMF colonisation among strawberry cultivars grown under field conditions has previously been reported (Robertson *et al.*, 1988). Moreover, studies conducted under glasshouse conditions showed that strawberry cultivars were either colonised equally (Robertson *et al.,* 1988) or differentially (Chávez & Ferrera-Cerrato, 1990; Vestberg, 1992b) by AMF. Therefore, further work is needed to characterise the origin of the lower root colonisation of 'Malling Centenary' growing in the peat-based substrate and to confirm the low AMF

colonisation level relative to other cultivars (e.g. ‘Elsanta’, ‘Red Glory’ and ‘Vibrant’). The next step is to assess root growth, morphology, exudation, and the rhizosphere microbial community to determine whether these traits are responsible for low AMF colonisation in strawberry roots. Previous reports have highlighted the presence of a relationship between root morphology and mycotrophy with AMF (Tawaraya, 2003). The root architecture of different strawberry cultivars and their root exudate composition have been reported to vary greatly (Vestberg, 1992b). The lack of AMF colonisation of some wheat varieties has previously been linked to the absence of root sugar exudates (Azcon & Ocampo, 1981). Therefore, a potential difference in root morphology and/or qualitative/quantitative differences in root exudates of ‘Malling Centenary’ may have influenced rhizosphere microbial communities or signalling pathways affecting AMF colonisation. The tripartite interaction between host plant, AMF and other microbes have been reported to influence positively (Rouphael *et al.*, 2015) or negatively AMF root colonisation (Germida & Walley, 1996). Characterisation of soil microbial community function remains at present limited, but the enhanced application of metagenomic tools will allow us to access such information in the near future.

The results from experiment 4 suggested that AMF species do not differ in their capacity to colonise roots of different sized plants; both plant size groups were colonised equally by AMF. This finding agrees with a previous report showing that total root colonisation by AMF and DSE were not affected by the difference in size of *Gentianella campestris* (L.) Börner plants (Piippo *et al.*, 2011).

3.4.3. Effect of AMF pre-inoculation on strawberry plug growth

This study showed that strawberry microplants and runner-tip derived plants could be AMF colonised during the weaning stage, however, the results indicated that early AMF

inoculation does not necessarily increase plug growth during propagation and indeed could even lead to growth reductions. AMF colonisation reduced the total fresh biomass of microplants (experiment 1), perhaps due to non-optimal conditions for photosynthesis resulting in a carbon drain on the host plant (Bethlenfalvay *et al.*, 1982). Despite the reduction of fresh weight, plants crown size was significantly increased by AMF inoculation although the reasons for this remained unclear. AMF colonisation may have resulted in changes in hormonal and/or nutritional conditions but further investigation would be required to characterise this. In a similar experiment (experiment 2), AMF colonisation did not affect plant crown size or total fresh biomass, but a significant disordinal interaction between AMF and substrates on the total plant dry biomass was observed. In coir, AMF root colonisation with *R. irregularis* reduced total plant biomass, whereas in vermiculite the AMF increased the total plant biomass. This result agreed with previous studies showing that the response of strawberry plants to AMF colonisation depends on specific host-AMF combinations (Chávez & Ferrera-Cerrato, 1990; Vestberg, 1992b), which may be linked to host preference and soil adaptation (Hayman, 1982).

In peat-based substrate AMF had no effect on development of runner-derived plants. In contrast, another study reported that certain combinations of strawberry cultivars and AMF species resulted in the increase of plant biomass in peat based substrate (Vestberg, 1992b). Perhaps, the neutral effect of AMF inoculation observed in the current experiment could be explained by the fact that nutrients were not limiting in the Irish dark peat/perlite mix used and/or the plant growth window may have been too short to detect an effect.

3.4.4. Effect of cold storage on the presence of AMF after plug re-growth

Experiment 5 was the first study to explore the effect of cold storage on the survival of AMF in pre-inoculated strawberry plug roots. The results indicated that AMF propagules

in pre-inoculated strawberry roots did not suffer from several months of storage at -2°C. AMF structures were observed in newly formed roots of both cv. 'Red Glory' and 'Vibrant' for all three AMF species screened (*F. mosseae, R. irregularis, C. claroideum*), irrespective of the duration of cold storage. Thus, propagules of the three AMF species were able to survive and to retain their infectivity after several months of cold storage. This result is in agreement with previous studies in which *Glomus* species propagules remained infective in frozen soil over winter (Addy *et al*., 1994; Addy *et al*., 1997) or after artificial freezing treatment at -12°C for 7 d in soil (Addy *et al*., 1998) and at -5°C for 4 weeks in silica sand (Klironomos *et al*., 2001). Addy *et al*. (1997) reported that extraradical mycelium of *R. irregularis* and *G. fasciculatum* survived freezing treatment and mycelia were much effective as inoculum compared to the spores of the same fungi. Therefore, AMF propagules can survive freezing temperature for several months given the environment in which they inhabit.

3.4.5. Effect of DSE and cold storage on AMF root colonisation level

Microscopic assessments showed that strawberry plug roots grown in peat were regularly associated with DSE. These DSE fungi have been described as 'miscellaneous fungi' that colonise the root tissue of a large array of plant species (Jumpponen & Trappe, 1998) without causing any noticeable damage to their host (Jumpponen, 2001) and have been frequently reported to co-exist with AMF (Urcelay, 2002). Lizarraga *et al.* (2015) reported the co-existence of DSE and AMF in *F.* x *ananassa* grown under field conditions. Hence, it is reasonable to expect that AMF and DSE share the same spatial niche and interact with each other. Microscopic analysis revealed that strawberry roots heavily colonised by DSE rarely contained mycorrhizal structures, suggesting niche competition for space. This observation agrees with previous reports showing that plants with the highest AMF colonisation generally showed the lowest DSE colonisation and *vice versa* (Kandalepas *et*

al., 2010; Urcelay *et al.*, 2011). Additional studies are needed to characterise the nature of AMF and DSE interaction in strawberry roots (e.g. competition for spaces, for carbon and/or mineral nutrients, and effect of DSE exudates) as well as their effects on the host fitness.

It was suspected that plant roots with high DSE colonisation were more likely to have lower AMF root colonisation due to competition for root space. Therefore, DSE root colonisation level was included as a covariate in ANOVA to remove its influence on AMF root colonisation. Against all expectations the colonisation by AMF remained different between plants cold stored for 1 and 4 months. Hence, the decline of DSE in the roots was not the only factor responsible for the increase of AMF root colonisation over time. Perhaps, prolonged cold storage resulted in the breakage of spore dormancy that might have resulted in additional spore germination and so a higher root colonisation. To study the effect of various cold storage periods on spore dormancy, future experiments need to occur in an environment that does not suppress or interfere with the strawberry–AMF interaction. Therefore, tests should be conducted in the absence of DSE (e.g. by using autoclaved peat substrates during the plug propagation). In addition, to verify if there is a correlation between better spore dormancy breakage and higher RLC, assessment of spore viability and a spore germination bioassay on minimal (M) medium should be run in parallel (Juge *et al.*, 2002).

Additionally, AMF species used in experiment 5 resulted in a different level of strawberry root colonisation. The plants pre-inoculated with *C. claroideum* presented a significantly higher level of root colonisation although the MPN test revealed the lowest number of viable propagules for this AMF species compared to *R. irregularis* and *F. mosseae.* Therefore, assuming that the three AMF species tested had similar colonisation capacities,

it was hypothesised that the cold storage treatment resulted in a higher breakage of spore dormancy for *C. claroideum* and/or *C. claroideum* spores presented a better tolerance to freezing injures. Previous studies have highlighted that the requirement to break spore dormancy varied greatly among AMF species (Douds & Schenk, 1991; Juge *et al.*, 2002) as well as their tolerance to freezing temperatures (Klironomos *et al*., 2001).

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3.5. Conclusion and prospects

The results presented in this chapter have confirmed that AMF propagules can colonise strawberry roots of several commercial cultivars when incorporated as a powder layer in an Irish peat/base mix, vermiculite and coir under misting conditions or plant propagators. Therefore, AMF inoculated during the weaning stage of microplants or runner tips can result in a high level of mycorrhizal colonisation, independently of plant material size and/or the strawberry cultivar inoculated (apart from 'Malling Centenary'). However, pre-inoculation of strawberry transplants with AMF does not necessarily translate to improved plant growth during weaning and propagation. In addition, AMF propagules can tolerate the formation of ice in the root ball induced by the cold storage at -2°C for several months. Therefore, commercial AMF inocula may be applied during strawberry tipping without reducing the mycorrhiza viability during subsequent cold storage of pre-colonised plants. Interestingly, this study has also highlighted the presence of DSE in Irish dark peat that may have competed with AMF root colonisation, but this requires further investigation. The main results of the five experiments were summarised in Table 3.9 (note the interaction terms were not presented for clarity).

Furthermore, with the respect to commercial introduction of AMF during weaning of strawberry transplants, the results of these five experiments are valuable for the strawberry nurseries that may consider introducing AMF via pre-inoculated strawberry plugs in their

fumigated field or soil-less substrate system, both systems often lacking beneficial microbes. Future research efforts should focus upon understanding the outcomes of the interaction among strawberry cultivars, AMF species (or strains), other root endophytes (e.g. DSE) and substrate (e.g. coir- and peat-based substrates). This will help the growers to select the best AMF-strawberry-substrate combinations to maximise AMF beneficial effects during propagation and after transplantation. Further research should also focus on testing lower amounts of AMF inoculum to reduce the costs for growers, improving the inoculation method to make the technology cheaper and less labour intense.

Table 3.9: Results summary of the five experiments presented in Chapter 3. Only the main factors are hereafter presented and interactions between factors are omitted for clarity.

	Experiment				
	1	**2**	**3**	**4**	**5**
Total no. treatments	5	4	10	48	30
AMF species and controls[a]	F.m, R.i, C.c, Cb^-, Cb^+	R.i, Cb^+	F.m, R.i, C.c, Cb^-, Cb^+	F.m, R.i, C.c, G.m, F.g, Cb^+	F.m, R.i, C.c
Plant cultivars[b]	EM-1996	V	RG, V	E, MC, RG, V	RG, V
Plant (runner) size categories	-	-	-	Small/Large	-
Weaning substrate	Vermiculite	Vermiculite or Coir	Peat/perlite mix	Peat/perlite mix	Peat/perlite mix
Re-growth substrate	-	-	-	-	Attapulgite clay
Cold storage duration at -2°C (months)	-	-	-	-	1, 2, 3, 4, 5
% RLC (range)	94%	40-76%	67-81%	12-64%	34-54%
Effect of AMF species on % RLC	ns	-	ns	***	***
Effect of plant cultivar on % RLC	-	-	*	*	ns
Effect of plant size on % RLC	-	-	-	ns	-
Effect of cold storage duration on % RLC	-	-	-	-	*
Effect of substrate on % RLC	-	*	-	-	-
Effect of AMF inoculation on plant survival	-	-	-	ns	-
Effect of AMF inoculation on plant crown size	***	ns	ns	ns	-
Effect of AMF inoculation on plant height	ns	-	ns	-	-
Effect of AMF inoculation on fresh biomass	*	ns	-	-	-

[a] Abbreviations F.m, R.i, C.c, G.m and F.g respectively stand for the single AMF species inoculated: *Funneliformis mosseae*, *Rhizophagus irregularis*, *Claroideoglomus claroideum*, *Glomus microagregatum*, *Funneliformis geosporum*. Cb^-: a control inoculated with autoclaved attapulgite clay; Cb^+: a control inoculated with an autoclaved equal mix of AMF species used and inoculated with bacterial washing.

[b] Abbreviations E, MC, RG, and V respectively stand for the strawberry cultivars: 'Elsanta', 'Malling Centenary', 'Red Glory' and 'Vibrant'.

'ns' stands for 'not significant', $*P \leq 0.05$, $**P \leq 0.01$, $*** P \leq 0.001$.

Chapter 4. Evaluation of the potential of pre-colonised strawberry plugs with mycorrhizal inoculants to increase tolerance to *Verticillium* wilt

4.1. Introduction

The soil-borne pathogen *Verticillium dahliae* (Kleb.) is a serious threat to strawberry production in soil (Pegg & Brady, 2002). This pathogen invades the vascular tissues of the roots and crown depriving the leaves and stems of water (Bhat & Subbarao, 1999; Lovelidge, 2004). The soil fumigant methyl bromide has been routinely applied for the past 40 years to control strawberry wilt; however, it was banned in Europe due to its high ozone-depleting potential and its chemical alternatives, chloropicrin, faces an uncertain future due to possible legislation (Ristaino & Thomas, 1997; Martin, 2003). Alternative measures, such as biofumigation, solarisation and crop rotation, contribute to disease control, but they are usually not as effective as commercial chemical fumigants (Tahmatsidou *et al.*, 2006; Korthals *et al.*, 2014). Consequently, extensive effort has gone into finding other economically effective alternatives to mitigate the threat of strawberry wilt (Martin, 2003; Goicoechea *et al.*, 2010). One approach is to exploit AMF as a bio-protectant against strawberry wilt. Inoculation of AMF at planting could increase strawberry plant tolerance to *V. dahliae* (Ma *et al.*, 2004; Tahmatsidou *et al.*, 2006; Sowik *et al.*, 2016). Pre-colonisation of horticultural plants with AMF before transplanting has been proposed as a method protecting plants against soil-borne pathogens (Azcón-Aguilar & Barea, 1997; Vestberg *et al.*, 2004). Although strawberry is an ideal production system to study such a control method, thus far there has not been any published information regarding the use of AMF pre-inoculated strawberry plug transplants to control *V. dahliae*.

The aim of this study was to investigate whether strawberry plugs pre-inoculated with commercially available AMF could enhance plant growth and reduce strawberry wilt incidence in contaminated soils. Several strawberry cultivars susceptible to wilt were screened to test the hypothesis that pre-inoculation with AMF can increase tolerance to wilt after transplantation under glasshouse or open field conditions.

4.2. Materials and methods

A total of seven experiments were conducted in 2014-2016. Three experiments were carried out with wilt conidial suspensions and one experiment with mycelia under controlled conditions. However, none of these inoculations resulted in wilt development; hence are not discussed further. The other three experiments were carried out with soils [naturally contaminated with *V. dahliae* inoculum] under glasshouse (Figure 4.1A) or open field (Figure 4.1B) conditions as summarised in Table 4.1.

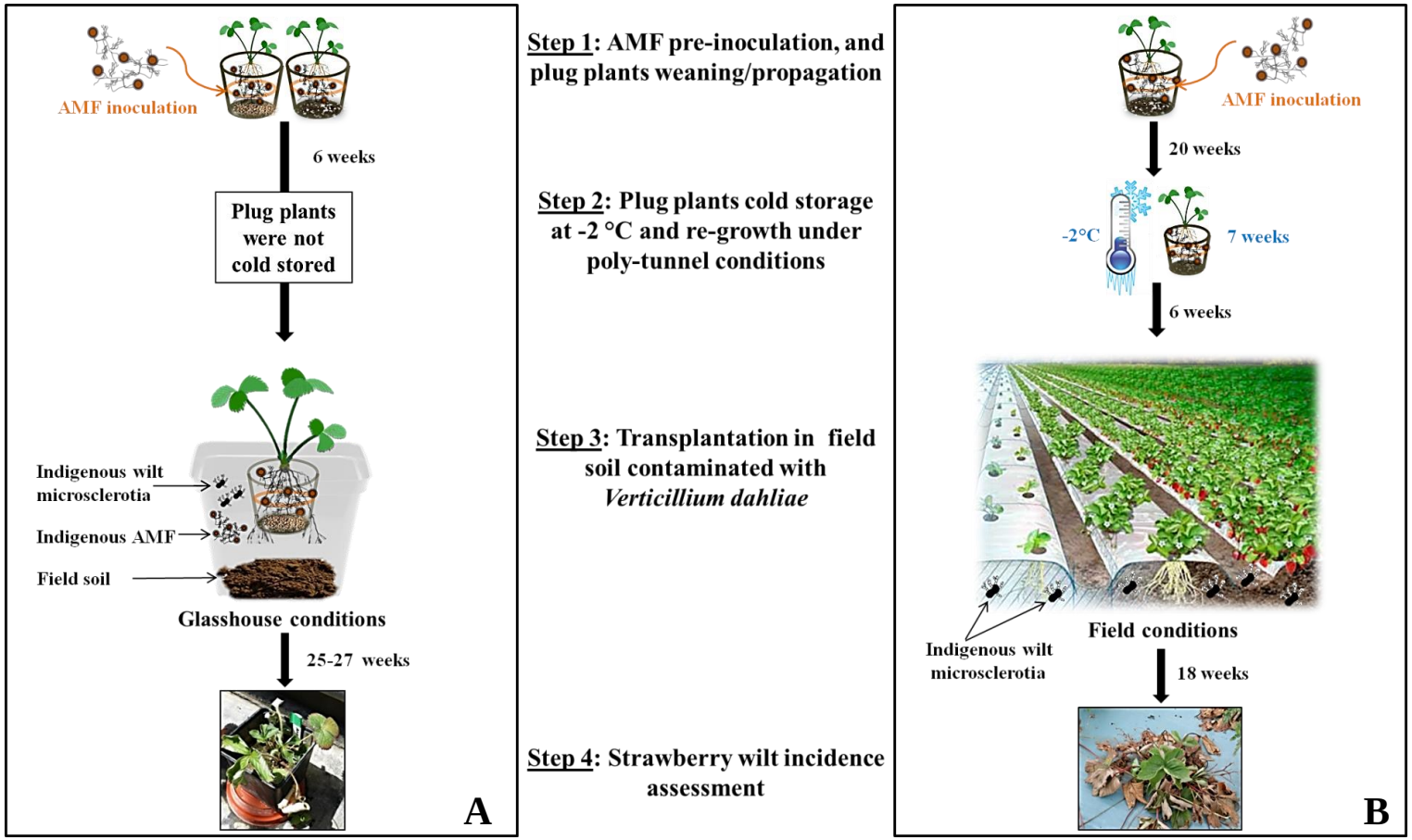

Figure 4.1: Schematic representation of the experimental setup to study the effect of arbuscular mycorrhiza fungi (AMF) pre-inoculation against *Verticillium dahliae*: (A) glasshouse experiments (experiment 1 and 2) and (B) field experiment (experiment 3). Microplants or runner-tips were AMF inoculated and propagated in soil-less substrates (Step 1). Plugs were cold stored for seven weeks at -2°C only for experiment 3, while plugs were kept under glasshouse conditions for experiment 1 and 2 (Step 2). Plugs were transplanted in field soil contaminated with *V. dahliae* microsclerotia (Step 3) and disease development assessed (Step 4).

Table 4.1: Summary of the three experiments to study the effect of early arbuscular mycorrhizal fungi (AMF) colonisation of strawberry plug transplants on the development of wilt caused by *Verticillium dahliae*.

AMF treatment	**Experiment number**		
	1	**2**	**3**
Total no. treatments	5	10	12
AMF species and controls[a]	F.m, R.i, C.c, Cb^-, Cb^+	F.m, R.i, C.c, Cb^-, Cb^+	F.m, R.i, C.c, Cb^+
Plant cultivars[b]	EM-1996	RG, V	MC, RG, V
Total no. of replicates	12	10	96
No. blocks	3	5	6
Type of plant material	Plug plant	Plug plant	Cold stored plug plant
Location	Glasshouse	Glasshouse	Field
Pathogens propagule density[c]	22.2 CFU g^{-1} of soil	22.2 CFU g^{-1} of soil	1.9 CFU g^{-1} of soil
Start date (i.e. transplantation)	19/03/14	16/04/14	22/05/15
Experimental duration	27 weeks	25 weeks	18 weeks

[a] Abbreviations F.m, R.i, C.c, G.m and F.g respectively stand for the single AMF species inoculated: *Funneliformis mosseae*, *Rhizophagus irregularis*, *Claroideoglomus claroideum*, *Glomus microagregatum*, *Funneliformis geosporum*. Cb^-: non-AMF control inoculated with autoclaved attapulgite clay; Cb^+: non-AMF control inoculated with an autoclaved equal mix of AMF species used and inoculated with bacterial washing.

[b] Abbreviations E, MC, RG, and V respectively stand for strawberry cultivars: 'Elsanta', 'Malling Centenary', 'Red Glory' and 'Vibrant'.

[c] The pathogen propagules were microsclerotia naturally present in the field soil. CFU stands for colony-forming unit.

4.2.1. Plant materials

All strawberry cultivars used are known to be susceptible to *V. dahliae*. The AMF pre-colonised strawberry plug transplants used in experiment 1 and 2 were derived from plugs produced in Chapter 3 (see Section 3.2.2 experiment 1 and 3). For experiment 3, AMF pre-colonised strawberry plugs were produced as described in Section 3.2.2 with the following modifications: (1) after cultivation for 82 d under glasshouse conditions, plants were transferred to a poly-tunnel with natural shorter days and cooler temperatures for 76 d to induce dormancy, (2) plants were acclimated to lower temperatures for 8 d in the dark at 2°C and (3) strawberry plugs were cold stored at -2°C for 7 weeks. At the end of the cold storage period, plants were transferred to a poly-tunnel for six weeks (spring time) to induce plant growth before field transplantation.

4.2.2. Determination of wilt inoculum density in field soils

Estimation of *V. dahliae* microsclerotia concentration in the soils was carried out using the Harris method (Harris *et al.*, 1993). In experiment 3, soil samples deriving from each of the 72 plots of the experiment were pooled together for this analysis. Microsclerotia concentrations are expressed in colony-forming unit (CFU) per gram of soil.

4.2.3. Plug transplantation in wilt contaminated soils

A randomised block design was used in all three experiments (Table 4.1). In experiment 1 and 2, plugs were re-potted into 1 L plastic pots (11 x 11 x 12 cm, Desch Plantpak Ltd, Essex, UK) filled up with soil collected from a non-fumigated commercial strawberry field at NIAB EMR, UK (N 51°17’20.93’’, E 00°27’11.52’’; soil: Barming series, loamy fine sand). The background nutrient status of the field soil was analysed before plant transplantation to identify potential elements that could negatively influence AMF

colonisation in this study (e.g. low pH and/or high P level; Table 4.2). The soil was sieved beforehand with a garden riddle (square hole wire mesh ca. 5 mm). Strawberry plugs were then kept under standard greenhouse conditions (22-23°C, ca. 40% RH, natural light:dark cycle, adequate pest control; Figure 4.2A, B) as outlined in Table 4.1. Plants from experiment 1 and 2 were watered daily with tap water and fertilised once a week with 50 mL of full-strength Rorison's nutrient solution but with phosphate omitted (Hewitt & Bureaux, 1966). To induce a moderate water stress to encourage wilt development for the last seven weeks of experiment 1 and 2, plants were watered only two or three times per week (one of those watering event included fertilisation).

Experiment 3 was carried out in raised double-row beds at NIAB EMR, UK (N 51°17'19.90'', E 00°27'13.38'' soil: Barming series, loamy fine sand) in open-field. The background nutrient status of the field soil was also analysed before transplantation in this experiment (Table 4.2). The soil was not fumigated before planting. Plastic drip irrigation was laid down in the middle of the bed, which was covered with blue plastic mulch (Figure 4.2C, D). A spacing of 30 cm between rows and between plants was used. There was a spacing of ca. 100 cm between neighbouring plots (each containing 16 plants) in the same bed (i.e. block). Irrigation, fertilisation and pest control followed standard commercial practices.

Figure 4.2: Strawberry plants were grown in pots filled up with field soil under glasshouse conditions in experiment (A) 1 and (B) 2. Strawberry plants in experiment 3 were grown in field plots at (C) one week and (D) 15 weeks post transplantation.

Table 4.2: Background nutrient status analysis[a] of the field soil (Barming series) used in the three experiments.

Experiment	pH	NO_3	NH_4	P	K	Mg
		ppm = mg kg^{-1}				
1, 2	5.9	8.1	0.9	31.1	198.6	69.6
3	7.9	2.4	2.0	30.4	188.5	52.1

[a] Available nutrient status of each growth medium was provided by NRM Laboratories (Berkshire, UK) in mg L^{-1} and it was converted to mg kg^{-1}. NO_3^- was determined by ion chromatography and NH_4^+ by colorimetric analysis. P, K, Mg and Ca were analysed by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy). Note that the nutrient status analysis of each growing medium could not be replicated due to high cost.

4.2.4. Root sample analysis and plant growth

Plant growth and percentage of AMF root colonisation were assessed at the end of experiment 1 and 2 and a subset of three samples (= three individual plants) was used for AMF colonisation assessments of each treatment. In experiment 3, percentage of AMF root colonisation was not assessed due to the presence of resident AMF confirmed in the soil samples used in experiment 1 and 2. Fresh roots were randomly sampled and stained

(Section 2.3.1) to assess AMF colonisation (Section 2.3.2). No attempt was made to distinguish between indigenous and pre-inoculated AMF.

The number of plants producing runners, crown size and plant dry weight were only assessed in experiment 1 and 2. To measure total dry weight, plants were harvested and washed with tap water to remove soil particles. The plants were then oven dried at 80°C for 3 d and then weighed. To calculate the root weight ratio (RWR: root dry weight as a fraction of the total plant dry weight), roots were weighed after being separated from the shoot. In experiment 1, nutrient content in strawberry shoots was analysed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) for P, K, Mg, Ca and micro-elements (Cu, Fe, Zn, B, Mn), while the total nitrogen and sulphur were measured with the Dumas method (AOAC, 1989). Each strawberry shoot sample analysed was pooled from four individual plants from the same block. The shoot nutrient analysis was conducted by NRM Laboratories, Berkshire, UK.

In experiment 1 and 2, fruit yield was not recorded because plants had not been subjected to adequate chilling and insect pollinators were not provided in the confined compartment (natural pollinators could not enter the compartment since the facility was completely sealed for the purpose of controlled isolation). In experiment 3, the fruit yield was also not assessed. Cultivars ‘Malling Centenary’ and ‘Vibrant’ showed an abnormal cropping behaviour (i.e. low flower production) for unknown reasons, while ‘Red Glory’ flowered normally but most of the fruits were eaten by birds before harvest.

4.2.5. Disease assessment

Wilt development was scored on the following scale: 0: no wilt symptoms and 1: presence of strawberry wilt symptoms such as wilted leaves, brown leaves, stunted or plant death.

4.2.6. Data analysis

All data were analysed using GenStat 13th edition (VSN International Ltd, Hemel Hempstead, UK). AMF root length colonisation (% RLC) and RWR data were analysed by ANOVA after arcsine square root transformation to satisfy normality. In experiment 1, there was only one treatment factor (AMF species). In experiment 2, there were two treatment factors (AMF treatment and strawberry cultivar); an unbalanced two-way ANOVA was used to analyse the data. Significant differences among individual treatments were determined by LSD post-hoc test if the overall treatment effect was significant ($P \leq 0.05$).

The number of plant producing runners (experiment 1) and disease incidence (experiment 3) were analysed using GLM with residual errors assumed to follow binomial distributions; the logit link function was used.

Only significant differences are reported in the text. For plant growth variables, i.e. crown diameter, RWR and plant dry weight, there were no significant differences among the AMF species tested, the data from different AMF species were hence pooled to form a single group (AMF inoculated). The non-mycorrhizal (NM) control (Cb^+ and Cb^-) data were treated similarly. Therefore, only the overall effect AMF c.f. NM for plant growth parameters was hereafter presented.

4.3. Results

4.3.1. Establishment of AMF inoculants after plug transplantation

After cultivation in non-sterilised field soil, microscopic assessment of strawberry roots from experiment 1 and 2 showed the presence of AMF structures in the roots of AMF pre-inoculated plants and NM controls (Figure 4.3). In experiment 1, at 27 weeks post transplantation all treatments were colonised by AMF to a similar extent (average % RLC across all treatments: ca. 93%; arbuscules and vesicles frequency ca. 32% and 46%, respectively).

In experiment 2, average % RLC at 25 weeks reached ca. 90% across all treatments including the NM controls. There was a significant difference in % RLC between strawberry cultivars 'Vibrant' and 'Red Glory' (Figure 4.4A; $F_{1,18} = 6.53$; $P = 0.020$); % RLC was 92% and 87% for 'Red Glory' and 'Vibrant', respectively. Arbuscule frequency differed with AMF treatment (Figure 4.4B; $F_{4,18} = 3.08$; $P = 0.043$) but not with strawberry cultivar. Plants pre-inoculated with *C. claroideum* had a higher frequency of arbuscules (14%) than the other AMF treatments (4%). However, AMF treatment and cultivar did not affect vesicle frequency (average ca. 46%). There were no significant interactions between AMF treatment and cultivar for any AMF parameters measured.

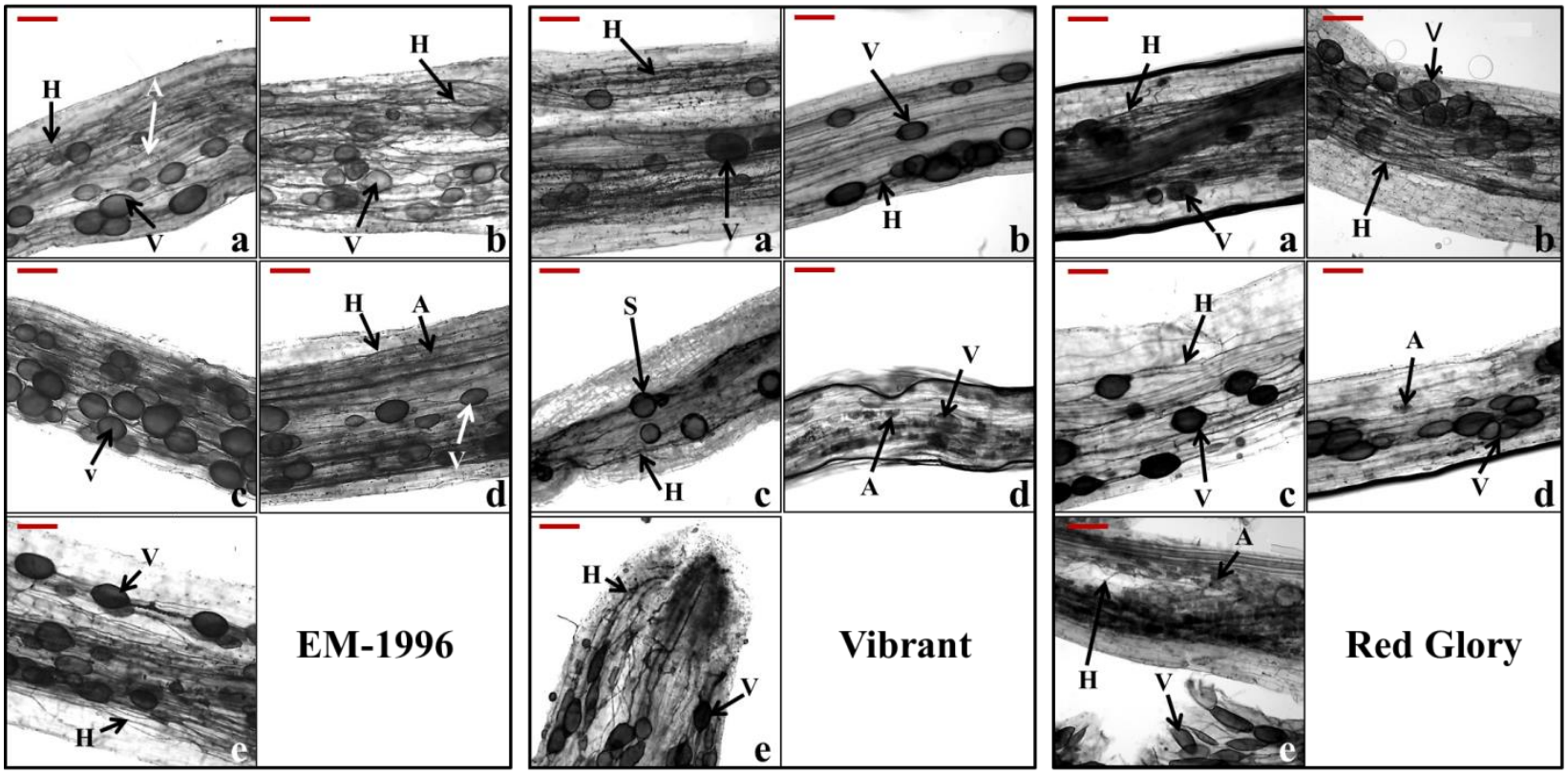

Figure 4.3: Root colonisation by arbuscular mycorrhiza fungi (AMF) of strawberry plants in experiment 1 (cv. 'EM-1966') and experiment 2 (cv. 'Vibrant' and 'Red Glory') respectively after 25 and 27 weeks of cultivation in pots filled up with field soils in a glasshouse compartment. Longitudinal squash of roots stained with trypan blue colonisation by AMF, in non-AMF plant controls (a) Cb^- and (b) Cb^+, as well as plants pre-inoculated with (c) *Funneliformis mosseae*, (d) *Rhizophagus irregularis* and (e) *Claroideoglomus claroideum*. Letters next to the arrows are A: arbuscule, H: hyphae, S: spore, V: vesicle (red scale bars represent 100 μm).

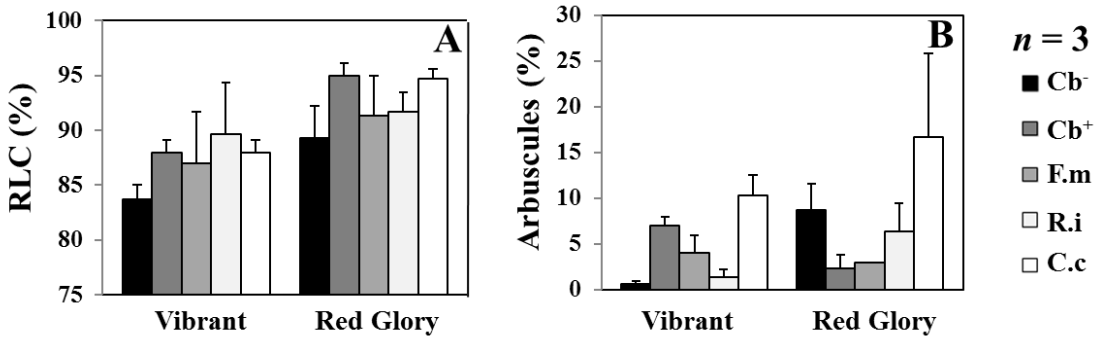

Figure 4.4: Percentage of strawberry root colonisation by (A) arbuscular mycorrhiza fungi (AMF, % RLC) and (B) arbuscules in experiment 2. The abbreviations Cb^-, Cb^+, F.m, R.i and C.c represent non-AMF control (Cb^-), non-AMF control with bacterial washing (Cb^+), *Funneliformis mosseae* (F.m), *Rhizophagus irregularis* (R.i) and *Claroideoglomus claroideum* (C.c). Bars are standard error (+ 1 SE), and *n* is the number of replicates per treatment. Strawberry cultivar had a significant effect on (A) % RLC ($F_{1,18}$ = 6.53; P = 0.020). AMF treatment had a significant effect on (B) % arbuscules ($F_{4,18}$ = 3.08; P = 0.043). Note that one of the scale does not start at 0

4.3.2. Effect of AMF pre-inoculation on plant growth

In experiment 1, all plants survived transplantation and grew normally. The AMF pre-inoculated plants appeared to have larger crown size (16.2 ± 0.4 mm; n = 36) than the NM control plants (14.8 ± 0.6 mm; n = 24); this difference was close to statistical significance ($F_{1,56}$ = 3.88; P = 0.054). Plant dry weight and RWR were not influenced by AMF pre-inoculation. The GLM analysis showed that the production of runners was affected by treatment (P = 0.032); the number of plants producing runners was less in the non-AMF control with bacterial washing (Cb^+) and *C. claroideum* treatments compared to the non-AMF control (Cb^-; Figure 4.5). Foliar concentrations of both macro-elements (N, S, P, K, Mg and Ca) and micro-elements (Cu, Fe, Zn, B and Mn) were not influenced by AMF pre-inoculation.

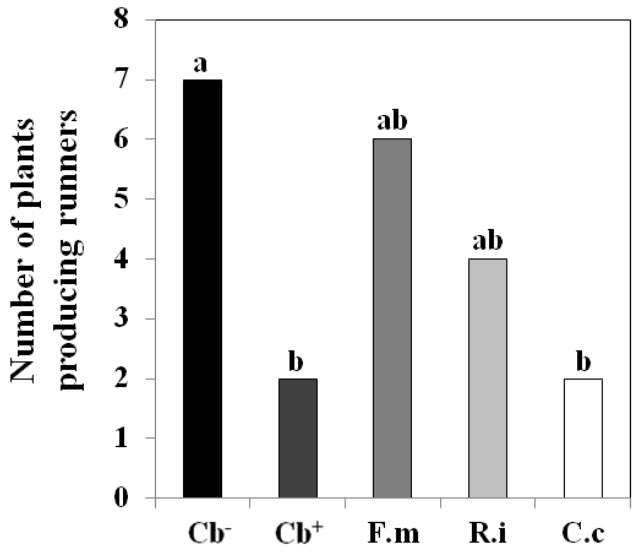

Figure 4.5: Results of generalised linear models fitting number of plants producing runners without pre-inoculated mycorrhiza (non-AMF control (Cb^-) and non-AMF control with bacterial washing (Cb^+)) or pre-inoculated with single AMF species: *Funneliformis mosseae* (F.m), *Rhizophagus irregularis* (R.i) and *Claroideoglomus claroideum* (C.c) in experiment 1. Data are number of plants producing runners (n = 12). The overall treatment effect was significant (GLM, P = 0.032). Treatments that did not differ significantly share at least one common letter (Pairwise comparisons, $P \leq 0.05$).

In experiment 2, crown diameter was not affected by AMF pre-inoculation but it was by cultivar ($F_{1,89}$ = 23.1; P < 0.001). Average crown size of 'Vibrant' (15.6 ± 0.3 mm; n = 47) was bigger than 'Red Glory' (13.8 ± 0.3 mm; n = 50). RWR was not affected by AMF pre-inoculation but by cultivar ($F_{1,89}$ = 6.6; P = 0.010). Average RWR value of 'Vibrant' (0.42 ± 0.01; n = 47) was higher than 'Red Glory' (0.38 ± 0.01; n = 50). Plant dry weight did not vary with AMF treatment, but the two cultivars differed significantly in total dry weight ($F_{1,89}$ = 49.4; P < 0.001) as 'Vibrant' produced more dry biomass (10.6 ± 0.4 g; n = 47) than 'Red Glory' (7.9 ± 0.2 g; n = 50). The interaction between AMF treatment and cultivar was not significant. None of the plants produced runners in experiment 2.

4.3.3. Effect of AMF pre-inoculation on strawberry wilt incidence

In experiment 1, 27 weeks after transplantation in soil with an average wilt inoculum density of 22.2 CFU g^{-1} of soil, only a few plants showed wilt symptoms (Figure 4.6a-d). In the plants without AMF pre-inoculation two out of 24 plants showed typical wilt symptoms; while in the plants pre-inoculated with AMF, five out of 36 plants were wilted. Such differences were not statistically significant.

In experiment 2, 25 weeks after transplanting in soil containing 22.2 CFU g^{-1} of soil, only a few 'Vibrant' plants showed wilt symptoms (Figure 4.6e-f), whilst all 'Red Glory' plants remained healthy (Table 4.3). The number of diseased plants was too low to allow for meaningful statistical comparison.

In experiment 3, 18 weeks after cultivation under field conditions with an average wilt inoculum density of 1.9 CFU g^{-1} of soil, wilt symptoms were observed (Figure 4.6g-i). AMF pre-inoculation increased or decreased the number of diseased plants depending on individual AMF species but differences were not statistically significant. The three strawberry cultivars differed significantly in the incidence of wilt (P = 0.029; Table 4.4). The wilt incidence was in the order of 'Malling Centenary' (41%: 158 out of 384) > 'Vibrant' (36%: 137 out of 384) > 'Red Glory' (21%: 82 out of 384; Figure 4.7). There were no significant interactions between AMF species and cultivar. The spatial map of strawberry wilt indicated two foci with high numbers of wilted plants (i.e. ≥ 10 diseased plants per plot indicated by orange to red colour; Figure 4.8).

Figure 4.6: Strawberry wilt symptoms observed across experiments were characterised by wilted leaves, brown leaves and stunted plants: (a-d) experiment 1; (e-f) experiment 2; (g-i) experiment 3.

Table 4.3: Number of wilted and healthy plants in experiment 2 for treatments without or with AMF pre-inoculation of strawberry cultivars 'Vibrant' and 'Red Glory'.

		No. of plants	
Cultivar	**AMF pre-inoculation**	**diseased**	**Healthy**
Vibrant	No	2	18
	Yes	3	24
Red Glory	No	0	20
	Yes	0	30

Table 4.4: Generalised linear model (GLM, analysis of deviance) of the number of diseased plants in experiment 3, 18 weeks after cultivation under field conditions. Significant differences are shown by bold font ($P \leq 0.05$).

Source of variation	**Number of diseased plants**		
	df	***Deviance***	***P***
Cultivar	2	42.6	**0.029**
AMF	3	11.8	0.559
Cultivar × AMF	6	20.9	0.716
Residual	55	310.6	

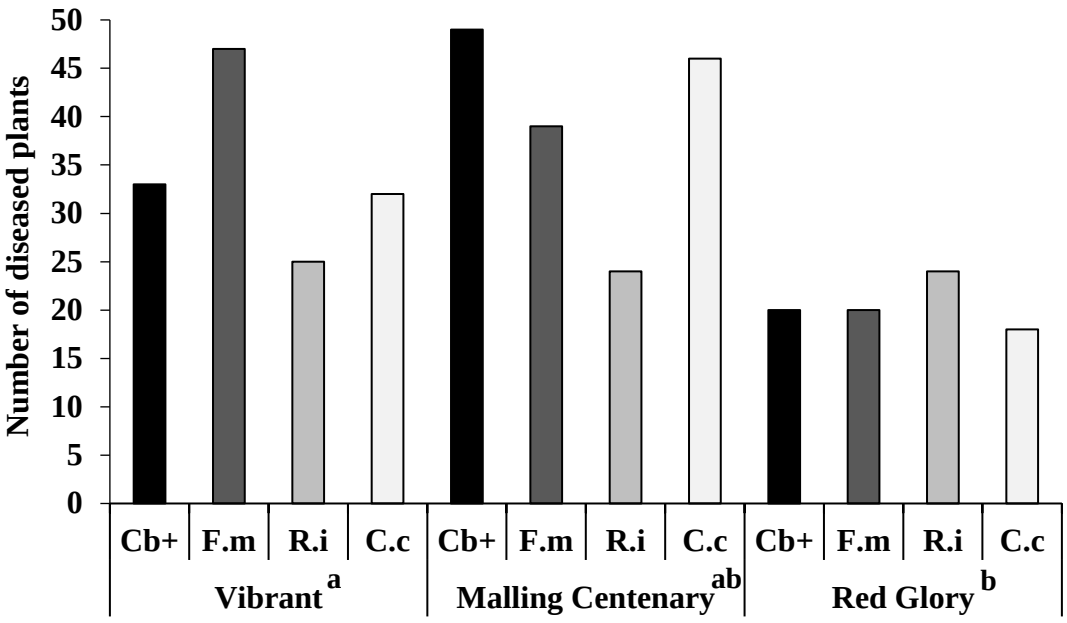

Figure 4.7: Number of diseased plants in experiment 3 without pre-inoculated mycorrhiza (Cb^{+}) or pre-inoculated with single AMF species (*Funneliformis mosseae* (F.m), *Rhizophagus irregularis* (R.i) and *Claroideoglomus claroideum* (C.c)) of three strawberry cultivars ('Vibrant', 'Malling Centenary' and 'Red Glory'), 18 weeks after cultivation under field conditions. Data are number of diseased plants (n = 96). Strawberry cultivars that did not differ significantly share at least one common letter (Pairwise comparisons, $P \leq 0.05$).

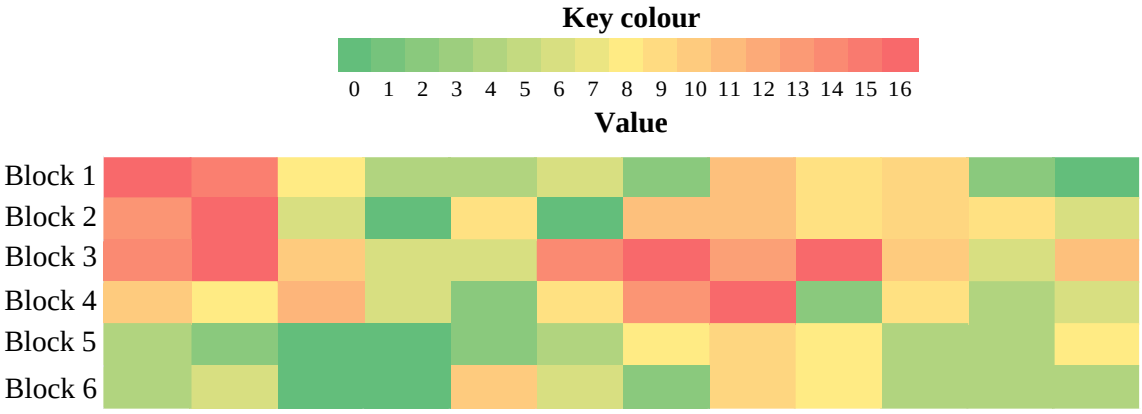

Figure 4.8: Spatial map of *Verticillium dahliae* on strawberry field at 18 weeks post transplantation showing two foci with high numbers of wilted plants per plot. The legend bar represents a colour key for the number of diseased plants per plot. There were six blocks (i.e. six planting beds) and 72 plots each with 16 plants (AMF treatment and strawberry cultivar for each individual plot are not presented for clarity).

4.4. Discussion

Since AMF have been shown to have positive effects on strawberry growth and health (Khanizadeh *et al.*, 1995; Norman *et al.*, 1996; Tahmatsidou *et al.*, 2006; Sowik *et al.*, 2016), there is an increasing interest to use them in commercial horticulture to increase yield and fruit quality while reducing fertiliser and biocide inputs. After several months in pots filled up with non-autoclaved field soil, AMF colonisation of strawberry roots reached on average 90% in experiment 1 and 2, agreeing with a previous study showing high % RLC (70%) in strawberry plants cultivated in soil (Santos-González *et al.*, 2011). This is the first report of such a high level of root colonisation for strawberry plants grown in pot filled up with non-autoclaved field soil. At the end of experiment 1 and 2, the controls and AMF pre-inoculated treatments showed similar levels of AMF root colonisation, indicating the presence of indigenous AMF propagules in the field soil used for both pot experiments. In addition, 'Vibrant' showed overall a lower AMF root colonisation level than 'Red glory' in experiment 2. This is in agreement with other studies showing that strawberry cultivars could differ in their response to AMF colonisation under both glasshouse or field conditions (Robertson *et al.*, 1988; Chávez & Ferrera-Cerrato, 1990; Vestberg, 1992; Khanizadeh *et al.*, 1995). However, it was not possible to confirm whether pre-inoculated AMF species persisted in the pot experiments due to the presence of AMF root colonisation in the controls. A number of studies have already discussed the fact that soils containing native AMF propagules are problematic in the sense that inoculated fungi cannot be distinguished easily from the indigenous AMF (Niemi & Vestberg, 1992; Tahmatsidou *et al.*, 2006; Rodriguez & Sanders, 2015). Therefore, a metagenomic approach may be used in the future to assess whether pre-inoculated AMF species persisted temporally and spatially and if they altered the composition of the native AMF community in the field soil (Rodriguez & Sanders, 2015). However, this will be only possible if there are significant genetic differences between field and inoculated AMF strains and species.

Pre-colonisation by AMF did not result as expected in an enhanced plant growth (e.g. plant dry biomass) after transplantation in pots containing non-sterile field soils. It was suggested that in such long-term experiments the indigenous AMF inocula present in the soil could have masked the initial positive effect on plant growth provided by AMF pre-colonisation. Nevertheless, future experiments with other strawberry cultivars and sampling times closer to the transplantation time may reveal whether the pre-inoculation was of any advantages for plant growth and nutrition in the early stage of establishment in the field. Moreover, non-mycorrhizal control plants inoculated with bacterial filtrate in experiment 1 indicated a negative effect of the background bacterial community of the commercial AMF inoculants on the number of plants producing runners. Several authors have discussed the potential of some bacterial strains to positively influence the production and quality of strawberry runners (Aslantaş & Güleryüz, 2004; Pirlak & Köse, 2010), but this is the first report of a negative effect of bacteria associated with the AMF inoculants on the production of strawberry runners. Therefore, additional work is needed to confirm this result and identify candidate microbe(s) responsible for this phenomenon.

It was not possible to confirm that early AMF colonisation can improve plant tolerance to wilt in pot experiments under glasshouse conditions (experiment 1 and 2). In fact, natural *Verticillium* inoculum (microsclerotia) only resulted in a very low level of wilt symptoms on susceptible strawberry cultivars despite the sufficient level of viable microsclerotia in the soil (22 CFU g^{-1} of soil). Even as little as 1 CFU g^{-1} of soil can normally lead to significant wilt symptoms on strawberry in naturally infested field soil (Harris and Yang 1996). However, the reasons for the low level of wilt symptoms observed in experiment 1 and 2 are unknown. The transfer of the wilt infected soil under pot conditions may have reduced the microsclerotia infectivity. Therefore, other inoculation methods using a mix

of wilt isolates were also tested: (a) drenching soil with wilt conidial suspension without root injury in soil or sandy compost, (b) direct injection of conidial suspension in strawberry crown, (c) root dipping in conidial suspension with artificial root injury before transplantation in autoclaved sandy compost (Bhat & Subbarao, 1999), (d) inoculation of wilt hyphae with colonised potato dextrose agar (PDA) media plates buried at the bottom of the pots filled up with attapulgite clay. However, none of these methods led to wilt symptoms under controlled conditions (data not presented). Hence, it is of great importance to optimise another wilt inoculation method to enable further research under controlled conditions.

Nonetheless, the results of pot experiments cannot be directly compared to open field conditions, where much more complex systems of invertebrates, microbes and nutrients prevail. When the field soil was transferred into pots (experiment 1 and 2), its structure was dramatically modified (e.g. by sieving) and glasshouse conditions were rather different from those in the open field. In the current study, these differences may have modified complex interactions between plants, soil borne pathogens and/or beneficial microbes resulting in the low number of wilted plants observed despite the high wilt propagule density in the soil tested.

In the open field study (experiment 3), all three susceptible strawberry cultivars tested suffered from *Verticillium* wilt. The typical strawberry wilt symptoms developed across plots contaminated in average with 1.9 CFU g^{-1} of soil. However, results showed that none of the pre-inoculated AMF species could significantly reduce wilt incidence under field conditions. Although AMF inoculants were not effective under the conditions tested, this does not rule out their utility with other strawberry cultivars and/or in other locations. In addition, there was a high degree of spatial aggregation of plants with *Verticillium* wilt

(Figure 4.8), with two apparent foci. This aggregation pattern, as observed with *V. dahliae* on other crops (Xiao *et al*., 1997; Johnson *et al*., 2006; Wei et *al*., 2015), is most likely due to the heterogeneity in soil pathogen inoculum. This aggregation may have masked any treatment effects – random assignment of treatments to individual plots is not able to reduce the negative influence caused by aggregated inoculum. If soil samples from each experimental plot had been tested for wilt propagule concentration (e.g. via wet-sieving plating or wet-sieving qPCR methods) before plantation, it could be possible to use these inoculum concentrations as covariates to analyse the data. Therefore, it is advisable for future open field experiments to carry out wilt propagule density tests before strawberry plugs transplantation.

4.5 Conclusion and prospects

In summary, the present study highlighted the difficulty in conducting strawberry wilt inoculation experiments in pots under glasshouse conditions even when using a field soil naturally contaminated with *Verticillium dahliae* microsclerotia. This study also showed that AMF pre-inoculation of strawberry plugs failed to enhance plant growth after transplantation in a soil already inhabited by native AMF and it did not improve plant tolerance to *V. dahliae* under field conditions.

The use of beneficial microbes such as AMF to control strawberry soil-borne diseases is still in its infancy, but it must be considered and studied as a potential alternative to chemical soil fumigants. Although few attempts have been made previously to select effective AMF species/strains, none of them have yet achieved a complete control of strawberry root diseases under field conditions (Martin & Bull, 2002; Tahmatsidou *et al.*, 2006). In fact, small changes in field conditions may result in greater changes in the

biological control abilities of non-native AMF inoculants (Vestberg, 1992; Vestberg *et al.*, 2005; Rodriguez & Sanders, 2015). The variation of AMF inoculants effectiveness against soil-borne pathogens under field conditions is context dependent because of (1) environmental factors, (2) production practices (e.g. use of fertilisers and biocides), (3) host species/cultivars, (4) competition with indigenous AMF communities, and (5) multiple pathogens. Another important aspect is that English strawberry cropping is currently moving away from traditional field cultivation toward production in soil-less substrate to mitigate the threat of strawberry wilt and other soil-borne pathogens. Therefore, strawberry wilt is expected to be less of a problem in strawberry production in the UK. Nevertheless, strawberry cultivation in substrate still relies on high water and fertiliser inputs and other types of root pathogens such as *Phytophthora* remain an issue. Therefore, the use of AMF inoculation for strawberry plants grown in soil-substrates may help to reduce chemical inputs, water use, and increase plant tolerance to root pathogens. In conclusion, management programs of strawberry root diseases using AMF should be specifically designed for soil-less production systems.

Chapter 5. Evaluation of the potential of arbuscular mycorrhiza fungi and plant growth promoting rhizobacteria to increase strawberry productivity and tolerance to *Phytophthora fragariae* and *Phytophthora cactorum* in soil-less substrates

5.1. Introduction

Control of soil-borne pathogens is a major problem in strawberry field production because of the withdrawal of methyl bromide, an effective broad-spectrum chemical fumigant (Ristaino & Thomas, 1997; Tahmatsidou *et al.*, 2006). Recently, UK strawberry production has been rapidly moving away from traditional field cultivation towards table-top system, where strawberry plants are grown in soil-less substrate (e.g. coir and/or peat) under protection (e.g. polythene tunnel or glasshouse; Boyer *et al.*, 2016). There are several advantages to the adoption of soil-less substrates in commercial strawberry production, including reduction in picking cost, extension of the growing season and reduced risk of soil-borne diseases (Paranjpe *et al.*, 2008; Martínez *et al.*, 2010; Lieten, 2013). Nevertheless, *Phytophthora fragariae* and *P. cactorum* continue to pose a serious threat to strawberry growing in soil-less substrate as they may have infected initial planting materials in nurseries (Schnitzler, 2004; Martínez *et al.*, 2010).

By inoculating strawberry rhizosphere with beneficial microbes, plants may be protected against biotic (e.g. pathogens) and abiotic (e.g. drought) stresses, while water and nutrient uptake could also be improved (Vestberg *et al.*, 2004; Boyer *et al.*, 2016). Strawberry is an ideal production system to study such methods as planting materials (e.g. micro-propagated or runner derived plug plants) can be easily inoculated with beneficial microbes during their propagation and/or at planting. Several biological inoculants have already been shown to reduce the threat of strawberry root diseases in soil-less substrates when

introduced at planting (Martinez *et al.*, 2013; Rouphael *et al.*, 2015). Amongst those beneficial microbes, AMF showed to enhance plant growth, increase fruit yield and/or improve fruit quality in soil-less substrates (Cekic & Yilmaz, 2011; Boyer *et al.*, 2015; Palencia *et al.*, 2015; Boyer *et al.*, 2016; Cecatto *et al.*, 2016) and/or increased tolerance to root pathogens such as *P. cactorum* and *P. fragariae* (Murphy *et al*., 2000; Vestberg *et al*., 2004). In addition, plant growth-promoting rhizobacteria (PGPR) are commonly growing on the root surface, or rhizoplane, and increase plant growth and/or productivity by one or more mechanisms such as improved mineral nutrition, protection against pathogens or production of phytohormones (Glick, 1995; Vestberg *et al.*, 2004). Various symbiotic (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*) and non-symbiotic (*Pseudomonas*, *Bacillus*, *Klebsiella*, *Azotobacter*, *Azospirillum*, *Azomonas*) PGPR are currently being used as bio-inoculants to promote plant health and productivity (Ahemad *et al*., 2014). Several studies have highlighted beneficial effects of PGPR on various crops including strawberry (Esitken *et al.*, 2010; Ipek *et al.*, 2014; Hautsalo *et al.*, 2016). For example, PGPR inoculation showed to increase growth, chlorophyll content, nutrient element content, yield of strawberry plants and even mitigated deleterious effects of salt stress (Karlidag *et al*., 2013). Some works have also reported a strong stimulatory impact of PGPR on AMF symbiosis and its functioning (Artursson *et al*., 2006). Interestingly, synergistic effects on strawberry growth following co-inoculation with PGPR and AMF were reported (Vosatka *et al.*, 1992) and PGPR were also found to stimulate AMF root colonisation (Vosatka *et al.*, 2000). Therefore, additional studies should strive towards an improved understanding of the functional mechanisms behind AMF-PGPR interactions, thus that optimized combinations of those beneficial microbes could be used as effective inoculants within sustainable strawberry production systems.

The first aim of this study was to investigate whether commercially available AMF and/or PGPR inoculants can reduce *P. cactorum* or *P. fragariae* development in soil-less substrates. Strawberry cultivars 'Malling Centenary' or 'Vibrant' were used to test the hypothesis that AMF pre-inoculation and/or inoculation of AMF and/or PGPR at planting time can enhance tolerance to *P. fragariae* in sandy compost. Then 'Malling Centenary' was used to study whether AMF pre-inoculation can increase tolerance to *P. cactorum*. The second objective was to assess whether commercially available AMF and PGPR inoculants can increase strawberry yield in coir. The strawberry cultivar 'Malling Centenary' was used to test the hypothesis that inoculation of AMF and/or PGPR at planting time can increase strawberry yield and plant growth.

5.2. Materials and methods

A total of four experiments were conducted in 2015-2016. Experiment 1 and 2 were carried out to study the protective effect of AMF and/or PGPR against *P. fragariae* under growth room conditions. Experiment 3 was carried out under glasshouse conditions to test whether AMF can reduce *P. cactorum* development. Finally, experiment 4 was conducted under glasshouse conditions to evaluate the effect of AMF and/or PGPR on strawberry plant growth and productivity in coir. Table 6.1 gives the summary of each experiment.

Table 5.1: Summary of the four experiments to study the effect of arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) on strawberry plant health or productivity in soil-less substrates under controlled conditions.

Microbial treatment	**Number of experiment**			
	1	**2**	**3**	**4**
Number of treatments	4	8	2	4
Treatments[a]	M, P, M+P, Cb^-	M, P, M+P, PreM, PreM+M, PreM+P, PreM+M+P, Cb^-	M, Cb^-	M, P, M+P, Cb^-
Plant cultivars	'Vibrant'	'Malling Centenary'	'Malling Centenary'	'Malling Centenary'
Total no. of replicates[b]	20	16	20	3
No. blocks	4	4	-	-
No. of experimental repeats[c]	1	1	2	2
Storage temperature of plugs	-2°C	+2°C	-2°C	-2°C
Location	Growth room	Growth room	Glasshouse (chilled)	Glasshouse
Growing substrate	Sandy compost	Sandy compost	Compost	Coir
Pathogen inoculated	*P. fragariae*	*P. fragariae*	*P. cactorum*	-
Pathogen inoculation method	Slurry	Slurry	Wound + zoospores	-
Pathogens concentration[d]	-	-	10^3 CFU mL^{-1}	-
Experimental duration	5 weeks	6 weeks	7 weeks	13 weeks

[a] **M**: commercial AMF mix inoculum (Rootgrow™) containing five species, *Funneliformis mosseae*, *F. geosporum Rhizophagus irregularis*, *Claroideoglomus claroideum*, *Glomus microagregatum*, (provided by Plantworks Ltd, Kent, UK). **P**: commercial bacterial preparation containing four PGPR species, *Rhizobium* (Agrobacterium) strain IRBG74, *Derxia lacustris* HL-12, *Bacillus megaterium* and *B. amyloliquefaciens* (disclosed by PlantWorks Ltd, Kent, UK). **M+P**: AMF and PGPR. **PreM**: plug plants pre-inoculated with Rootgrow™ during tipping. **PreM+M**: AMF pre-inoculated plugs inoculated with Rootgrow™. PreM+P: AMF pre-inoculated plugs were inoculated with PGPR. **PreM+M+P**: AMF pre-inoculated plugs were inoculated with Rootgrow™ and PGPR. **Cb^-**: negative control without AMF pre-inoculation and no microbial inoculum. All microbial treatments were added at planting, except for treatment PreM.

[b] In experiment 4, each coir bag (replicate) was planted with 10 strawberry plants.

[c] The number of time the experiment was conducted.

[d] CFU: colony-forming unit.

5.2.1. Plant material

Cold stored (-2°C) strawberry plugs used in experiment 1, 3 and 4 (Table 6.1) were obtained from Hargreaves Plants, Norfolk, UK; plants derived in this way have shown in previous work to be free from AMF colonisation (Xu, pers. comm.) and several plants were tested prior to the main experiments to confirm this.

For experiment 2, AMF pre-inoculated and non-mycorrhizal strawberry plugs were obtained from R W Walpole Ltd, Norfolk, UK. Plastic trays (40 cells, ca. 132 cm^3 per cell; PG Horticulture Ltd, Northampton, UK) were filled up with a peat/perlite/coir mix (7:2:1, v/v; fertilised by the supplier with Osmocote® [added at 3 kg m^{-3}] and Micromax® premium [trace elements and magnesium fertiliser added at 0.3 kg m^{-3}]; Legro Beheer b.v., Asten, The Netherlands) using an automatic Javo tray filler (Javo b.v., Noordwijkerhout, The Netherlands; Figure 6.1A-C). For the AMF inoculated plants, Rootgrow™ (PlantWorks Ltd, Kent, UK) was mixed homogeneously with the potting mix at 10% (v/v), while only the potting mix was used for the non-mycorrhizal treatment. On 7th July 2015, runner tips of cv. 'Malling Centenary' were harvested and then pinned-down under glasshouse conditions at R W Walpole Ltd, Norfolk, UK. The plantlets were firstly weaned (using a misting system spraying water every hour for the first 5 d aiming for RH 80% and then every three hours for the next 5 d, while plants were protected from direct sunlight; Figure 5.1D). The misting system was then switched off. The plants were irrigated as needed, no additional fertiliser was added and direct sunlight was progressively introduced. The plug plants were grown for seven weeks (Figure 5.1E). On 27th August 2015, plug plants were sent to NIAB EMR, where they were cold stored at 2°C for 14 weeks. A few plants were tested prior to the experiment to determine whether the pre-inoculation was successful (10 samples, each with five plants); the results indicated the presence of a low level of AMF structures in root (Figure 5.2) with average % RLC of ca. 3%.

Figure 5.1: (A) Bale breaker loading the tray filler machine with potting substrate (i.e. peat/perlite/coir mix: 7:2:1, v/v), here inoculated with the commercial AMF inoculum Rootgrow™. (B) Javo tray filler loading the plastic trays with potting mix. (C) View of a plastic tray filled with potting mix. (D) Strawberry plugs weaned under a misting system. (E) Plug plants grown under glasshouse conditions seven weeks post transplantation.

Figure 5.2: Root colonisation by arbuscular mycorrhiza fungi (AMF) of strawberry plugs in experiment 2 after 21 weeks (i.e. after 7 weeks under glasshouse conditions and 14 weeks in a dark storage compartment at 2°C) of cultivation. Letters next to the arrows are A: arbuscule, H: hyphae, V: vesicle (red scale bars represent 100 μm).

5.2.2. Beneficial microbe inoculations

Inoculum of AMF and PGPR were provided by PlantWorks Ltd, Kent, UK. The AMF granular formulation was applied as commercially available Rootgrow™, a clay/pumice/zeolite mix containing spores, mycelium, and colonised host plants root fragments of five different AMF species (*Funneliformis mosseae*, *F. geosporum Rhizophagus irregularis*, *Claroideoglomus claroideum*, *Glomus microagregatum*). Rootgrow™ contained ca. 350 propagules mL^{-1} as determined by MPN analysis (Section 2.1.2; Cochran, 1950). The PGPR inoculum contained 10^8 CFU mL^{-1} and was supplied as a fine grade (0.5-1.0 mm) pumice containing four different rhizobacterial species (*Rhizobium* strain IRBG74, *Derxia lacustris* HL-12, *Bacillus megaterium* and *B. amyloliquefaciens*). This PGPR mix was used because it was commercially available and because it contained bacteria species (i.e. Bacillus) reported to have beneficial effects on strawberry productivity and to inhibit the growth of the strawberry pathogen

Colletotrichum acutatum in vitro (Erturk *et al*., 2012; Ipek *et al*., 2014, Es-Soufi *et al*., 2017). In each experiment, the negative control (Cb^-) was not pre-inoculated or inoculated at planting. At time of planting the granular AMF and/or PGPR inoculum were placed into each planting hole before transplantation of the strawberry plug. In experiment 1 and 2, the volume of granular inoculum added per planting hole was ca. 7.6 mL for AMF and PGPR. In experiment 3, each pot received 25 mL of granular AMF inoculum. In experiment 4, each planting hole received 20 mL of AMF inoculum and/or 2 mL of PGPR inoculum.

5.2.3. Pathogen inoculation

A mixture of three *P. fragariae* isolates (BC-1, BC-16 and Nov-9), from the pathogen collection of NIAB EMR, Kent, UK was used to inoculate plants in experiment 1 and 2. The isolates were cultured separately in Petri dishes on sterile modified kidney bean agar (KBA) for at least 30 d in the dark at 18 ± 1°C (Wynn, 1968; Maas, 1972). For each isolate, one piece (1-4 mm^2) of colonised KBA (from each stock culture) was then transferred to new Petri dishes containing fresh KBA and incubated separately as above. Mycelia were then harvested 30-60 d after plate inoculation. The excised colonies of the three isolates (including the agar beneath) were put into a blender with ice H_2O (1 g culture: 1 g ice H_2O) in equal ratio and blended twice for 2-5 s. The resulting inoculum slurry was transferred to a cooled beaker, which was kept on ice during the entire inoculation procedure. Before transplantation, roots were gently washed with tap water to remove substrate particles. Plants were then inoculated by dipping the roots into the inoculum slurry.

One *P. cactorum* isolate (P414; known to be pathogenic against 'Malling Centenary') from NIAB EMR was used in experiment 3. The stock culture was cultured in Petri dishes on a

sterile V8 (Campbell's V8 juice) agar for 7 d in the dark at 18 ± 1°C (Harris *et al.*, 1997). Then a sterilised cork borer was used to cut 10 mm discs from the margins of actively growing cultures. Discs were immersed in a non-sterile compost extract (2 L distilled water drained through 50 g compost and diluted two-fold before usage) and incubated for 2 d at 20°C in an illuminated incubator. A suspension of 10^3 zoospores mL^{-1} was then produced following the method described by Harris *et al.* (1997). A vertical slit (ca. 10 mm long) was made using scalpel blade at the base of an internal leaf (close to the crown). The inoculum was then directly sprayed onto the wounded area using a garden sprayer, 5 mL per plant. Inoculated plants were placed into a chilled glasshouse compartment (ca. 20°C) and covered with a clear polythene sheet for 48 h to prevent the zoospores from drying out.

5.2.4. Transplantation

In experiment 1, there were four treatments: plug inoculated at planting with (1) AMF [M], (2) PGPR [P], (3) both AMF and PGPR [M+P], and (4) a negative control without microbial inoculation [Cb^-]. Each treatment contained 20 replicate plants giving 80 plants in total; a randomised block design was used. About three weeks prior to the start of the experiment, ca. 100 cold stored (-2°C) plugs of cv. 'Vibrant' were transferred to a poly-tunnel with natural temperature and light conditions for ca. two weeks to induce plant growth. Plants were watered once per day with tap water. No additional fertiliser was added.

All plants were inoculated with *P. fragariae* as described in Section 5.2.3. Immediately after pathogen inoculation, plants were transplanted into 500 mL plastic pots (9 x 9 x 10 cm, Desch Plantpak Ltd, Essex, UK) filled up with ca. 400 mL of autoclaved (two cycles at 121°C for 20 min with about 4 d between cycles) sandy compost (Table 5.2). The potting

mix consisted of one part sand (Sinclair horticulture Ltd, Lincoln, UK) and one part sieved compost (reduced peat mixes with added bark and grit from Sinclair Pro, Cheshire, UK) fertilised with Multi-Mix® (added at 1 kg m^{-3}; Sinclair Pro, Cheshire, UK). Pots were placed in a growth cabinet (Meridian Refrigeration Ltd, Croydon, UK; constant 15°C, ca. 72% RH, light: dark 16 h/8 h, PPFD of 40 μmol m^{-2} s^{-1}). Plants stood in a shallow layer of water (2-7 mm) during the entire experiment. The experiment was terminated five weeks after transplanting.

In experiment 2, eight inoculation treatments were tested. There were plants inoculated at planting time with (1) AMF [M], (2) PGPR [P] and (3) both microbes [M+P]. There were also (4) AMF pre-inoculated plant [PreM] and AMF pre-inoculated plants inoculated at planting with (5) AMF [PreM+M], (6) PGPR [PreM+P], and (7) both AMF and PGPR [PreM+M+P]. A negative control (8) without microbes inoculated [Cb^-] was also included. Each treatment contained 16 replicate plants giving 128 plants in total; a randomised block design as used. About two weeks prior to the start of the experiment, ca. 320 cold stored (2°C) plugs of cv. 'Malling Centenary' were transferred to a growth cabinet (Meridian Refrigeration Ltd, Croydon, UK; day and night 15°C, ca. 72% RH, light: dark 16 h/8 h, PPFD of 40 μmol m^{-2} s^{-1}) to induce plant growth. Plants were watered once per week with tap water and no additional fertiliser was added. Finally, plugs were inoculated with *P. fragariae* and treated as in experiment 1. The experiment was terminated after six weeks.

In experiment 3, there were two treatments: (1) plug pre-inoculated with AMF [PreM] and (2) negative control without AMF pre-inoculation [Cb^-]. Each treatment contained 20 replicate plants giving 40 plants in total. This experiment was conducted on two separate occasions (Table 5.1). Before pathogen inoculation, cold stored (-2°C) plugs of cv. 'Malling Centenary' were transplanted into 500 mL plastic pots (9 × 9 × 10 cm, Desch

Plantpak Ltd, Essex, UK) filled up with ca. 450 mL of standard compost mix (reduced peat with added bark and grit, fertilised with Multi-Mix® [added at 1 kg m^{-3}] and Osmocote® [added at 4.44 kg m^{-3}], from Sinclair Pro, Cheshire, UK; Table 5.2). Plants were then transferred to a poly-tunnel under natural temperature and light conditions for ca. five weeks to induce plant growth and AMF colonisation. Plants were manually watered once per day with tap water and no additional fertiliser was added. All plants were then inoculated with *P. cactorum* as described in Section 5.2.3. Finally, each plant was randomly placed into a chilled glasshouse compartment (temperature set at 20°C during the day and 15°C during the night, with natural light cycle). Plants were watered as above. The experiment ran for seven weeks before destructive sampling.

In experiment 4, there were four treatments: plug inoculated at planting time with (1) AMF [M], (2) PGPR [P], (3) both AMF and PGPR [M+P], and (4) a negative control with no microbes inoculated [Cb^-]. This experiment was conducted on two separate occasions. There were three replicate coir bags and a randomised design was used (Table 5.1). Cold stored (-2°C) plugs of cv. 'Malling Centenary' were planted in coir bags (BotaniCoir, London, UK), 10 plants per bag and inoculated with AMF and/or PGPR at planting as described in Section 5.2.2. Irrigation was delivered to plants via four irrigation lines using drippers (four per bag), controlled by Galcon irrigation timer (DC15: City Irrigation Ltd, Kent, UK). Three were three coir bags per irrigation line. The volume of irrigation was adjusted over time and reached 1 L per day per bag at 6 weeks from plantation. Concentrated nutrient solution of Vitafeed 102 (100 g L^{-1}; Vitax Ltd, Leicester, UK) was injected into the irrigation lines by a dosatron injector (D3 Green line: City Irrigation Ltd, Kent, UK) set at a dose rate of 0.5% (v/v) for two weeks from plantation and then adjusted at a dose rate of 1% (v/v) and thereafter remained at this rate. After the onset of flowering a mini hive of bumblebees (*Bombus terrestris* audax; Agralan, Wiltshire, UK) was

introduced to the compartment to pollinate flowers. Plants were grown in a glasshouse compartment set at 23°C day/20°C night with natural light cycle. Experiment 4 was terminated after the final fruit harvest (i.e. 13 weeks post transplantation).

Table 5.2: Background nutrient status analysis[a] of the substrate used in experiments.

Experiment	Substrate	pH	NO_3	NH_4	P	K	Mg
			ppm = mg kg^{-1}				
1	Sandy compost	5.8	75	27	16	200	45
2	Sandy compost	4.9	175	131	62	267	51
3	Compost	4.7	236	75	101	380	130
4	Coir	6.6	< 4	20	< 6	31	< 1

[a] Available nutrient status of each growth medium was provided by NRM Laboratories (Berkshire, UK) in mg L^{-1} and it was converted to mg kg^{-1}. NO_3^- was determined by ion chromatography and NH_4^+ by colorimetric analysis. P, K, Mg and Ca were analysed by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy). Note that the nutrient status analysis of each growing medium could not be replicated due to high cost.

5.2.5. Disease assessment, plant productivity and root sample analysis

In experiment 1, 2 and 3, plants were assessed once a week for aboveground disease symptoms on a rating scale:

1 – no symptoms,

2 – flaccid foliage,

3 – totally collapsed and dead.

In experiment 3, after the final aboveground disease assessment, the crowns were cut longitudinally and the extent of internal necrosis was recorded:

1 – no necrosis,

2 – up to 25% necrosis,

3 – 25 to 50% necrosis,

4 – 50 to 75% necrosis,

5 – 75 to 100% necrosis.

In experiment 4, ripe fruits were picked twice weekly. Total and Class I fruits (> 18 mm diameter) were obtained for individual coir bags and the number of fruit was recorded for every pick. After the final fruit pick, total fresh shoot weight of the plants from individual bags was also determined. A few plants died during the experiment; average number of fruits (total and Class I fruit), average plant yields (total and Class I fruit), and average fresh shoot weight were calculated per plant for each individual coir bag and used in subsequent statistical analysis.

At the end of each experiment, a composite sample of roots was taken for each pot or coir bag (i.e. roots deriving from three plants out of 10 were pooled together) for assessment of AMF root colonisation (preferentially on younger roots). The roots were then cleared with 2% KOH before being stained with Trypan blue (Section 2.3.1) and microscopically assessed for root length colonisation (RLC; Section 2.3.2).

5.2.6. Data analysis

All data were analysed using GenStat 13th edition (VSN International Ltd, Hemel Hempstead, UK). Only significant differences were reported in the text. For experiments with more than one factor, the interactions were statistically tested. In experiments 1-3, the disease data were all analysed using generalised linear models (GLM) with residual errors assumed to follow Poisson distribution; the log link function was used. There were two treatments factors (M and P) in experiment 1, three factors in experiment 2 (PreM, M and P) and one factor in experiment 3 (M). In experiment 3, individual experiments conducted at different times were treated as a blocking factor. In experiment 4, for each bag average fruit yields (total and Class I fruit), average number of fruits (total and Class I fruit) and average plant fresh shoot weight per plant were analysed by two-way ANOVA. There were

two treatment factors (M and P). Individual replicate experiments were treated as a blocking factor. Common diagnostic plots (e.g. q-q plots, residual-fitted value plot) did reveal apparent violation of the normality and homoscedasticity assumption. Hence, average number of fruit data were square root transformed, while average yield and fresh shoot weight were both log transformed to satisfy normality. If ANOVA indicated significant effects of a specific treatment factor or interaction, pairwise comparison was performed based on the LSD test. In all experiments, the AMF root colonisation data were not statistically tested due to a very low level of AMF within roots.

5.3. Results

5.3.1. Establishment of AMF in the substrates

Experiment 1 and 2 were carried out to study the protective effect of AMF and/or PGPR against *P. fragariae*, while experiment 3 was undertaken to test whether AMF can control *P. cactorum*. Finally, experiment 4 was conducted to evaluate the effect of AMF and/or PGPR on strawberry plant growth and productivity in coir. In experiments 1-3, there was no AMF colonisation observed in the roots of AMF treated plants. In experiment 2, AMF pre-colonised plugs showed an average of 3% RLC before transplantation; but no AMF colonisation was observed at the end of the experiment. In experiment 4, 13 weeks post inoculation, AMF colonisation was observed at a low level (average 15% RLC) and varied greatly among samples; there was no AMF colonisation in many AMF inoculated root samples. There were no obvious differences in RLC between M and M+P treatments.

5.3.2. Effect of beneficial microbes against red core and crown rot

The effects of AMF and PGPR inoculations against *P. fragariae* (red core) were studied in experiments 1 and 2. In experiment 1, the first wilting symptoms appeared eight days

after pathogen inoculation. In general, the number of plants with visible symptoms remained stable after 20 d from inoculation (Figure 5.3A, B); 60 out of 80 plants showed disease symptoms at the end of the experiment. The number of diseased plants was decreased by AMF inoculation at planting (P = 0.020; Figure 5.4), while PGPR treatment did not affect the number of diseased plants. There was no significant interaction between AMF and PGPR.

In experiment 2, the first wilting symptoms appeared in less than a week after inoculation. No more symptoms appeared after 20 d from pathogen inoculation and the overall number of wilted plants was lower than in experiment 1; 40 out of 128 plants showed disease symptoms. However, there were no significant treatment (PreM, M, P) effects on the number of diseased plants; neither were there significant interactions among treatment factors.

Figure 5.3: Plants inoculated with *Phytophthora fragariae* in experiment 1 (view of block 4 only) at two different time points: (A) at planting and (B) three weeks after planting. Diseased plants were wilted and showed brown leaves (red arrows).

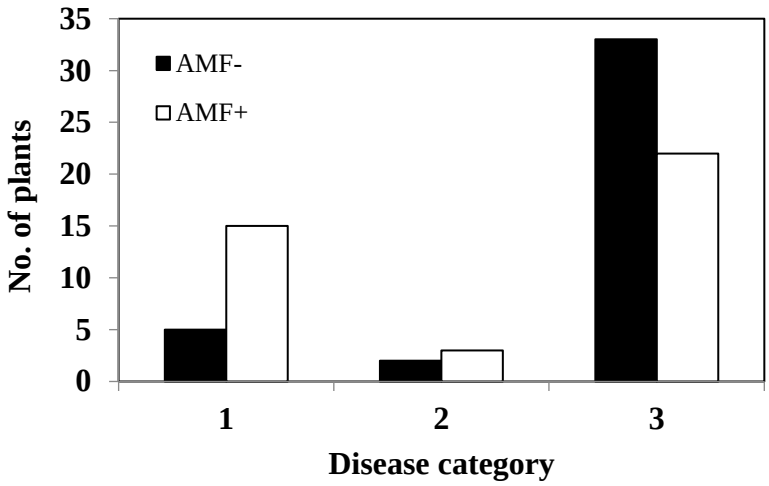

Figure 5.4: Influence of arbuscular mycorrhiza fungi (AMF) addition in experiment 1 on disease score 35 d after inoculation of *Phytophthora fragariae*. Data are the total number (n = 40) of plants in each of the following disease category: 1 - no symptoms, 2 - floppy foliage, 3 - totally collapsed and dead. White bars show plants inoculated with mycorrhiza at planting (AMF+; i.e. M and M+P treatments) and black bars show plants without mycorrhiza (AMF-; i.e. P and Cb$^-$ treatments).

The effect of mycorrhizal inoculation against *P. cactorum* (crown rot) was studied in experiment 3. The first wilting symptoms appeared about two weeks after inoculation (Figure 5.4A) and the number of plants with visible symptoms remained stable after five weeks from pathogen inoculation. In total, 31 and 33 out of 40 plants showed crown necrosis for the 1st and 2nd replicate experiment, respectively (Figure 5.5A, B). However, there were no significant effects of AMF pre-inoculation on the number of diseased plants and crown necrosis level.

Figure 5.5: Plant inoculated with *Phytophthora cactorum* in experiment 3 (replicate 1) seven weeks after pathogen inoculation: (A) severely wilted plant and (B) observation of crown necrosis (level 4, see Section 5.2.5).

5.3.3. Effect of AMF and PGPR on strawberry production in coir

Strawberry plants in experiment 4 grew normally and there were no visual differences in plant growth among treatments (Figure 5.6). A single plant in each coir bag produced on average nine and ten fruits for the 1st and 2nd replicate experiment, respectively; the corresponding average fruit weight was 60 and 67 g. There were no significant differences in the average plant yields (total and Class I fruit) and average plant number of fruits (total and Class I fruit) among treatments. The interaction involving the factors M and P was not statistically significant. For the average plant fresh weight, none of the treatments resulted in significant differences and there was no significant interaction. Despite the absence of a significant effect on the average plant yields and number of fruits (total and Class I fruit) followed the same treatment order: P > M+P > M > Cb$^-$ (Figure 5.7A), whereas the treatment order for average plant shoot fresh weight was: M > M+P > P > Cb$^-$ (Figure 5.7B).

Figure 5.6: Strawberry cv. 'Malling Centenary' plants in experiment 4 growing in coir bags, 13 weeks after plantation.

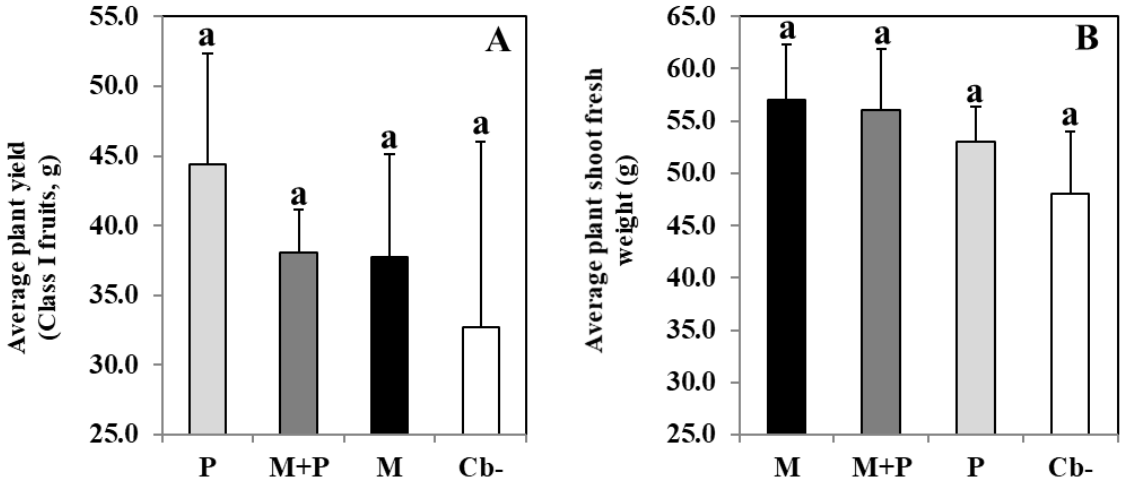

Figure 5.7: Average plant Class I fruit yield (A) and average plant shoot fresh weight (B). The treatments were plugs inoculated at planting time with AMF (M), PGPR (P), both AMF and PGPR (M+P) and a negative control with neither AMF nor PGPR added (Cb^-). Note the scales do not start at 0. Treatments that did not differ significantly share at least one common letter (Pairwise comparisons, $P \leq 0.05$). Note the scales do not start at 0.

5.4. Discussion

The soil-less substrates used in horticultural production usually lack beneficial microbes (Postma *et al.*, 2008). By inoculating commercial AMF and PGPR alone or together to the plant rhizosphere, it might be possible to reduce chemical inputs and grow strawberry more sustainably (Boyer *et al.*, 2016). Bacteria and mycorrhizal fungi occurring naturally in plant rhizosphere could be excellent candidates for the development as a biocontrol agent or a biofertiliser because they are already part of the balance between plants, pathogens and soil (Whipps, 2004). The main objective of this study was to investigate whether commercially available AMF and/or PGPR inoculants could reduce development of root pathogens such as *P. fragariae* (red core), and *P. cactorum* (crown rot) in peat-based composts and test whether AMF and/or PGPR can improve strawberry productivity in coir under controlled conditions.

In experiment 1-3, AMF inoculation in compost mixes containing peat and fertilisers did not result in strawberry root colonisation. However, a low level of root colonisation (average 15%) was detected in coir bags in experiment 4. Therefore, soil-less substrates used in those experiment appeared not to be conducive for AMF root colonisation, in contrast to the results obtained in Chapter 3. Nevertheless, previous studies reported negative effects of certain peat types and coir on strawberry RLC (Vestberg *et al.*, 2004; Boyer *et al.*, 2016). For example, fertilised compost mixes were previously used to test the effect of AMF against *Phytophthora* on strawberry plants and results were rather disappointing in term of disease severity reduction and/or AMF root colonisation that was associated to the high level of available P measured in the substrates (Murphy *et al.*, 2000; Vestberg *et al.*, 2004). In addition, high inputs of fertilisers under commercial practices have been shown to reduce AMF root colonisation in strawberry (Niemi & Vestberg, 1992). For example, excessive P inputs showed to often reduce AMF colonisation in

several crops when applied to the substrate in pot experiments under controlled conditions (Olsson *et al.*, 1997). Consequently, the absence of root colonisation in the compost mixes used in experiment 13 may have been the results of a high concentration of available P (16-101 ppm; Table 5.2) and a low pH (i.e. < 5.3; Table 5.2) or the combination of other chemical, physical and biological properties of the substrates themselves. In contrary, even low, AMF root colonisation was observed in coir used in experiment 4, that presented a relatively neutral pH (6.6) and a very low P level (i.e. < 6 ppm; Table 5.2). Therefore, further investigations on the role of commercial fertilisers and substrate properties on AMF colonisation are needed.

In experiment 1, the number of diseased plants was reduced by AMF inoculation at planting time. This agrees with several studies highlighting the positive effect of AMF inoculation against red core (Mark & Cassells, 1996; Norman *et al.*, 1996; Norman & Hooker, 2000). However, PGPR alone and co-inoculation with AMF did not result in reduced disease development in experiment 1. In contrast, Vestberg *et al.* (2004) reported an increase in disease development of *P. fragariae* after AMF inoculation (alone or in mixture with PGPR). However, it should be noted that although the AMF inoculation showed a positive effect against red core in experiment 1, AMF colonisation was not detected in the roots. Either the sampling method failed to detect a low level of AMF colonisation or the substrate containing the AMF inoculum (i.e. attapulgite clay/pumice/zeolite mix) could have achieved this as attapulgite clay has previously been found to limit the development of *Phytophthora* symptoms in strawberry plants (Hautsalo *et al.*, 2016; pers. obervation). In experiment 2, neither AMF pre-inoculation, nor microbial (i.e. AMF and/or PGPR) inoculations at planting, nor the combination of both inoculation methods reduced disease development. Fewer plants in experiment 2 suffered from red core (31%) than in experiment 1 (75%). It is, however, unclear whether the lower level of

diseased plants in experiment 2 was due to the differences in susceptibility between the two cultivars and/or an effect of the growing season. Previous studies have highlighted the difference of red core susceptibility between strawberry cultivars (Van de Weg, 1997) and the growing season has also been shown to modify the effect of the beneficial microbes against *Phytophthora* (Vestberg *et al.*, 2004). The reasons for the difference in disease development may be the physiological status of the strawberry plants that may modify the root exudation and the microbial community composition in the rhizosphere, either introduced or natural. In experiment 3, disease reduction was also not achieved by AMF pre-inoculation during the season favourable for strawberry growth. The reason for this is unknown. Nevertheless, in experiment 2 and 3, the absence of AMF root colonisation might have explained the absence of the biological control effect expected. It is now essential to establish a reliable experimental system to study the potential bioprotective effect of AMF against *P. fragariae* and *P. cactorum*.

In experiment 4, inoculation of plants with AMF and/or PGPR at planting time in coir had a consistent positive trend of increasing shoot fresh weight, fruit yield and number of fruits produced. These observations agree with previous reports of AMF inoculation of strawberry in coir (Boyer *et al.*, 2016). Co-inoculation of AMF and PGPR did not give better results than the inoculation of either AMF or PGPR alone for strawberry growth and yield, supporting a previous study (Vestberg *et al.*, 2004). However, the positive effects of AMF and PGPR observed in the current study were not statistically significant. The nature of the experimental design (i.e. random design) could have been responsible for large experimental residual errors, leading to the absence of significant treatment effects. Four irrigation lines were used for this study to deliver water into coir substrate. Although the irrigation lines are supposed to provide equal amounts of water and fertiliser, they are known to often introduce systematic differences in experimental results (Xu pers. Comm.).

Consequently, the use of a randomised block design with a single irrigation line as a block may have been more appropriate than a randomised design to minimise experimental residual errors.

5.5. Conclusion

Use of AMF and PGPR to reduce disease development and to improve crop production in commercial strawberry production remains a challenge despite extensive evidences of their beneficial impacts. In fact, for biological agents to have real potential to be developed as a commercial product and used by strawberry growers, their positive effects need to be consistent and reliable. Unfortunately, the beneficial impact of AMF and PGPR appear to be context dependent and variable, highlighting the complexity of plant-microbe interactions and so the difficulty to establish AMF symbiosis in different soil-less substrates. This study highlighted the difficulty for AMF to establish in soil-less substrates containing high P level and/or presenting a low pH. Therefore, strawberry cultural practices such as the amount of fertiliser, the irrigation regime and the type of substrate may have to be adjusted to improve AMF and/or PGPR colonisation. In addition, AMF and PGPR inoculum production methods may need to be improved for better application and establishment of those beneficial microbes in commercial soil-less strawberry production systems. To take this research forward, it is imperative to find a substrate that could be commercially relevant and conducive for AMF and/or PGPR. Although microbial inoculation did not result in significant positive effects on strawberry health and yield in this study, further optimisations and understanding of the strawberry-microbe interactions in soil-less substrates is needed before this technology can be adopted by the strawberry industry.

Chapter 6. Can an axenic autotrophic *in vitro* system be established to explore the nature of interactions between AMF and soil-borne pathogens in strawberry plants?

6.1. Introduction

Among other benefits, AMF colonisation is known to alter strawberry plant response to biotic stresses, leading to increased tolerance to attacks by root pathogens (Norman *et al.*, 1996; Tahmatsidou *et al.*, 2006; Sowik *et al.*, 2016). However, the interactions among AMF and pathogens are complex; a further complicating factor is that most of these studies have been conducted under glasshouse or field conditions where changes in the environmental conditions also affect the nature of these interactions. Therefore, investigating the nature of the interaction between pathogens and AMF under *in vitro* conditions may help to reveal the role of AMF in increased tolerance/resistance to strawberry soil-borne pathogens.

The application of autotrophic systems (i.e. *in vitro* culture systems with photosynthetic active plant tissues) may therefore be a useful approach to study various aspects of plant–AMF interactions. The AMF symbiosis has been successfully established under axenic or semi-axenic controlled conditions with several plant species (e.g. banana, barrelclover, clover, ficus, potato and vine; Hepper, 1981; Voets *et al.*, 2005; Koffi *et al.*, 2009; Voets *et al.*, 2009; Nogales *et al.*, 2010; Lovato *et al.*, 2014), including strawberry (Elmeskaoui *et al.*, 1995; Cassells *et al.*, 1996). Autotrophic systems may facilitate the study of biochemistry, molecular and physiological aspects of the plant–AMF–pathogen interaction, allowing more accurate assessments than in conventional pot systems.

Recently, several autotrophic systems have been developed and demonstrated the protective effect of AMF against pathogens (Nogales *et al.*, 2010; Koffi *et al.*, 2013; Lovato *et al.*, 2014; Oye Anda *et al.*, 2015). In addition, the *in vitro* system developed by Sowik *et al.* (2008) allowed the successful association of strawberry plantlets with *Verticillium dahliae* under axenic conditions, speeding up screening for wilt resistance. Although strawberry roots were successfully associated with AMF under *in vitro* conditions (Elmeskaoui *et al.*, 1995; Nuutila *et al.*, 1995; Cassells *et al.*, 1996), the study of the interactions among strawberry cultivars, AMF and pathogens has not been reported yet under axenic conditions.

The objective of this study was to establish a simple experimental culture system associating micropropagated strawberry plantlets with AMF and a single root pathogen under a controlled and axenic environment. Two autotrophic systems developed by Müller *et al.* (2013) and Voets *et al.* (2009) were adapted into a single *in vitro* system to study the effects of AMF against strawberry root pathogens. *Rhizophagus irregularis* (MUCL 43194) was used as the AMF inoculant and two strawberry pathogens were tested separately: *V. dahliae* was inoculated onto *Fragaria vesca* (one diploid parent of cultivated strawberry) and *P. fragariae* was inoculated onto *F. ananassa* cv. 'Calypso'. AMF spore germination, spread of the pathogen on the medium, disease scores and intraradical root colonisation by AMF and the pathogen were assessed.

6.2. Materials and methods

This study aimed to investigate the protective effects of AMF against *V. dahliae* or *P. fragariae* under an axenic *in vitro* culture system. Two experiments were carried out and a schematic representation of the experimental setup is shown in Figure 6.1.

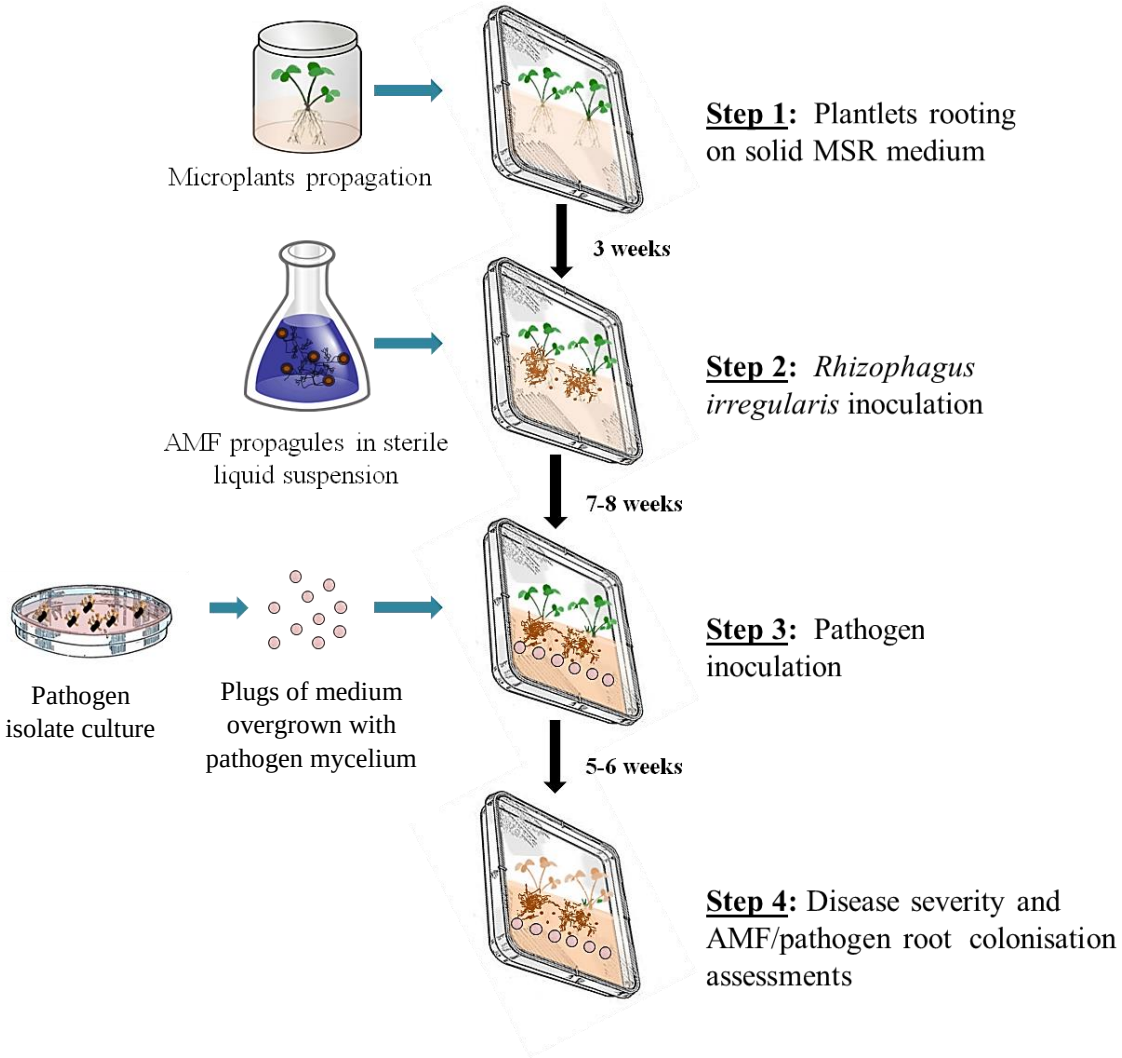

Figure 6.1: A schematic representation of the experimental setup to study the interaction of arbuscular mycorrhiza fungi (AMF) and *Verticillium dahliae* or *Phytophthora fragariae* under *in vitro* culture: (1) strawberry microplants were rooted on Modified Strullu Romand (MSR) medium; (2) microplants were then inoculated with commercial sterile *Rhizophagus irregularis* spores; (3) microplants were inoculated with plugs of medium overgrown with pathogen mycelium, and (4) disease severity in addition to AMF or pathogen root colonisation was assessed.

6.2.1. Plant materials

Tissue culture plants of an *F. vesca* clone VSI and *F.* x *ananassa* cv. 'Calypso' produced as described in Section 2.2.1 were used in experiment 1 and 2, respectively.

6.2.2. Arbuscular mycorrhiza fungus

Rhizophagus irregularis (N.C. Schenck & G.S. Sm.) C. Walker & A. Schuessler 2010, strain MUCL 43194, was purchased from Premier Tech Biotechnologies (Mycorise® ASP, Rivière-du-Loup, Canada) as a sterile suspension of spores (400 spores mL^{-1}) and used to inoculate *in vitro* strawberry plants.

6.2.3. Strawberry plantlets establishment in axenic conditions

The autotrophic *in vitro* system was adapted from axenic culture systems developed by Müller *et al.* (2013) and Voets *et al.* (2009). Square Petri dishes (12 x 12 cm) were used as in Müller *et al.* (2013) to maintain axenic conditions. The plates were filled with Modified Strullu Romand (MSR) medium (Declerck *et al.*, 1998), lacking sucrose and vitamins as in as in Voets *et al.* (2009), but the medium was solidified with 5 g L^{-1} Phytogel™ instead of 4 g L^{-1} Gel Gro™. After solidification, the upper half of the medium was removed from the Petri dishes. Two microplants were then transferred into each square plate, with the roots placed on the surface of the MSR medium and the shoot extending into the empty part of the plate. The square plates were then sealed with parafilm and incubated vertically in a growth room (21°C, light:dark 16 h/8 h, PPFD of 40 μmol m^{-2} s^{-1}) for 3 weeks to allow plants to produce new roots.

6.2.4. Strawberry plantlets inoculation with AMF

After 3 weeks on the MSR medium, each plant was inoculated with 250 µL (ca. 100 spores) of the sterile *R. irregularis* spore suspension. For each experiment, 20 square plates with AMF (+AMF treatment) and 20 plates without AMF (-AMF treatment) were set up. The plates were sealed again with parafilm and the root area was covered with aluminum foil to allow the roots and AMF to grow under darkened conditions. The plates were incubated for 7-8 weeks after AMF inoculation.

6.2.5. Soil-borne pathogens

Three isolates of both *V. dahliae* and *P. fragariae* from the collection held at NIAB EMR (Kent, UK) were used.

In experiment 1, three *V. dahliae* isolates (12251, 12252 and 12253) were recovered from NIAB EMR cryostore and cultured separately on sterile prune lactose yeast agar (PLYA) medium (Talboys, 1960) for ca. 1 month in the dark at 22 ± 1°C. Sterile purified water was pipetted onto each Petri dish (5 mL) and the medium surface gently rubbed to make a conidial suspension. Finally, 0.2 mL conidial suspension of each isolate was pipetted onto the same fresh PYLA plate; the plates were then incubated for 18 d at ambient conditions close to a natural light source.

In experiment 2, three *P. fragariae* isolates (BC-1, BC-16 and Nov-9) were cultured separately on sterile modified KBA medium (Maas, 1972) for 1-2 months in the dark at 18 ± 1°C. Then one piece (1-4 mm^2) of colonised KBA was transferred to a fresh KBA medium plate, and then incubated in the dark at 18 ± 1°C for ca. 3 weeks.

6.2.6. Inoculation of strawberry plantlets with pathogens

In experiment 1, after 8 weeks of growth in plates with or without AMF, the plantlets were randomly divided in four treatments: inoculated with (1) wilt and AMF (+AMF+Wilt), (2) AMF only (+AMF-Wilt), (3) wilt only (-AMF+Wilt), and (4) no AMF or wilt (-AMF-Wilt). Each treatment had ten replicate plates each with two plantlets. Inoculation with *V. dahlia* was as follows: hyphal plugs, 5 mm in diameter, were harvested from 18 d old colonised PLYA Petri dishes using a sterilised cork borer and transferred onto the roots (three plugs per plant). The plates were then sealed, shaded with aluminium foil and incubated in a growth room (Meridian Refrigeration Ltd, Croydon, UK; day and night 21-22°C, light: dark 16 h/8 h, PPFD of 40 μmol m^{-2} s^{-1}). The position of each plate in the growth room was randomised.

In experiment 2, after 7 weeks of growth, the plantlets were randomly divided in four treatments: inoculated with (1) *P. fragariae* and AMF (+AMF+Pf), (2) AMF only (+AMF-Pf), (3) *P. fragariae* only (-AMF+Pf), and (4) no AMF or *P. fragariae* (-AMF-Pf). Inoculation with *P. fragariae* was as follows: Hyphal plugs, 5 mm in diameter, were cut from the growing edge of three weeks old colonised KBA Petri dishes and transferred onto the plants roots (one plug from each of the three isolates per plant). The plates were sealed, covered with foil, and incubated in a growth room (Meridian Refrigeration Ltd, Croydon, UK, day and night 15-16°C, light: dark 16 h/8 h, PPFD of 40 μmol m^{-2} s^{-1}; Figure 6.2); the position of each plate in the growth room was randomised.

Figure 6.2: A photo depicting the square plates of experiment 2 incubated in a growth room at 15-16°C after inoculation of plantlets with *Phytophthora fragariae* hyphal plugs.

6.2.7. Estimation of disease severity and assessment of root colonisation by AMF and pathogens

In both experiments, disease severity was assessed five weeks after inoculation with hyphal plugs. The symptom was assessed on a rating scale from 0 to 5: 0 - no symptoms, 1 - shoot with a single leaf showing symptoms (yellowish-brown appearance), 2 - up to 25% of leaves showing symptoms, 3 - up to 50% of leaves showing symptoms, 4 - up to 75% of leaves showing symptoms, and 5 - plant death.

After disease severity assessment, AMF root colonisation (i.e. hyphae, arbuscules and vesicles) was assessed on the plantlets in the mycorrhizal treatments (+AMF). Similarly, pathogen structures (i.e. hyphae and microsclerotia for *V. dahliae* and oospores for *P. fragariae*) were assessed in those plantlets inoculated with the pathogens. The plantlet roots were removed from the plates with a pair of forceps and stained as described in Section 2.3.1. Each root sample was pooled from four individual plantlets (i.e. two plates).

6.2.8. Data analysis

All data were analysed using GenStat 13th edition (VSN International Ltd, Hemel Hempstead, UK). In both experiments, AMF colonisation was not observed in the mycorrhizal treatments. Therefore, to analyse the disease severity, data from the AMF treatments were pooled with their respective non-AMF treatments. The disease severity data were all analysed using a generalised linear model with the Poisson distribution and a log-link function. There was no evidence of over-dispersion in the residual deviance.

6.3. Results

Fragaria vesca and *F.* x *ananassa* cv. 'Calypso' plantlets were able to produce new roots and leaves on modified MSR medium in a completely sealed environment (Figure 6.3A, B). In addition, *R. irregularis* (MUCL 43194) could germinate on modified MSR medium (Figure 6.4A, B), but no AMF intraradical colonisation was observed after trypan blue staining of *F.* x *ananassa* and *F. vesca* roots. The mycelium of both pathogens was able to grow and spread on MSR medium in the presence of plant roots (Figure 6.5A, B). Staining of *F. vesca* roots with trypan blue failed to detect wilt infection (i.e. presence of hyphae and/or microsclerotia). Nevertheless, the shoot showed symptoms of wilt (Figure 6.6A). *F.* x *ananassa* cv. 'Calypso' plantlets were highly infected by *P. fragariae* (Figure 6.6B) with the presence of abundant oospore in the root tissues (Figure 6.5C) and the petiole of the leaves (Figure 6.6D). For both experiments, the number of diseased plants was increased by pathogen inoculation ($P < 0.001$; Figure 6.7A, B),

Figure 6.3: (A) *Fragaria vesca* and (B) *F.* x *ananassa* cv. 'Calypso' plantlets with well-established root systems and healthy shoots after one month of culture on Modified Strullu Romand (MSR) medium under axenic conditions.

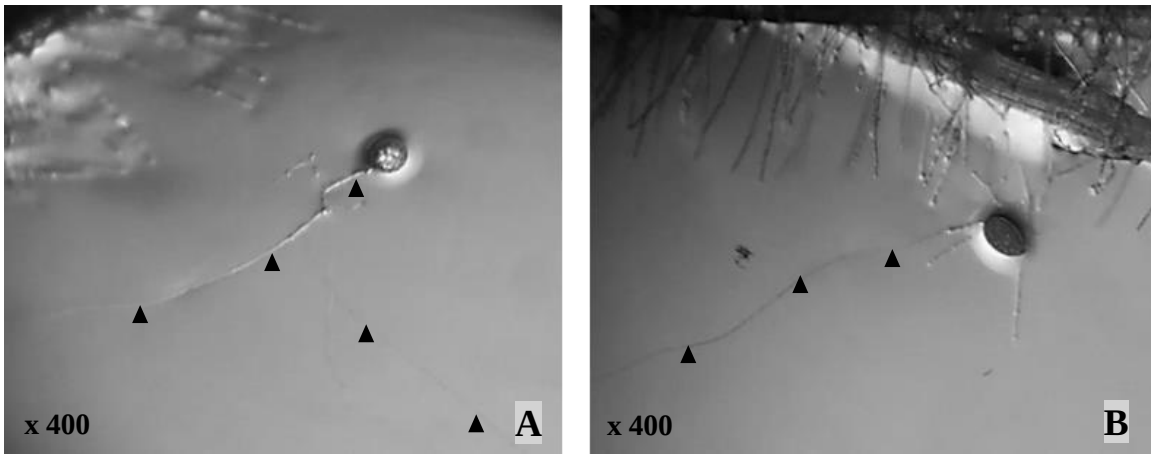

Figure 6.4: Germinated *Rhizophagus irregularis* spores (black arrow = mycorrhizal hyphae) on Modified Strullu Romand (MSR) medium. Photos were taken after two months of culture under axenic conditions with (A) *Fragaria vesca* and (B) *F.* x *ananassa* cv. 'Calypso' plantlets. The scale was not available in the camera used for image acquisition, thus the magnification is reported instead.

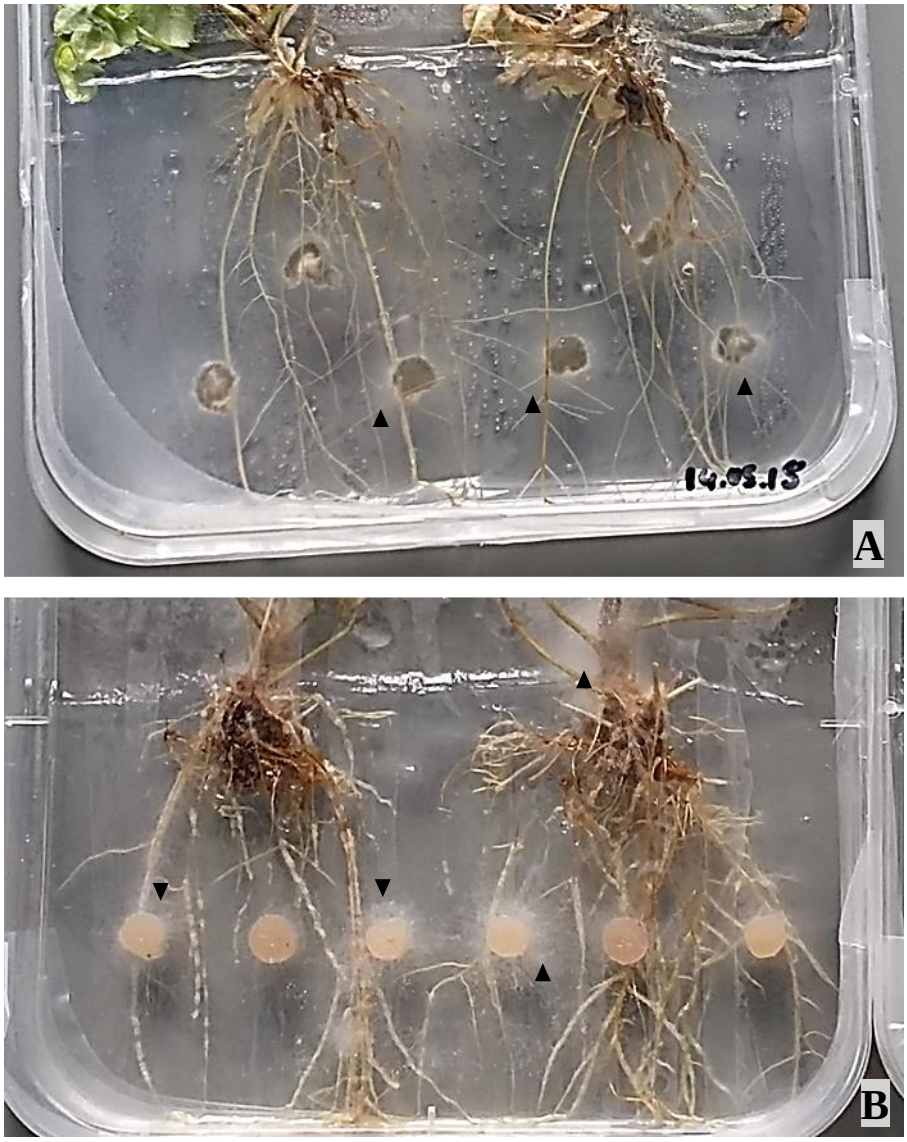

Figure 6.5: (A*) Fragaria vesca* plantlets inoculated with hyphal plugs of *Verticillium dahliae* and (B) *F.* x *ananassa* cv. 'Calypso' plantlets inoculated with hyphal plugs of *Phytophthora fragariae* 5 weeks after pathogen inoculation. Both pathogens could spread and establish on the surface of Modified Strullu Romand (MSR) medium (black arrows highlight areas where the mycelium grew around the mycelial plugs).

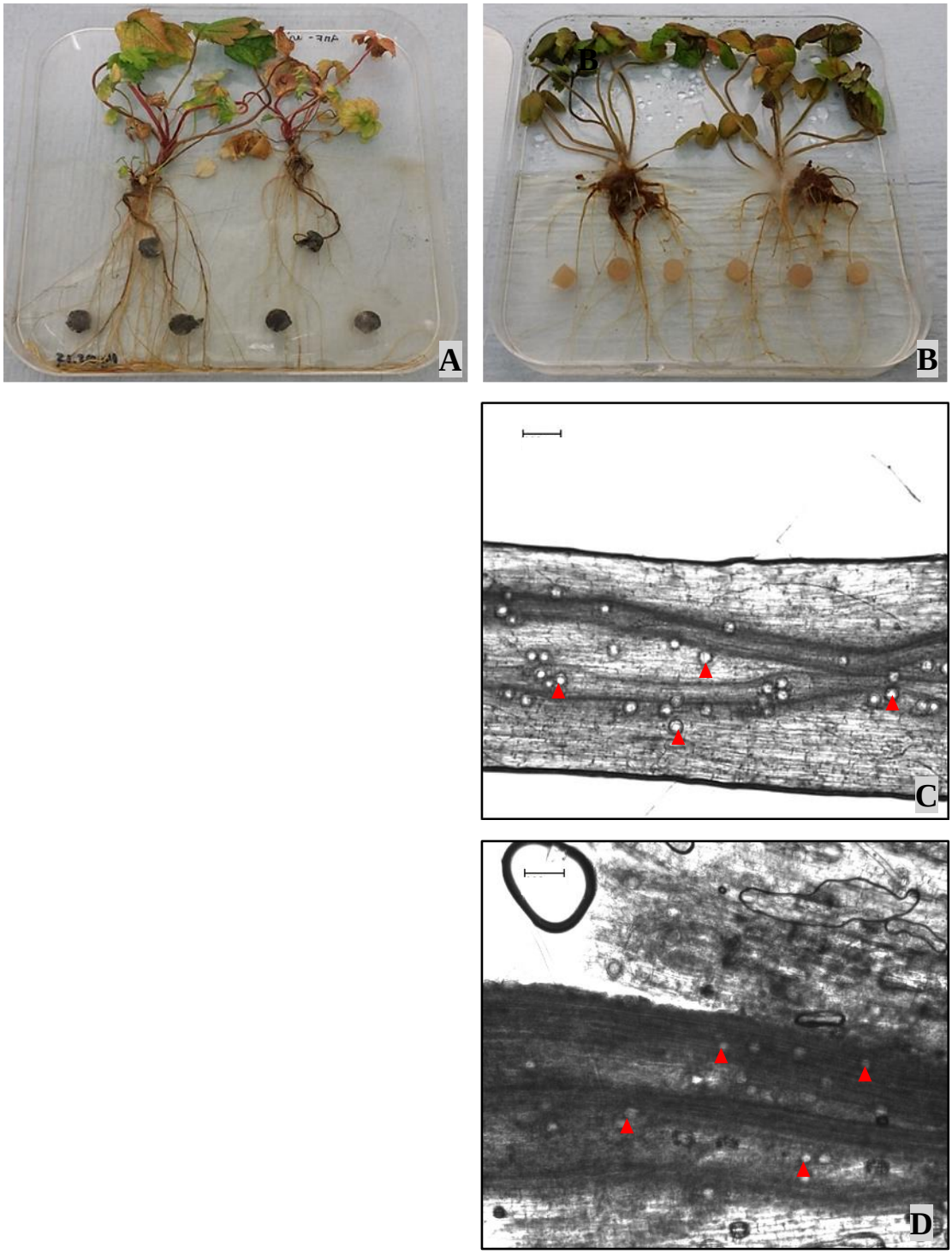

Figure 6.6: Disease symptoms of (A) *Fragaria vesca* plantlets inoculated with *Verticillium dahliae* and (B) *F.* x *ananassa* cv. 'Calypso' plantlets inoculated with *Phytophthora fragariae*, 5 weeks after pathogen inoculation. Longitudinal squash of 'Calypso' (C) root and (D) leave petiole stained with trypan blue showing the presence of *P. fragariae* oospores (red arrows). The black scale bars represent 100 µm.

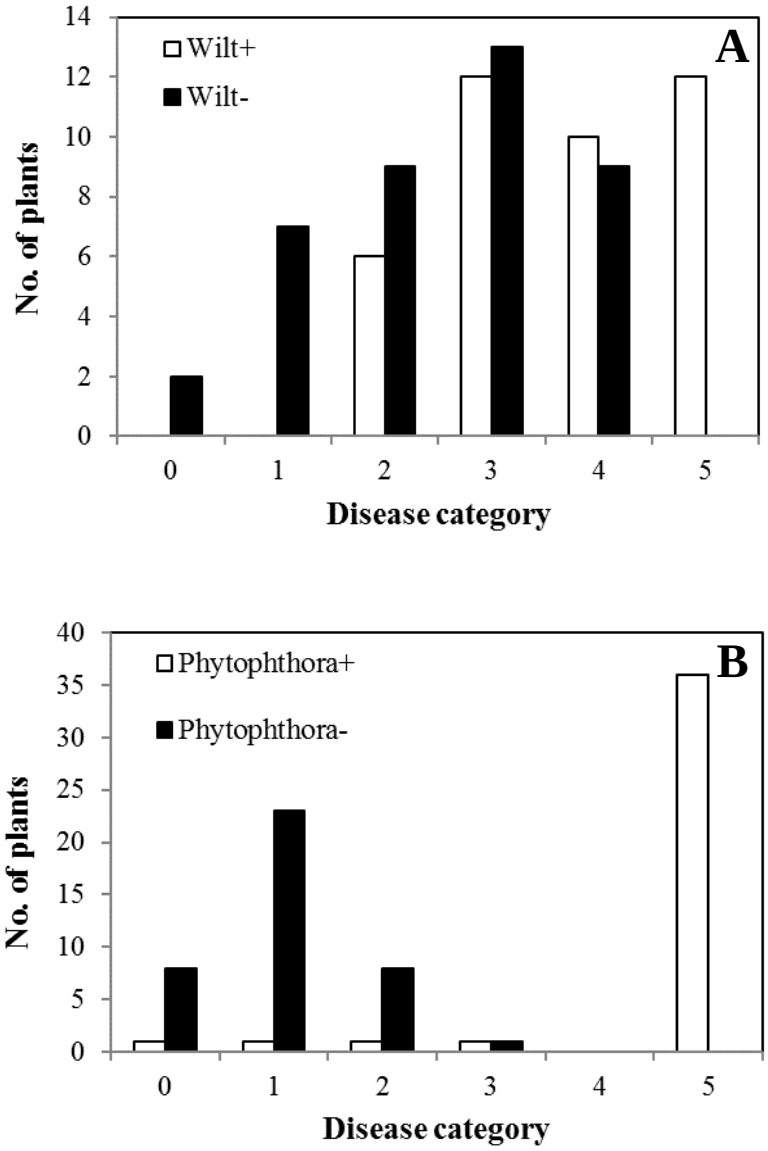

Figure 6.7: Influence of pathogen inoculation on disease score of (A) *Fragaria vesca* plantlets inoculated with *Verticillium dahliae* and (B) *F.* x *ananassa* cv. 'Calypso' plantlets inoculated with *Phytophthora fragariae*, 5 weeks after pathogen inoculation. Data are the total number (n = 40) of plants in the following disease categories: 0 - no symptoms, 1 - shoot with a single leaf showing symptoms, 2 - up to 25% of leaves showing symptoms, 3 - up to 50% of leaves showing symptoms, 4– up to 75% of leaves showing symptoms, and 5 - plant death. White bars show plant inoculated with pathogen (Wilt+ or Phytophthora+) and black bars show plants without pathogens (Wilt- or Phytophthora-).

6.4. Discussion

In this study, both *V. dahlia* or *P. fragariae* could induce disease symptoms of strawberry plantlets grown *in vitro* on MSR medium. However, the root colonisation by *V. dahlia* could not be confirmed by microscopic observations. The absence of observable pathogen colonisation could be the result of sampling issues or the trypan blue stain was not adequate to detect the presence of the pathogens in the strawberry root tissues. Therefore, other detection methods should be tested when strawberry wilt is inoculated *in vitro* on MSR medium. For example, the use of WGA-AF488/Propidium Iodide has been suggested to be suitable to stain *V. dahliae* within *in vitro* plant roots (Liang, 2012; Taylor pers. comm.); alternatively, a PCR bioassay could be also used to confirm the presence of pathogen DNA within the root tissues (Mirmajlessi *et al*., 2015). The presence of plants with yellow and/or brown leaves was observed in both experiments, but to a lesser extent in culture plates without pathogens. Those observations might be the result of natural aging and/or nutrient depletion of the growing medium. Therefore, to improve this culture system and the precision of disease severity assessments, fresh MSR medium could be added to the plates to avoid nutrient depletion in the *in vitro* system (Voets *et al.*, 2009; Oye Anda *et al*., 2015).

Unfortunately, the potential protective effect of AMF against *V. dahliae* or *P. fragariae* could not be tested using the current culture system as AMF root colonisation was not achieved. The reason for this is unknown. The sterile spores of *R. irregularis* were viable as observed by their germination on the MSR medium in both experiments. In parallel, *R. irregularis* spores were also inoculated onto microplants of *F. vesca* and *F.* x *ananassa* cv. 'Calypso' during their weaning stage on attapulgite clay to confirm they viability. *R. irregularis* was able to highly colonise plantlets roots (data not shown). In the context of *in vitro* culture conditions, several factors may have affected the interaction between the plant and AMF. For example, the limited gas exchange in this completely sealed *in vitro*

environment may have been responsible for high humidity that may have lower PPFD due to water condensation (see Figure 6.3A). In addition, the PPFD provided to plants in the growth room was low in comparison to previous studies (300 µmol m^{-2} s^{-1}, Voets *et al.*, 2005; 200 µmol m^{-2} s^{-1}, Müller *et al.*, 2013). Therefore, the combination of water condensation and low PPFD may have prevented the establishment of the symbiosis. In addition, substrate conditions should also be considered. For example, inadequate pH, low porosity and/or high thickness have also been mentioned to altered AMF hyphal development and function *in vitro* (Mosse, 1988; Liu & Yang, 2008; Costa *et al.*, 2013).

Other culture autotrophic systems may be able to overcome the problem of water condensation and lack of light that were potentially responsible for the absence of AMF colonisation in this study. The adaptation to strawberry plantlets of the semi-axenic systems developed by Voets *et al*. (2005) and improved by Koffi *et al*. (2009) appear to be an interesting solution. However, semi-axenic systems are more complex to manipulate than axenic ones. Semi-axenic systems are more sensitive to contamination due to additional interventions on the growing plates (i.e. small opening on the plate lid) and there is a higher risk of microplant death during acclimatisation. Nevertheless, in semi-axenic systems, because the shoots of the plantlets can grow outside of the culture plate, risks of water condensation and reduction of PPFD will be reduced. Furthermore, a modified mycelium donor plant (MDP) semi-axenic system may also be used to allow a faster and homogenous colonisation of strawberry roots. Voets *et al*. (2009) developed the MPD semi-axenic system and they showed a successful AMF colonisation of *Medicago truncatula* roots transferred on already actively growing extraradical hyphae extending from *M. truncatula* donor plants. This method was also successfully adapted by Koffi *et al*. (2012) to study the interactions between *R. irregularis*, banana and the nematode pathogen *Radopholus similis*.

6.5. Conclusions

In this study, micropropagated strawberry plantlets were successfully infected by *P. fragariae* on MSR medium with the corresponding disease symptoms, while infection with *V. dahlia* resulted in corresponding symptoms but root infection could not be confirmed. Despite AMF spores germinating on MSR, AMF-strawberry symbiosis could not establish in the present axenic autotrophic system. The reasons for the absence of AMF root colonisation are unknown, but the future adaptation of the MDP *in vitro* growing system and increase of PPFD might be able to solve this problem. As far as autotrophic *in vitro* cultivation systems are concerned, there are very limited data about interactions among environmental factors, plant cultivars, AMF species and species of pathogens. In conclusion, this first attempt to use a simple axenic autotrophic system with strawberry plantlets opens new avenues to explore the role of AMF in affecting major strawberry root pathogens under *in vitro* conditions.

Chapter 7. General discussion

7.1. Overview

This is to the best of our knowledge, the first research focusing on the AMF-strawberry interaction as a model system to study the possibility of pre-colonising strawberry plug materials with AMF to increase plant growth, productivity and tolerance against major strawberry root diseases. This study aimed to investigate the following hypotheses:

- H1: It is feasible to apply AMF inoculum during strawberry tipping in different soil-less substrates under misting conditions and obtain highly AMF-colonised strawberry plugs. (Chapter 3)
- H2: AMF in colonised strawberry plug roots can survive a prolonged period of storage at -2°C. (Chapter 3)
- H3: AMF pre-colonisation of strawberry plants increases plant tolerance against *Verticillium dahlia* under glasshouse and open field conditions. (Chapter 4)
- H4: AMF pre-colonisation and/or inoculation of AMF and/or PGPR at planting increases plant tolerance against *Phytophthora fragariae* and *P. cactorum* in soil-less substrates. (Chapter 5)
- H5: AMF and/or PGPR inoculations increase strawberry productivity in coir bags. (Chapter 5)
- H6: In an attempt to control for the influence of fluctuating environmental conditions that occur under both field and glasshouse conditions a simple *in vitro* autotrophic system can be established to investigate strawberry-AMF-pathogen interactions. (Chapter 6)

Figure 7.1 gives an overview of the results obtained on the impact of AMF on plant health and/or productivity at different stages of various strawberry growing systems.

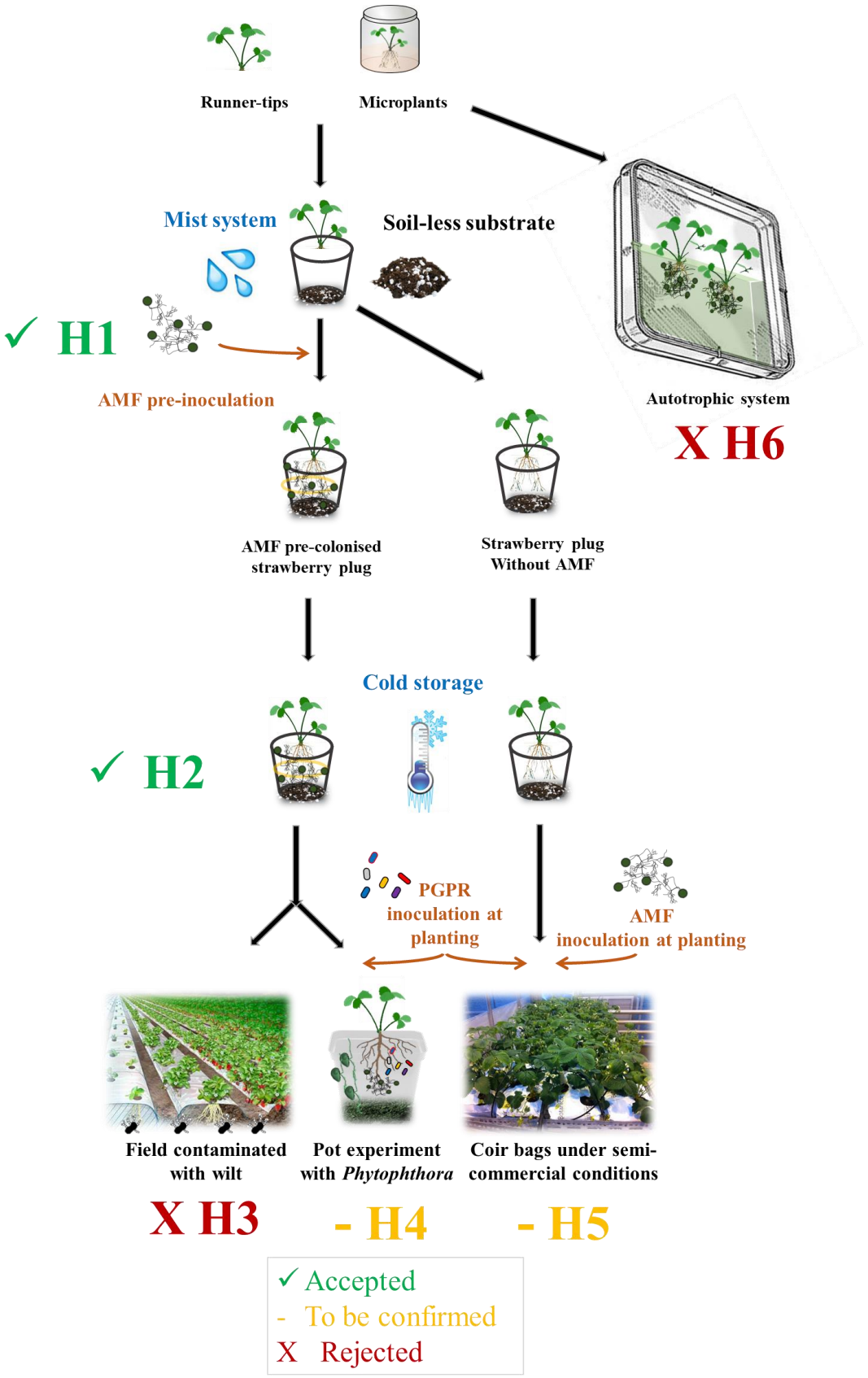

Figure 7.1: Overview of the impact of arbuscular mycorrhizal fungi (AMF) on strawberry health and productivity in growing systems. Hypotheses are displayed as 'accepted', 'rejected' or validation/rejection 'to be confirmed'. Hypotheses are not fully stated for clarity (Section 7.1).

7.1.1. Strawberry plug weaning practices and freezing cold storage do not prevent AMF root colonisation deriving from early inoculation

Pre-inoculation of strawberry plants with AMF before trans-planting has been proposed as an environmentally-friendly method to improve plant growth and health by alleviating biotic and abiotic stresses. Strawberry production systems make AMF pre-inoculation at the weaning stage relatively straightforward for both *in vitro* and runner derived plantlets. The results presented in Chapter 3 confirmed the feasibility of applying AMF inoculum during strawberry tipping in different soil-less substrate media commonly used in commercial situations. AMF propagules can in fact colonise strawberry roots of several commercial cultivars when incorporated as a powder layer in an Irish peat/base mix, vermiculite and coir under misting conditions and/or plant propagators. Therefore, AMF pre-colonisation of strawberry planting material can be commercially adopted by strawberry nurseries. A larger scale study was also designed to investigate the feasibility of incorporating AMF pre-inoculation in a commercial strawberry nursery, which confirmed that strawberry roots could be colonised by AMF. However, the level of RLC remained low (average 3%). This result highlighted the necessity to identify the limiting factors that reduce AMF colonisation under commercial productions. Different cultivation practices and AMF inoculation methods should be tested separately and together to identify the best combinations and growing conditions. To achieve this, it may be necessary to incorporate AMF inoculum as a powder layer, select a soil-less substrate suitable for AMF colonisation and adjust the cultivations practices such as the amount of fertiliser and/or irrigation regimes.

AMF inoculated during the weaning stage of microplants or runner tips resulted in a high level of RLC (average 70%), independently of plant material size and/or the strawberry cultivar inoculated (with the exception of ‘Malling Centenary’). However, early AMF

inoculations of strawberry plugs did not necessarily translate to improved plant growth during weaning and propagation phase. Plant genotype has also been shown to have a selective effect on the microbes that colonise the rhizosphere (Edel *et al*., 1997). To a limited extent this was observed in strawberry, with different cultivars displaying either a negative or positive interaction with specific AMF species (Khanizadeh *et al*., 1995). Therefore, to completely exploit the beneficial effect of AMF on strawberry in soil-less substrates, further work is also required to explain to what extent the ability of specific strawberry genotypes being colonised by AMF in soil-less substrate is heritable. If this trait is controlled genetically, this could be exploited in breeding programmes to breed strawberry plants that can be easily colonised by AMF in substrate to increase their cropping potential and tolerance to root pathogens (Boyer *et al.,* 2016; Hohmann *et al*., 2017).

Strawberry plugs need to be cold stored (-2°C) for various length of time to achieve chilling requirements and to schedule cropping. Therefore, it was necessary to investigate whether AMF could survive freezing temperatures for several months. In chapter 3, results showed that AMF propagules could tolerate the formation of ice in the root ball during cold storage at -2°C up to five months. This was the first evidence that commercial AMF inocula can be applied during strawberry tipping without reducing the mycorrhiza viability during subsequent cold storage of pre-colonised plants. This study has also indirectly highlighted the potential presence of DSE propagules in Irish dark peat; the extent of DSE in the root was negatively associated with AMF root colonisation after cold storage. Further studies are needed to identify the origin and identity of the DSE and the nature of their relationship with AMF and/or the plant host under commercial conditions.

7.1.2. Evaluation of the potential of pre-colonised strawberry plugs with mycorrhizal inoculants to increase tolerance to *Verticillium* wilt

AMF have been shown to be able to reduce the negative effects of strawberry soil-borne diseases (Vestberg *et al*., 2004). There are clear examples of how AMF could alleviate strawberry wilt in non-fumigated soil and so considered as a potential alternative to chemical soil fumigants (Ma *et al.*, 2004; Tahmatsidou *et al.*, 2006; Sowik *et al.*, 2016). For unknown reasons, it was very difficult to induce wilt symptoms under growth chamber and glasshouse conditions when using naturally contaminated field soil or soil-less substrate mixes artificially inoculated with different types of wilt propagules (Chapter 4). Therefore, it was very challenging to study the effect of AMF inoculations against strawberry wilt under pot conditions. This was not an isolated case; other studies conducted at NIAB EMR on the interaction between strawberry and *V. dahliae* also experienced similar issues (Cockerton pers. comm.). The strawberry cultivars used for those trials have all been selected for their susceptibility to *V. dahliae*. Therefore, the reasons for the absence or very low levels of wilt symptoms in pot experiments remains unknown. Unfavourable culture conditions when using naturally contaminated field soil or prolonged culture of wilt isolates on artificial media may have affected fungal pathogenicity. Further studies are needed to optimise experimental cultural conditions, select very susceptible strawberry cultivars (e.g. 'Elsanta', 'Emily'), use more virulent wilt strains and/or improve preparation of wilt inoculum and inoculation methods. By contrast, in the field experiment wilt symptoms were observed on the same susceptible strawberry cultivars used in some of the pot experiments. However, AMF pre-inoculation did not reduce the incidence of plants with wilt symptoms. Variability in the effectiveness of AMF inoculants against soil-borne diseases under field conditions depends on various environmental factors (Vestberg, 1992; Vestberg *et al.*, 2005; Rodriguez & Sanders, 2015). Therefore, small changes in field conditions may have resulted in greater changes in the biological control abilities of the

non-native AMF inoculants used in this study. Indigenous AMF inoculum present in the field could have also negated the initial positive effects of AMF pre-colonisation against strawberry wilt. Nevertheless, future experiments with other strawberry cultivars and sampling times closer to the transplantation time may reveal whether AMF pre-inoculation can be of any advantage for plant growth and nutrition in the early stage of establishment in the field. Further studies based on soil metagenomics should also be carried out to find the functional roles of the microbial communities that respond to AMF pre-inoculations. This would enable us to understand the mechanisms underlying the changes in community composition in strawberry rhizosphere. The experimental design may also need to be adapted and include soil samplings to identify the level of pathogen inoculum and its distribution before plant transplantation. In fact, there was a high degree of spatial aggregation of plants with *Verticillium* wilt. This aggregation pattern was observed with *V. dahliae* on other crops (Xiao *et al*., 1997; Johnson *et al*., 2006; Wei et *al*., 2015), which is most likely due to the heterogeneity in soil pathogen inoculum. This aggregation may have also masked any treatment effects – random assignment of treatments to individual plots is obviously not able to reduce this negative influence caused by aggregated inoculum.

Most importantly, strawberry wilt is expected to become less of a problem for strawberry production in the UK. The current restriction on methyl bromide, the necessity to mitigate the threat of strawberry wilt, the requirements to extend growing season and increase the ease of picking are leading to new strawberry production systems in the UK. Strawberry cropping is currently moving away from traditional field cultivation toward production into soil-less substrates under protection (glasshouse or polythene tunnel). Therefore, the management programs of strawberry root diseases and increase of productivity need to focus now on soil-less production systems.

7.1.3. Effects of AMF and PGPR on strawberry productivity and tolerance to *Phytophthora* diseases in soil-less substrates

Other root pathogens such as *P. fragariae* and *P. cactorum* continue to pose a serious threat to strawberry growing in soil-less substrates even on a table-top system (Schnitzler, 2004; Martínez *et al.*, 2010). In fact, strawberry plugs can be initially infected by both pathogens in nurseries or during cultivation via contaminated water supply. In addition, strawberry table-top systems rely heavily on high input of fertiliser, pesticides and water; the soil-less substrates used (e.g. coir or peat) are usually devoid of beneficial microbes which can also affect plant growth and productivity even in the absence of pathogens. Therefore, inoculations of AMF and/or PGPR in soil-less substrate such as coir were proposed as a sustainable method to improve strawberry health and yield (Boyer et *al.,* 2016). A series of pot experiments were carried out to test whether commercially available AMF and/or PGPR inoculants could reduce development of *P. fragariae* and *P. cactorum* in peat-based composts and improve strawberry productivity in coir under controlled conditions. Results of the current study showed in one occasion the potential of AMF inoculation alone to reduce symptoms caused by *P. fragariae*, although the presence of AMF colonisation was not here confirmed. Unfortunately, neither AMF root colonisation nor typical red core symptoms were observed in a repeat experiment. Thus, it was not possible to conclude whether AMF could reduce red core severity or incidence during those two experiments. The absence of AMF symbiosis was also observed in the *P. cactorum* experiment conducted under glasshouse conditions in commercially used compost. The difficulty to establish AMF colonisation during those three experiments was probably the result of chemicals (i.e. high P level and/or low pH), physical and/or biological properties of the compost mixes used, but the reasons for this remained unknown. Regarding the microbial inoculations in the coir bag study, AMF root colonisation was successfully established under semi-commercial conditions but remained quite low (average 15% RLC). Despite

the presence of AMF root colonisation, AMF did not significantly influence strawberry yield or plant growth although there was a trend of higher fruit yield associated with AMF inoculation at planting. The PGPR inoculation alone or coupled with AMF also failed to provide a significant increase of fruit yield and plant growth in this occasion. The nature of the experimental design (i.e. random design) was identified to be potentially responsible for large experimental residual errors, leading to the absence of significant treatment effects. It was also unfortunate that the AMF pre-colonised plugs could not be used in this work. In fact, they were not properly cold stored by the supplier and resulted in the death of most of the plants that could not be used. Further optimisations and understanding of the strawberry-microbe interactions in soil-less substrates on table-top system are needed to ensure consistent beneficial effect of AMF and/or PGPR inoculations.

7.1.4. The use of an axenic autotrophic *in vitro* system to explore the nature of interactions between AMF and soil-borne pathogens

Plant-microbe interactions involve very complex mechanisms and pathways that are very sensitive to environmental conditions. The use of a simple autotrophic system was presented as a useful tool to dissect various aspects of strawberry-AMF interactions by reducing the influences of environmental factors and interaction with other microbes occurring in the field and glasshouse conditions. Micropropagated strawberry plantlets were for the first time successfully grown with *V. dahlia* or *P. fragariae* on MSR medium. Unfortunately, the symbiotic interaction between AMF and strawberry did not establish in the present axenic system. The reasons for the absence of AMF root colonisation are unknown, but they were most likely associated with the low level of light quality provided during the tests. Nevertheless, plantlets were successfully associated with *V. dahlia* or *P. fragariae* on MSR medium with the corresponding disease symptoms, which was difficult to obtain in various pot experiments and never attempted before. Regarding the absence of

establishment of the AMF symbiosis, future adaptations of this system are necessary. The modified mycelium donor plant (MDP) *in vitro* growing system and the increase of light quality are both expected to provide better conditions for the establishment of the AMF symbiosis and should be the second step forward to improve this *in vitro* system. This study may open new avenues to explore the strawberry-AMF-root pathogens interaction under axenic controlled conditions on MSR medium.

7.2. Recommendations and future work

Plant-microbe interactions play an important role in plant health and productivity (Jeffries *et al*., 2003). The interest of strawberry growers in the role that AMF and other beneficial microbes could play in the control of root diseases has increased over recent years. However, the mechanisms involved in the mycorrhizal protection against plant pathogens are still poorly understood (Pozo & Azcon-Aguilar, 2007). Investigations of this field are made complex because of different parameters and growing conditions that may affect AMF symbiosis quantitatively and qualitatively: i) the model system is a combination of interactions between three different partners (strawberry-AMF-root pathogens), ii) the growth conditions should be favourable for the plant and AMF but also meet commercial standards, and iii) the identity of the partners defines the specificity of the system. Therefore, understanding each interaction independently is a prerequisite for improving our knowledge in this field and for identifying processes behind the bio-protective effects of the AMF symbiosis under commercial situations. I have successfully demonstrated the possibility to inoculate strawberry plugs at an early stage in different soil-less substrates and I have tested several methods that allow the study of the effect of AMF inoculation against different strawberry root pathogens. Those protocols could easily be adapted to study the interaction between other AMF species, other beneficial microbes, other strawberry cultivars, and other pathogens in different growing conditions. The methods

used here could also be applied to other important horticultural crops grown in soil and/or soil-less substrates. Many interesting observations in this study have resulted in a number of thought-provoking recommendations for the use of AMF in commercial strawberry production and future research in the area:

- The potential benefits of AMF in strawberry production seem to be currently ignored by the strawberry industry. This may be partially due to inadequate methods for large-scale inoculum production and the price of the commercial inocula. Thus, it is important to improve inoculum production techniques for introducing AMF to strawberry production systems and other horticultural crops. In addition, research should also focus on testing lower amounts of AMF inoculum to reduce the costs for growers and improving inoculation methods. Other AMF formulations or inoculation methods should be considered. For instance, a liquid formulation delivered through the fertigation system or mixing the granular AMF formulation in the potting mix are both expected to be cheaper but will need further optimisations to avoid losses of inoculum and to ensure they are reachable by strawberry roots.

- Pre-inoculation of strawberry plugs with AMF resulted in high levels of root colonisations in different soil-less substrates (e.g. vermiculite, peat/perlite mix and coir) when incorporated as a powder layer during the weaning stage (Chapter 3). However, when thousands of strawberry plugs would need to be pre-inoculated with AMF, the ‘powder layer’ inoculation method appears to be difficult to implement in a commercial situation, and it may not be the most cost-effective option for the grower. Therefore, the design of a new tray filling machinery that

could form an inoculum powder layer in the compost in pots/cells may help strawberry nurseries to optimise the plant pre-inoculation with AMF.

- Approximately 230 species of AMF have been recognized so far (Krüger *et al*., 2012) and this is surely an underestimate of the real diversity (Young 2012). Sequences within individual AMF spores and isolates have also shown to present multiple variants, as well as within and between species of the *Glomeromycota* (Rodriguez *et al.,* 2001). It is imperative to identify how different growing conditions and different strawberry cultivars could influence AMF genomic changes and consequently their positive effects on plant health and productivity. This type of research may help to design new AMF products and use them under the most appropriate conditions to maximise their positive effects.

- The potential of AMF to increase strawberry plant growth and health might be partially controlled by host and AMF genotypes and their interactions. Genotype-dependent plant reaction has been demonstrated in strawberry and other crops (e.g., wheat) that showed differences in the level to which they form a symbiotic relationship with AMF (Vestberg, 1992b; Al-Karaki & Al-Raddad, 1997). Further work is required to determine the extent to which mycorrhizal responsiveness of strawberry cultivars in a soil-less substrate is heritable. If this feature is controlled genetically, it could be exploited for breeding strawberry that could be easily colonised by AMF in substrate (e.g. coir) to enhance their productivity and tolerance to pests and pathogens.

- The presence of DSE together with AMF was observed inside the root of strawberry plants when grown in Irish peat-based substrate. Therefore, additional

studies are needed to characterise the identity and origin of the DSE and the nature of AMF and DSE interactions in strawberry roots (e.g. competition for root space, for carbon and/or mineral nutrients, and effect of DSE exudates). It will be also necessary to investigate the effect of DSE colonisation on the host fitness. For example, DSE inoculations on tomato has showed to improve plant growth, to influence fruit yield and fruit quality parameters and to decrease the negative effect of *V. dahliae* (Andrade-Linares *et al.*, 2011). If similar positive effects are identified for strawberry, DSE could also be used a beneficial microbe that will complement the current biological agent armoury.

- A single biocontrol agent is often inoculated to control a single pathogen (Wilson & Backman, 1999). However, this commonly used procedure might be responsible for the inconsistent performance provided by AMF. In fact, a single AMF species or strain is not active in all substrates and/or against all root pathogens. Therefore, it is advisable to mix different AMF species together and/or with other types of beneficial microbes (e.g. PGPR, *Trichoderma*) presenting different colonisation patterns. This might be useful to increase strawberry tolerance against pathogens via different mechanisms of disease suppression, biogeochemical cycling of nutrients and by creating a more stable rhizosphere community. For example, co-inoculations of AMF and PGPR have been reported to enhance plant growth and/or health in many occasions (Esitken *et al.*, 2010; Ipek *et al.*, 2014; Hautsalo *et al.*, 2016). Interestingly, synergistic effects on strawberry growth following co-inoculation with PGPR and AMF have been reported (Vosatka *et al.*, 1992) and PGPR were also found to stimulate AMF root colonisation (Vosatka *et al.*, 2000). This mixture may coexist without exhibiting adverse effects on each other and suitable combinations of AMF and PGPR may improve plant health and

productivity. Further studies are now required to investigate the functional complementarity between the AMF and PGPR when co-inoculated in substrate (e.g. coir) with different strawberry cultivars to develop suitable biocontrol of strawberry pathogens.

- Strawberry root colonisation by AMF can establish in coir during weaning stage or cultivation on table-top, which is of great importance for strawberry production. However, AMF colonisation was generally shown to be reduced in coir compared to other substrates (e.g. vermiculite). AMF inoculation in coir has been shown to increase plant biomass as well as the size and the number of Class I fruits (Chapter 6), which was consistent with a previous study (Boyer *et al.*, 2016). However, the positive effects of AMF inoculations were not statistically significant in this occasion. The nature of the experimental design (i.e. random design) was identified to be responsible for large experimental residual errors, leading to the absence of significant treatment effects. Further studies are needed to shed light on the AMF-strawberry interaction in coir and find the best cultivation practices to establish an increased level of root colonisation in this substrate and potentially more consistent protection against root diseases.

- To confirm whether pre-inoculated AMF species can persist temporally and spatially in soil or substrate and if they altered the composition of the native AMF and/or microbial community metagenomic approach should be used in the future. There have been many studies that have examined the effects of biological control agents on pathogen populations, and other microbial communities. This has been done in both the soil and inside the roots. However, the effects of AMF on microbial communities in soil-less substrate have not been the focus of most studies.

Therefore, metagenomic analysis should be used to investigate changes in microbial communities in the rhizosphere or within the root of strawberry plant cultivated in coir.

- In the context of strawberry table-top systems, cropping sequences and high use of fertilisers and pesticides may affect AMF root colonisation under commercial situations. Several reports of pesticide/AMF interactions showed that pesticides (e.g. fungicides, fumigants, herbicides and insecticides) fertilisers and irrigation regimes all affect AMF symbiosis (Johnson & Pfleger, 1992). Nevertheless, it is hard to make simple generalisations from those studies because of the variability in pesticide formulations, fertiliser compositions and experimental conditions. Specifically, a series of experiments are needed to answer the following questions: Are pesticides and fertilisers responsible for the difficulty of AMF to establish in coir and other peat-based substrates when cultivated on a table-top system?

- Finally, strawberry plants are also the target of many other pests and pathogens occurring above- and/or below-ground. The effect of AMF has been reported previously against foliar and systemic pathogens as well as insects and nematodes on several other crops (Comby *et al*., 2017). For example, strawberry plants root colonisation by *F. mosseae* and *G. fasciculatum* reduced larval survival and biomass of black vine weevil (*Otiorhynchus sulcatus*) when inoculated singly, but not together (Gange, 2001). Additional studies like those are needed to increase our knowledges in this field.

- AMF inoculation has showed effects against insect pests but few studies have addressed the impact upon beneficial insects such as pollinators and insect

predators (e.g. parasitoids). Studies in these areas are still scarce and need further attention.

The future success of the biological control in horticultural crops will benefit from research like this. With the respect to commercial introduction of AMF in strawberry production systems, the results of this work demonstrated that AMF pre-colonisation with commercially available inocula is possible under misting conditions and can result in the production of highly colonised strawberry plugs. In addition, pre-inoculated strawberry plugs could be cold stored for several months at -2°C without losing their AMF root colonisation. Those results are very encouraging for the strawberry growers interested in AMF biotechnology. However, the early establishment of the AMF symbiosis when using AMF Plantworks Ltd inocula did not promote strawberry health, growth or productivity under controlled and/or open field conditions. Although the experiments were set up in a robust way, it seemed that the initial AMF and/or the pathogens inocula used and/or cultivation conditions were not appropriate. Therefore, several experiments were not conclusive. It is clear that strawberry production systems are currently not designed to host AMF symbiosis (i.e. high fertilisation and/or irrigation regimes, use of substrates not conducive for AMF and dependency on high pesticides inputs) and the commercial AMF inocula quality is often variable. In conclusion, it is essential to optimise AMF inocula quality, inoculation techniques and AMF growing conditions before strawberry growers should use AMF biotechnology to control pathogens and/or increase plant productivity.

List of abbreviations

Ammonium	NH_4^+
Arbuscular mycorrhizal fungi	AMF
Analysis of Variance	ANOVA
Association of Official Agricultural Chemists	AOAC
Besloten Vennootschap	b.v.
Boron	B
Calcium	Ca
Centimetre	cm
Circa	ca.
Colony-forming unit	CFU
Confer (compare)	cf.
Copper	Cu
Cultivar	cv.
Dark septate endophytes	DSE
Day	d
Department for Environment Food & Rural Affairs	DEFRA
Degrees Celsius	^oC
Degrees of freedom	*df*

Deoxyribonucleic acid	DNA
Doctor	Dr
Doctor of Philosophy	PhD
et alia	*et al.*
East Malling Research	EMR
European and Mediterranean Plant Protection Organization	EPPO
European Bank of the *Glomeromycota*	BEG
Ex. gr.	*e.g.*
Food and Agriculture Organization	FAO
Generalised linear model	GLM
Glomeromycota in vitro collection	INVAM
Gibberellic acid	GA_3
Gram	g
Hour	h
Hydrochloric acid	HCl
Indole-3-butyric acid	IBA
Inductively Coupled Plasma-Optical Emission Spectroscopy	ICP-OES
Id est `	i.e.
Iron	Fe
Kidney bean agar	KBA

Kilogram	kg
Least significant difference	LSD
Litre	L
Magnesium	Mg
Manganese	Mn
Mating type	MAT
Metre	m
Microlitre	µL
Micromole	µmol
Micrometre	µm
Milligram	mg
Millilitre	mL
Millimetre	mm
Modified mycelium donor plant	MDP
Modified Strullu Romand	MSR
Most probable number	MPN
Murashige & Skoog	M&S
Mycothèque de l'Université catholique de Louvain	MUCL
National Institute of Agricultural Botany	NIAB
Nitrate	NO_3^-

Nitrogen	N
Number of replicates	*n*
Number	no.
Parts per million	ppm
Percent	%
Phosphorus or PGPR treatment in Chapter 5 (see context)	P
Photosynthetic photon flux density	PPFD
Plant growth-promoting rhizobacteria	PGPR
Polymerase chain reaction	PCR
Polyvinyl alcohol-lactic acid-glycerol	PVLG
Potassium	K
Potassium hydroxide	KOH
Pound sterling	£
Power of hydrogen	pH
Prune lactose yeast agar	PLYA
Quantitative polymerase chain reaction	qPCR
Relative humidity	HR
Root length colonisation	RLC
Root weight ratio	RWR
Second	s

Species pluralis	spp.
Standard error	SE
Sulphur	S
Tonne	t
United Kingdom	UK
Volume/Volume percent	v/v
Watt	W
Weight/Volume Percent	w/v
Wheat Germ Agglutinin, Alexa Fluor 488 Conjugate	WGA-AF488
Zinc	Zn

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